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Mecanismos de Ação da Bradicinina na Diferenciação Neural *in Vitro*

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"Mecanismos de ação da bradicinina na diferenciação neural *in vitro*"

MICHELI MAINARDI PILLAT

Tese de Doutorado submetida ao Instituto de Química da Universidade de São Paulo como parte dos requisitos necessários à obtenção do grau de Doutora em Ciências - Área: Bioquímica.

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Dedicatória

À minha família, que tanto amo.

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"Para os crentes,
Deus está no princípio de todas as coisas.
Para os cientistas, no final de toda reflexão"
Max Planck

"Ó Espírito Santo,
Amor do Pai e do Filho,
Inspirai-me sempre aquilo que devo pensar,
aquilo que devo dizer, como eu devo dizê-lo,
aquilo que devo calar, aquilo que devo escrever,
como eu devo agir, aquilo que devo fazer,
para procurar a Vossa glória, o bem das almas
e minha própria santificação"
Cardeal Verdier

RESUMO

Pillat, M.M. Mecanismos de Ação da Bradicinina na Diferenciação Neural *in vitro*: 2013. 152p. Tese de Doutorado – Programa de Pós-Graduação em Ciências Biológicas (Bioquímica). Instituto de Química, Universidade de São Paulo, São Paulo.

Durante o desenvolvimento do sistema nervoso, as células têm a tarefa de proliferar, migrar, diferenciar, morrer ou amadurecer de modo altamente preciso para formar estruturas complexas. Tal precisão é alcançada em decorrência da interação perfeita entre as células que se comunicam por meio de mensageiros químicos no ambiente extracelular. Nesse contexto, nosso grupo tem reportado o envolvimento da bradicinina (BK) em processos do desenvolvimento neural. Recentemente, observou-se que a BK desempenha um papel importante na determinação do destino neural, favorecendo a neurogênese em detrimento da gliogênese em diversos modelos de diferenciação, além de potencializar a migração celular observada no modelo de neuroesferas de rato (Trujillo et al, 2012). Essas descobertas motivaram, como objetivo geral dessa tese, a investigação dos mecanismos subjacentes à BK que determinam seus efeitos. Dessa forma, o principal modelo de diferenciação utilizado foi as células precursoras neurais (CPNs) isoladas do telencéfalo de embriões de camundongos. Estas células proliferam na presença dos fatores de crescimento (GFs) EGF + FGF2, mantendo-se multipotentes e formando as neuroesferas, ao passo que migram e diferenciam em neurônios e glias pela remoção desses GFs, com boa proximidade aos eventos do desenvolvimento do cortex *in vivo.* Como resultados do presente trabalho, observou-se, inicialmente, que a BK também influencia efetivamente na diferenciação neural no modelo de CPNs murinas. Ao término da diferenciação, observou-se que esta cinina favoreceu a migração e promoveu o enriquecimento neuronal, evidenciado pelo aumento da expressão das proteínas β3-Tubulina e MAP2. Constatou-se também, que se observa uma baixa taxa de proliferação ao término da diferenciação na presença de BK (Trujillo et al, 2012), em consequência da grande proporção de neurônios em cultura estimulada por esta cinina. Esta relação causal foi evidenciada pelo ensaio de incorporação de EdU e concomitante imuno-detecção dos marcadores β3-Tubulina, GFAP e Nestina. Fatores que promovem a neurogênese podem promovê-la suprimindo a proliferação celular em CPNs indiferenciadas, mais especificamente, alongando a fase G_1 do ciclo celular que resulta na divisão de diferenciação. Assim, investigou-se também se a BK influencia nesse processo. Análises por citometria de fluxo demonstraram que esta cinina suprimiu a proliferação estimulada pelos GFs, levando ao acúmulo de células na fase G_1 do ciclo celular. Esse acúmulo não provém do bloqueio do ciclo, uma vez que se observam grandes proporções de células nas fases subsequentes à G1, indicando que essa fase foi apenas prolongada pela BK e, assim, corroboraria no favorecimento da neurogênese. Outra face dos mecanismos adjacentes à BK para seus efeitos na diferenciação neural se refere às vias de sinalização disparadas por esta cinina. Observou-se que a BK induz a produção de AMPc por intermédio de proteínas G sensíveis à toxina pertussis (TP) (provavelmente através da subunidade βγ de proteínas G_i) e promove a mobilização de cálcio dos estoques intracelulares, evidenciando o envolvimento da família de proteínas G_q. Esses resultados sugerem que o receptor B2 de cinina acopla-se tanto às proteínas G_i quanto às proteínas G_q em CPNs. A exposição dessas células à BK também ativou as vias da

PI3K/Akt e da MAPK p38, mas não influenciou na ativação de STAT3 e JNK. Destaca-se o potencial da rota da MAPK ERK como uma das principais cascatas responsáveis por decodificar sinais de mensageiros externos em respostas celulares. O tratamento com BK em CPNs ativou a ERK por tempo prolongado e estimulou sua translocação para o núcleo. O efeito de BK na glio- e neurogênese de CPNs foi dependente da atividade de ERK, porque o bloqueio farmacológico dessa enzima impediu esse efeito de BK. Por outro lado, o favorecimento da migração induzido por esta cinina foi dependente da atividade da p38, enquanto, o seu efeito antiproliferativo foi condicionado à atividade das suas duas MAPKs, ERK e p38. Além disso, a via da PI3K/Akt ativada por BK não influenciou nos três eventos avaliados. Finalmente, utilizou-se nessa tese uma abordagem reducionista da diferenciação, porém amplamente utilizada por estudos mecanísticos de neurogênese, as células PC12. Assim, observou-se que a BK também ativa a ERK por tempo prolongado e com translocação nuclear, sendo que tal forma de ativação dessa quinase é proposta na literatura como necessária e suficiente para induzir a neurogênese dessas células. Demonstrou-se ainda que o bloqueio apenas da ativação sustentada de ERK, pela inibição das atividades das PKCs clássicas, impede o favorecimento da neurogênese por BK em células PC12. Juntos, esses resultados contribuem para elucidação dos mecanismos de ação da BK na regulação da diferenciação neural, colaborando para melhor entender esse processo e prevendo possíveis aplicações em terapias de reparo neuronal em pacientes com doenças, por exemplo, de Parkinson, Alzheimer, Esclerose Múltipla e lesões isquêmicas.

Palavras-chave: diferenciação neural, proliferação, migração, bradicinina, vias de sinalização e ERK.

ABSTRACT

Pillat, M.M. Mechanisms of Bradykinin in Neural Differentiation. 2013. 152p. PhD Thesis – Graduate Program in Biochemistry. Instituto de Química, Universidade de São Paulo, São Paulo.

During CNS development cells perform the task of proliferating, migrating, differentiating, dying or maturing in highly accurate patterns. Such accuracy is reached as a result of the perfect interaction among the cells that constantly communicate with each other through cell-cell contact or through chemical messengers present in the extracellular medium. In this context, our group has reported the involvement of bradykinin (BK) in neural differentiation of stem cell models (Trujillo et al, 2012). Recently, it has been observed that BK plays an important role in determining neural destination, favoring neurogenesis over gliogenesis in several models of differentiation, besides potentializing cell migration observed in the model of rat neurospheres. These discoveries have motivated, as the general objective of this thesis, the investigation of the mechanisms underlying BK-promoted effects on neural differentiation using neural precursor cells (NPCs) isolated from the telencephalon of mice embryos. These cells proliferate in the presence of growth factors (GFs) EGF + FGF2, remaining multipotent and forming neurospheres, while they migrate and differentiate in neurons and glias following removal of these GFs, resembling in simplified conditions events of the development of the cortex *in vivo.* As results of the present thesis, it was initially observed that BK also effectively influences neural differentiation fate of the mouse NPC model. This kinin favored migration and promoted neuronal enrichment, evidenced by increased expression of β3-Tubulin and MAP2 marker proteins. Moreover, proliferation rates were largely decreased following differentiation in the presence of BK (Trujillo et al, 2012), due to the large proportion of neurons in the culture stimulated by this kinin. This causal relation was evidenced by the EdU incorporation assay and the concomitant immunodetection rates of β3-Tubulin, GFAP and Nestin markers. Factors which promote neurogenesis can promote it by suppressing cell proliferation in undifferentiated NPCs, more specifically, prolonging the G_1 phase of the cell cycle that result in the division of differentiation. Thus, it was further investigated whether BK influences this process. Flow cytometry analyses showed that this kinin suppressed the proliferation stimulated by GFs, resulting in the accumulation of cells in the G_1 phase of the cell cycle. This accumulation is not caused by a cycle block, since wide proportions of cells are observed in phases subsequent to the G_1, indicating that this phase was only prolonged by BK, thus corroborating for favoring neurogenesis. Another aspect of the mechanisms adjacent to BK for its effects on neural differentiation refers to the signaling pathways triggered by this kinin. Here, we show that the kinin B2 receptor couples to both G_i and G_q proteins in NPCs. BK induced the production of intracellular cAMP by activation of G proteins sensitive to pertussis toxin (PT) (probably through βγ subunit of G_i proteins) and promoted the mobilization of calcium from intracellular stocks, demonstrating the involvement of YM-254890-sensitive G_q proteins. Exposure of these cells to BK also activated PI3K/Akt and MAPK p38 pathways, but did not affect the activation of STAT3 and JNK. It is important to note the potential MAPK-ERK route as one of the main cascades responsible for decoding signals from external messengers

into cellular responses. NPC treatment with BK activated ERK for prolonged time and stimulated its translocation into the nucleus. The effect of BK on glio- and neurogenesis of NPCs depended plainly on ERK activity, because the pharmacological blockade of this enzyme prevented the BK-exerted effects. On the other hand, the favoring of migration induced by this kinin was dependent on p38 activity, while its antiproliferative effect was conditioned to the activity of both the MAPKs ERK and p38. In addition, the PI3K/Akt pathway activated by BK did not affect any of the three evaluated events. Finally, we used in this thesis a reductionist approach of differentiation based on the use of PC12 cells, which has been widely used for mechanistic studies of neurogenesis. Thus, it was observed that BK also activated ERK for prolonged time and with nuclear translocation, considering that such form of kinase activation is proposed in the literature as necessary and sufficient to induce neurogenesis in these cells. This study also demonstrated that blockade only of the sustained ERK activation, through the inhibition of the activity of classic PKCs, prevents the favoring of neurogenesis by BK in PC12 cells. Together, these results compose novel mechanisms of action of BK on events of neural development *in vitro*, contributing to the better understanding of this process and foreseeing possible applications in the future for neuronal repair strategies.

keywords: Neural Differentiation, proliferation, migration, bradykinin, signaling pathways e ERK.

LISTA DE ABREVIATURAS E SIGLAS

AC: Adenilato Ciclase

AF: Alexa Fluor

BDNF: *Brain-derived Neurotrophic Factor*

BK: Bradicinina (*bradykinin*)

BrdU: 5-bromo-2-deoxiuridina

BSA: Albumina do soro bovino

cDNA: DNA complementar

CPN: Células Precursoras Neurais

CTL: Controle

DAPI: *4',6-diamidino-2-phenylindole*

DAG: Diacilglicerol

DMEM: *Dulbecco's Modified Eagle Medium*

EdU: 5-etinil-2-deoxiuridina

EGF: Fator de Crescimento Epidérmico

FGF: Fator de Crescimento de Fibroblasto

GAPDH: Gliceraldeído 3-fosfato Desidrogenase

GAP: *GTPase Activating Protein*

GDP: Guanosina Difosfato

GEF: *Guanine Nucleotide Exchange Factors*

GFAP: *Glial Fibrillary Acidic Protein*

GFs: Fatores de Crescimento (*Growth Factors)*

GPCR: Receptor acoplado à proteína G

GTP: Guanosina Trifosfato

HMWK: Cininogênios de alta peso molecular (*High-molecular-weight kininogen*)

iPS: Células-tronco induzidas (*Induced Pluripotent Stem Cell*)

IP3: Inositol-1.4.5-trifosfato

IgG: Imunoglobulina G

JNK: *c-Jun N-terminal kinases*

LMWK: Cininogênios de alta peso molecular (*Low-molecular-weight kininogen*)

MAP-2: *Microtubule-associated protein-2*

MAPK: *Mitogen-activated protein kinase*

MEK: *ERK activator kinase*

MFI: Intensidade de Fluorescência Mediana

NGF: *Nerve Growth Factor*

NEP: Endopeptidase Neutra

PAGE: Eletroforese Poliacrilamida-dodecil sulfato de sódio

pb: pares de base

PBS: Tampão fosfato salino

PCR: Reação em cadeia da polimerase

PI: Iodeto de Propídio

PKC: Proteína Quinase C

PLC: Fosfolipase C

PKA: Quinase ativada por AMPc

PMA: *Phorbol 12-myristate 13-acetate*

PI3K: *Phosphoinositide 3-kinase*

PACAP: *Pituitary Adenylate Cyclase-activating Polypeptide*

PFA: Paraformaldeído

RNA: Acido Ribonucleico

RNAi: RNA de interferência

RNAm: RNA mensageiro

RT: Transcrição Reversa

RTK: Receptor Tirosina Quinase

SC: Soro de Cavalo

SDS: Dodecil Sulfato de Sódio

SFB: Soro Fetal Bovino

SNC: Sistema Nervoso Central

STAT3: *Signal transducer and activator of transcription 3*

TC: Toxina Cólera

TP: Toxina Pertussis

SUMÁRIO

INTRODUÇÃO 1

A presente tese abrange vários tópicos das áreas de diferenciação neural e vias de sinalização intracelular. Por esta razão, essa sessão apresentará questões importantes para a compreensão do trabalho realizado e suas contribuições relacionadas aos seguintes temas:

1.1 Células-Tronco e Progenitoras Neurais: Essa subseção descreve tais células e seus tipos de divisão celular (proliferativa e de diferenciação). Os modelos celulares, bem como os marcadores de células neurais indiferenciadas e especializadas, utilizados nessa tese, também são discutidos.

1.2 Respostas celulares a sinais externos no desenvolvimento embrionário: Essa subseção introduz a importância de mensageiros extracelulares na comunicação entre as células, direcionando-as a proliferar, migrar e/ou diferenciar de forma ordenada durante o desenvolvimento neural. Essa tese estudou as influências e os mecanismos da bradicinina nestes três processos celulares *in vitro.*

1.3 Vias de Sinalização: A decisão de proliferação, migração e/ou diferenciação em resposta a um mensageiro, como a bradicinina, é tomada pelas vias de sinalização, que interpretam os sinais do meio externo e interno. Essa subseção apresenta tópicos básicos sobre as vias de sinalização.

1.3.1 Vias de sinalização classicamente acionadas por receptores acoplados à proteína G (GPCR): Visto que a bradicinina atua através do receptor B2 de cininas e este é um GPCR, essa subseção apresenta as subunidades da proteína G, as principais famílias dessas proteínas, suas possíveis vias a jusante e as toxinas que modulam suas atividades.

1.3.2 Cascatas das MAPKs: Essa subseção apresenta as MAPKs e as vias de sinalização acionadas por GPCR que as ativam. A mesma destaca a cascata de ERK, visto que esta integra várias vias de sinalização, decodificando-as em respostas celulares, como a neurogênese, conforme sua forma/topologia de ativação.

1.4 Bradicinina: Essa subseção introduz o sistema calicreína-cinina e as vias de sinalização acionadas por intermédio de proteínas G acopladas ao receptor B2 de cininas. Além disso, a mesma aborda a integração dessas vias acionadas pelo receptor B2 na cascata de ERK.

1.4.1 Bradicinina na Diferenciação Neural: Essa subseção discute as recentes descobertas de nosso e de outros grupos que motivaram o presente trabalho. Entres estas descobertas, destaca-se o papel da bradicinina no favorecimento da neurogênese e migração, bem como na supressão da proliferação após diferenciação de diferenciação de CPNs e células pluripotentes (P19 e iPS)

1.1 – CÉLULAS-TRONCO E PROGENITORAS NEURAIS

Uma célula-tronco é uma célula não diferenciada (não especializada) que foi assim nomeada devido sua capacidade de se diferenciar em outras células e de se auto-renovar indefinidamente. Existem inúmeros tipos de células-tronco, classificados quanto

a sua origem e potencial de diferenciação. Considerando o potencial, as células-tronco pluripotentes e multipotentes são as mais importantes. As primeiras possuem uma grande capacidade de diferenciação e podem originar todos os tipos celulares de um organismo adulto, já as últimas possuem uma capacidade de diferenciação mais limitada e só formam tipos celulares presentes no mesmo tecido de sua origem (Oliveira et al, 2013; Zhu & Huangfu, 2013).

As células-tronco neurais são classicamente consideradas células multipotentes, já que se diferenciam em neurônios e células da linhagem glial, sendo responsáveis pela formação do sistema nervoso na fase fetal e pós-natal e manutenção da sua integridade fisiológica na fase adulta. Entretanto, estudos recentes demonstram que as células-tronco neurais murinas possuem um potencial muito mais amplo do que o esperado, não se restringindo somente a capacidade de originar células do sistema nervoso central (SNC). Essas células, expostas a condições específicas *in vitro* e *in vivo*, são capazes de se diferenciar em células de linhagens mesodérmicas, tais como células hematopoiéticas e musculares (Bjornson et al, 1999; Clarke et al, 2000; Oishi et al, 2002). O grande potencial dessas células torna o estudo do seu comportamento e propriedades ainda mais relevante.

As células-tronco neurais podem ser isoladas do cérebro em desenvolvimento e sua manipulação *ex vivo* é um excelente modelo para o estudo de sua auto-renovação, diferenciação neural e migração, bem como da influência de fatores externos nestes processos. Na presença dos fatores de crescimento EGF (*Epidermal Growth Factor*) e FGF2 (*Fibroblast Growth Factor-2*), as células-tronco neurais se multiplicam em cultura em monocamadas ou agregados em suspensão, chamados de neuroesferas (Gage, 2000;

McKay, 1997; Oliveira et al, 2013; Reynolds & Weiss, 1996). Estas células proliferam, originando outras células-tronco ou células progenitoras neurais com limitado potencial de auto-renovação (Bebien et al, 2012; Reynolds & Weiss, 1996). As células progenitoras, por sua vez, na presença dos mesmos fatores de crescimento (*growth fators* - GFs), EGF e FGF2, originam somente a outras células progenitoras. Deste modo, a neuroesfera é constituída por células-tronco e células progenitoras e a terminologia "células precursoras neurais" (CPNs) é utilizada para descrever ambos os tipos celulares (Reynolds & Weiss, 1992; Svendsen et al, 1998).

O desenvolvimento do sistema nervoso central (SNC) é resultado de múltiplos eventos. Este se inicia com a proliferação de CPNs, seguido pela especificação da identidade da célula neural (neurônio ou glia), migração, apoptose e maturação celular. Devido à importância e complexidade do SNC, estes cinco processos que resultam na sua formação são altamente controlados e dependem de uma interação perfeita entre fatores do meio extracelular e da programação intrínseca da célula (Bebien et al, 2012).

A proliferação de CPNs dá-se durante todo o desenvolvimento do SNC, tendo, inicialmente, a finalidade principal de expandir o número de células que o constituem (Frisen et al, 1998). Entretanto, esta não é a única finalidade da proliferação. Os precursores neurais podem realizar dois tipos diferentes de divisão: (1) Divisão proliferativa e (2) divisão para diferenciação (Calegari et al, 2005; Lako et al, 2009; Lukaszewicz et al, 2002) A primeira origina dois precursores, completa-se em aproximadamente 11 a 16 horas e tem a finalidade de expandir a população de CPNs (auto-renovação). Além disso, a divisão proliferativa é sempre simétrica, ou seja, o citoplasma é dividido igualmente, originando duas células-filhas idênticas. Por outro lado,

a divisão para diferenciação origina duas células-filhas, sendo que uma ou as duas células deixam o ciclo celular e sofrem diferenciação. Esse tipo de divisão é mais lento que a divisão proliferativa, completando-se em aproximadamente 24 horas. Além disso, a divisão para diferenciação pode ser assimétrica (quando somente uma célula-filha sofre diferenciação) ou simétrica (quando as duas células-filhas sofrem diferenciação) (Calegari et al, 2005; Lako et al, 2009; Lukaszewicz et al, 2002). O controle do balanço entre a divisão proliferativa e a divisão para diferenciação determina a geração do número apropriado de CPNs e neurônios no SNC (Caviness et al, 1995; Polleux et al, 2001).

A decisão se as CPNs irão continuar somente se auto-renovando ou também diferenciar é tomada nas fases iniciais do ciclo celular, com base nos sinais internos e externos obtidos do microambiente (Lako et al, 2009). Basicamente, o ciclo celular é dividido em quatro fases (Figura 1.1). Na primeira delas, denominada Gap 1 e usualmente conhecida como G_1, a célula prepara-se para a fase seguinte, sintetizando, por exemplo, proteínas específicas necessárias para a replicação do DNA. Nessa fase, as células-tronco e progenitoras neurais também tomam decisões importantes sobre o destino das células filhas, decidindo se a divisão realizada será do tipo proliferativa ou para diferenciação (Lako et al, 2009; Steensgaard et al, 2004). Sabe-se que células precursoras neurais apresentam a fase G_1 do ciclo celular abreviada enquanto estão realizando divisão proliferativa (Figura 1.1) (Orford & Scadden, 2008). Por outro lado, estudos recentes demonstraram, *in vitro* e *in vivo*, que todas as CPNs que apresentam um alongamento da fase G_1 do ciclo celular irão realizar a divisão para diferenciação (Calegari et al, 2005; Lukaszewicz et al, 2002). Além disso, demonstrou-se que a indução do alongamento da fase G_1 dessas células resulta no comprometimento com a diferenciação neuronal *in vitro*

e da neurogênese prematura em embriões *in vivo* (Calegari & Huttner, 2003; Neganova et al, 2009). Portanto, esses dados suportam a hipótese que o alongamento da fase G_1 do ciclo celular em CPNs trata-se de um mecanismo indutor da neurogênese (Lako et al, 2009).

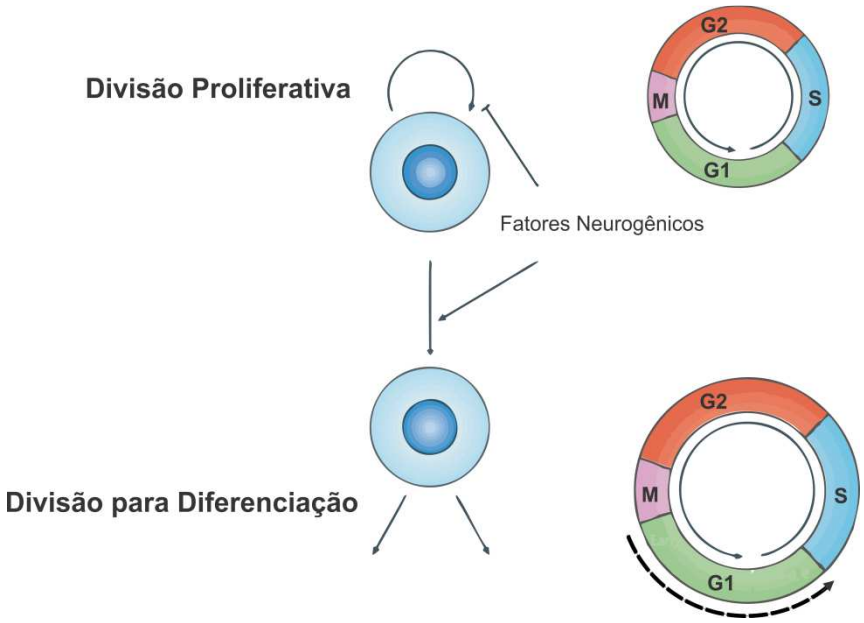


Figura 1.1: Divisão proliferativa e para diferenciação de células precursoras neurais. As células precursoras neurais apresentam um ciclo celular mais curto quando estão realizando divisão proliferativa uma vez que possuem uma fase G_1 abreviada. Essas células, quando estimuladas por determinados fatores neurogênicos, prolongam a duração da fase G1 e, por conseguinte, realizarão divisão para diferenciação. Modificada de Orford (2008).

A segunda fase do ciclo celular chama-se fase de Síntese, usualmente conhecida como fase S, e é o período no qual as células duplicam o seu material genético. A terceira fase denomina-se Gap 2 ou G_2 consiste, basicamente, de uma preparação para a fase seguinte, realizando síntese de proteínas específicas. Na quarta e última fase, chamada Mitose ou fase M, ocorre a separação dos componentes celulares entre as duas células filhas. A separação dos componentes em partes iguais caracteriza a divisão simétrica, já a separação em partes diferentes caracteriza a divisão assimétrica. As células também podem parar de proliferar e sair do ciclo celular, porém em um estado quiescente. Nestes casos, diz-se que as células estão na fase G_0. Os neurônios são exemplos conhecidos que

permanecem toda a sua vida nessa fase, já outros tipos celulares podem entrar e sair do ciclo conforme os sinais do meio extracelular (Ali et al, 2011; Guma et al, 2011).

Há um aumento progressivo na proporção de células na fase G_1 fazendo divisões de diferenciação, bem como na proporção de células que abandonam o ciclo celular (neurônios) durante a corticogênese (Caviness et al, 1995). O controle regional e temporal desses processos determina o número de neurônios nas diferentes áreas do SNC (Polleux et al, 1997). Dessa forma, de acordo com Lukaszewicz e colaboradores (2002), estes dados sugerem que fatores que controlam a corticogênese/neurogênese o fazem influenciando na duração do ciclo celular e no modelo de divisão.

Para se avaliar a diferenciação neural *in vivo* e *in vitro*, são necessários marcadores específicos de CPNs, bem como de neurônios e glias recém-originados. Nesse contexto, sabe-se que a diferenciação celular está intimamente conectada a alterações morfológicas decorrentes de modificações de proteínas que compõem o citoesqueleto, como os filamentos intermediários e os microtúbulos. Essas proteínas são expressas em padrões específicos do tipo celular, permitindo o acompanhamento da diferenciação e desenvolvimento neural (Bebien et al, 2012; Herrmann & Aebi, 2000). Entre esses marcadores, destacam-se: (1) Nestina, (2) β 3-tubulina, (3) MAP2 e (4) GFAP.

A Nestina é um filamento intermediário do tipo VI. Apesar de ser expressa em outros tipos celulares, durante o desenvolvimento neural essa proteína é expressa somente em células precursoras neurais (células-tronco neuroepiteliais, glias radial e progenitores neurais) (Lendahl et al, 1990). Por outro lado, a β 3-tubulina é um subtipo de tubulina, expressa especificamente em neurônios durante o desenvolvimento neural, inclusive em neurônios imaturos. Como todas as tubulinas, a β 3-tubulina forma os

microtúbulos. Já a MAP2 é da família de Proteínas Associadas aos Microtúbulos (MAP). Ela é especificamente expressa em neurônios e atua estabilizando o crescimento dos microtúbulos, passo essencial na neurogênese/neuritogênese. Por fim, o GFAP (Proteína Glial Fibrilar Ácida) é um filamento intermediário do tipo III e, como o próprio nome descreve, é uma proteína presente em células gliais. O GFAP também pode ser expresso em glias radiais (um tipo de célula-tronco precursora neural), mas estas últimas também expressam Nestina (Bebien et al, 2012; Li et al, 2011; Oliveira et al, 2013).

As neuroesferas, principal modelo de diferenciação neural utilizado nessa tese, abrangem boa parte dos processos complexos que ocorrem nos estágios iniciais do desenvolvimento neural, tais como proliferação de CPNs, migração e neuro- gliogênese. Similarmente ao observado *in vivo* (Tropepe et al, 1999), os fatores de crescimento (GFs) FGF2 e EGF induzem a proliferação de CPNs também *in vitro* (Oliveira et al, 2013; Reynolds & Weiss, 1996). Além disso, outra vantagem desse modelo é que após a remoção dos GFs e exposição à superfície aderente, as CPNs migram e diferenciam-se em neurônios e glias, espontaneamente. Assim, as neuroesferas refletem o desenvolvimento neural com boa proximidade dos processos complexos que ocorrem *in vivo* e, portanto, são um excelente modelo para avaliar a influência de fatores externos (Para revisão ver Trujillo (2009); (Lameu et al, 2012).

Entretanto, dependendo do ponto de vista analisado, a complexidade do modelo experimental torna-se uma desvantagem. Inúmeros estudos mecanísticos que avaliam a neurogênese isoladamente optam por utilizar as células PC12 como modelo de diferenciação (Santos et al, 2007; Sasagawa et al, 2005; von Kriegsheim et al, 2009). Essa é uma linhagem celular imortalizada (cultura homogênea), derivada de um

feocromocitoma que se desenvolve na região medular da glândula suprarrenal. As células PC12 passam por uma diferenciação terminal quando expostas a fatores externos, tais como o NGF (*Nerve Growth Factor*) e PACAP (*Pituitary Adenylate Cyclase-activating Polypeptide*), ou quando cultivadas sobre uma matriz aderente de laminina (Bouschet et al, 2003; Graner et al, 2000; Santos et al, 2007). Assim, esse modelo de diferenciação neural também foi utilizado na presente tese, uma vez que o mesmo oferece maior controle sobre as variáveis experimentais e permite comparações com os inúmeros trabalhos mecanísticos de neurogênese previamente publicados, que utilizam células PC12.

1.2 - RESPOSTAS CELULARES A SINAIS EXTERNOS NO DESENVOLVIMENTO EMBRIONÁRIO

Na formação de um organismo multicelular ou, mais restritamente, na formação de um órgão como o cérebro, todas as células têm a tarefa de proliferar, migrar, diferenciar, morrer ou amadurecer de modo espaço-temporal altamente preciso para formar estruturas e organismos extremamente complexos com funções e atividades tão complexos quanto. Tal precisão é alcançada em decorrência da interação perfeita entre as células que constantemente comunicam-se pelo contato célula-célula ou por meio de mensageiros químicos, tais como hormônios, fatores de crescimento, agentes quimiotáxicos, etc. Estes mensageiros podem ser produzidos pela própria célula que interpretará a mensagem, por células adjacentes ou por células extremamente distantes. Dessa forma, dependendo da concentração que um dado mensageiro atinge uma célula específica (especificidade espacial), ele pode diferencialmente influenciar no posicionamento (direção da migração), divisão ou diferenciação das células.

Adicionalmente, o momento em que o mensageiro encontra a célula (especificidade temporal) também é muito importante para a determinação da resposta celular. Isto se dá, uma vez que durante a embriogênese as células estão constantemente sofrendo estímulos que podem resultar em mudanças intracelulares permanentes (memória celular) e, dessa forma, fazem com que a célula reaja de maneira diferente a um dado mensageiro conforme o momento em que ela entra em contato com o mesmo. As células reconhecem os mensageiros por meio de proteínas específicas inseridas na membrana plasmática (proteínas transmembrana), conhecidas como receptores. A ligação do mensageiro no seu receptor específico, por sua vez, irá disparar vias de sinalização intracelulares (Caviness et al, 1995; Polleux et al, 2001).

1.3. Vias de Sinalização

Alguns anos atrás, as vias de sinalização eram descritas linearmente e compostas por poucos elementos, os quais eram classificados em: um receptor, um segundo mensageiro e um efetor responsável pela modulação de um grupo seleto de alvos. O segundo mensageiro foi assim nomeado porque ele é produzido em resposta ao primeiro mensageiro extracelular. Os segundos mensageiros intracelulares clássicos eram o AMP cíclico (AMPc) e Ca^{2+}, que ativam diretamente as suas respectivas proteínas efetoras, ou seja, enzimas quinases que fosforilam proteínas-alvo específicas regulando-as. Dado que se observava a via de sinalização de forma linear, pensava-se que a mesma atuava simplesmente como condutora da informação (Figura 1.2A).

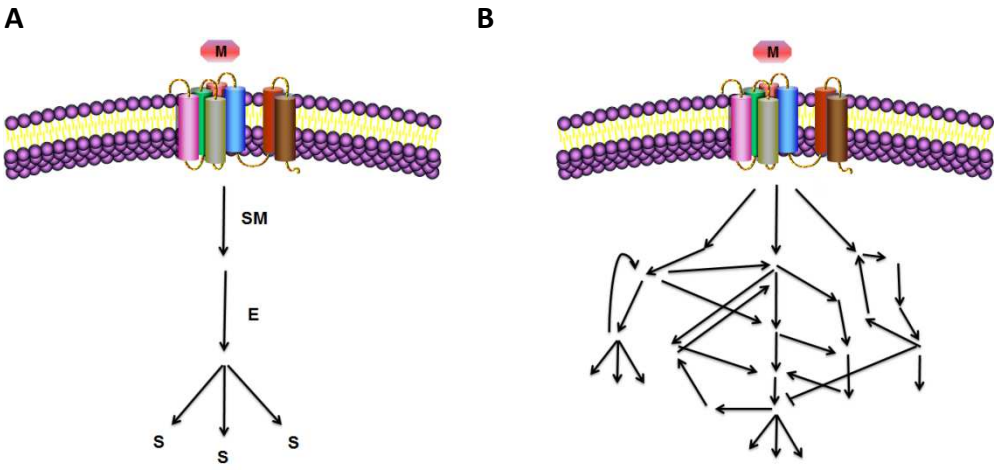


Figura 1.2: Representações das formas de descrição das vias de sinalização. Na letra A observa-se como as vias de sinalização eram descritas no passado, já na letra B observa-se uma representação de como as vias são entendidas atualmente.

Atualmente, sabe-se que as vias de sinalização são extremamente complexas e que suas funções vão muito além da condução de informação. Como representado na Figura 1.2B, as vias de sinalização apresentam vários pontos de retroalimentação, modulação cruzada (*crosstalk*), redundância e integração (von Kriegsheim et al, 2009). Tais características as deixam com funções integradoras e adaptadoras de informações, envolvendo-as na compreensão de informações químicas de mensageiros, sem desconsiderar o contexto celular (Figura 1.2B). Por tais razões, as redes das vias de sinalização são consideradas o cérebro da célula, além, de se assemelharem as conexões da circuitaria neural (Bhalla & Iyengar, 1999). A complexidade das vias de sinalização torna impossível classificar um dado elemento como segundo, terceiro ou quarto mensageiro, visto que em uma via ele pode atuar como segundo mensageiro, mas em outra ele pode atuar como terceiro ou quarto.

1.3.1 Vias de sinalização classicamente acionadas por receptores acoplados a proteínas G (GPCR)

A superfamília de receptores acoplados à proteína G (GPCR) forma o maior e mais complexo grupo de proteínas integrais da membrana plasmática envolvido na transdução de sinal, sendo constituído por mais de mil diferentes tipos de receptores. Estes últimos podem ser ativados por estímulos externos, tais como fatores de crescimento, peptídeos vasoativos, agentes quimiotáxicos, hormônios, entre muitos outros tipos de mensageiros (Blaukat et al, 2000). Todos os GPCRs possuem sete domínios transmembranares hidrofóbicos, conectados entre si por alças de aminoácidos hidrofílicos. A porção N-terminal do receptor localiza-se no lado extracelular da membrana plasmática, enquanto a porção C-terminal localiza-se intracelularmente. O mensageiro extracelular, também chamado de agonista do receptor, liga-se às alças extracelulares do GPCR disparando sinais intracelulares por interações de proteínas específicas na segunda ou terceira alça intracelular, ou ainda, no C-terminal (Bourne, 1997). Entre essas proteínas específicas, destacam-se as proteínas G heterotriméricas que, conforme o próprio nome sugere, contêm três subunidades, α, β e γ (Figura 1.3). A subunidade α está conectada ao GDP no seu estado inativo (Figura 1.3 à esquerda), mas quando o mensageiro liga-se ao GPCR, este último atua como um fator de troca de nucleotídeos de guanina (GEF - *Guanine Nucleotide Exchange Factors*), catalisando a troca de GDP por GTP. Esta troca provoca a dissociação da subunidade α das outras duas subunidades, β e γ (Figura 1.3 à direita). As subunidades das proteínas G no estado dissociado G_{α}-GTP e $G_{\beta\gamma}$, ou seja, no estado ativo, podem então ativar proteínas efetoras, destacando-se a adenilato ciclase (AC), fosfolipase Cβ e PI3K (Hall et al, 1993; Hamm, 1998).

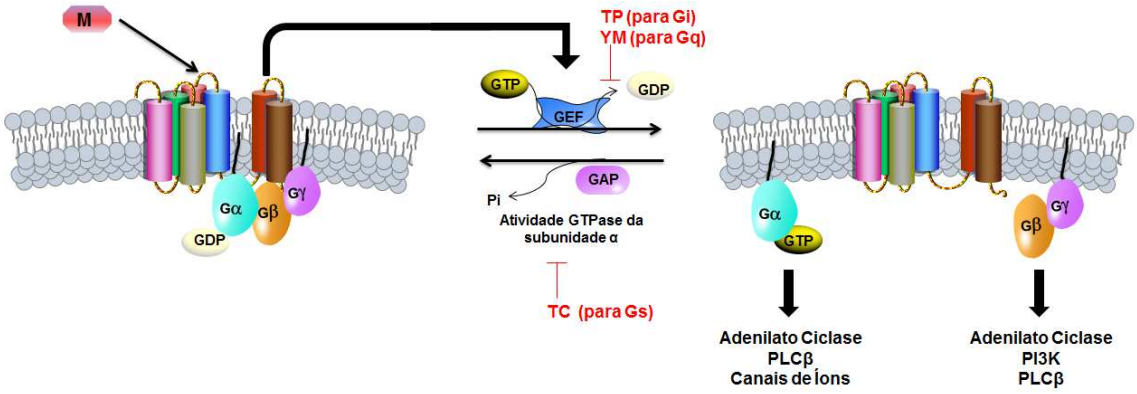


Figura 1.3: Ciclo de ativação e desativação da proteína G. O mensageiro liga-se ao receptor acoplado à proteína G, que passa a atuar como um fator de troca de nucleotídeos de guanina (GEF – *Guanine Nucleotide Exchange Factor*), catalisando a troca de GDP por GTP na subunidade α. Esta troca provoca a dissociação da subunidade α, anteriormente ligada às subunidades βγ, com a posterior ativação de vias a jusante. A desativação da subunidade α das proteínas G dá-se pela sua atividade GTPásica intrínseca, que pode ser catalisada por proteínas ativadoras de GTPase (GAP – *GTPase Activating Protein*).

Atualmente, são conhecidos vinte e três subtipos da subunidade α, seis subtipos da subunidade β e doze subtipos da subunidade γ. As proteínas G são classificadas em quatro principais famílias, G_s, $G_{i/o}$, $G_{q/11,}$ e $G_{12/13}$ de acordo com semelhanças na sequência de aminoácidos, efetores a jusante e sensibilidade a toxinas (Hamm, 1998). A família de proteínas G_s ($G_{\text{stimulatory}}$) classicamente é conhecida por ativar vários tipos da enzima AC, enquanto a subunidade α da proteína G_i ($G_{\text{inhibitory}}$) é conhecida por inativar alguns tipos desta mesma enzima. A família de enzimas AC é composta por nove membros, que convertem, especificamente, ATP em AMPc. Este último foi o primeiro segundo mensageiro a ser descoberto (Sutherland & Rall, 1958) e até os tempos atuais continua sendo intensamente estudado devido a sua ampla modulação nas redes de sinalização intracelular e suas conseguintes influências em processos fisiológicos, tais como proliferação, migração e crescimento de neuritos (Erhardt et al, 1995; Nicol et al, 2005).

Nesse contexto, toxinas bacterianas são ferramentas indispensáveis para investigações envolvendo proteínas G. Sabe-se que a subunidade α das proteínas G possuem atividade GTPásica intrínseca, que catalisa a hidrólise do GTP em GDP, levando,

desta forma, a sua autodesativação. No entanto, a toxina cólera (TC; Figura 1.3), secretada pela bactéria *Vibrio cholerae*, impede essa atividade GTPásica especificamente das subunidades α das proteínas Gs, mantendo-as na forma ativa ($G_{\alpha S}GTP$). Já a família de proteínas $G_{i/o}$ é sensível à toxina pertussis (TP), que bloqueia a troca de GDP por GTP, mantendo-as na forma inativa. O bloqueio da troca GDP por GTP, consequentemente, bloqueia também a dissociação da subunidade α das subunidades βγ, impedindo que estas últimas desempenhem suas funções (Hall et al, 1993; Hamm, 1998).

Anos atrás, acreditava-se que as subunidades βγ desempenhavam apenas uma função de suporte à subunidade α. Entretanto, atualmente, sabe-se que as subunidades βγ, sensíveis à TP, também podem ativar efetores a jusante, como por exemplo, a AC do tipo 2, 4 e 7, PI3K e PLCβ3 (Chen et al, 1995; Singer et al, 1997; Zamponi et al, 1997). As proteínas G_o (G_{other}) são também sensíveis à TP, no entanto, elas não modulam a atividade de ACs e a concentração de AMPc intracelular, mas ativam canais de íons na membrana plasmática, tais como canais de Ca^{2+}, $C1^-$ e K^+ (Liu et al, 1994)

A outra família de proteínas G é a família de proteínas Gq. Estas não modulam diretamente a atividade de AC, mas ativam diretamente a PLCβ. Essa fosfolipase cliva fasfatidil inositois localizados na membrana plasmática originando diacilglicerol (DAG) e inositol 1,4,5 trifosfato (IP3). A PLCβ também atua na retroalimentação negativa da via, funcionando como uma proteína ativadora de GTPase (GAP - *GTPase activating protein*) para a subunidade α da proteína Gq (Singer et al, 1997). O DAG, por sua vez, pode ativar diversas isoformas da proteína quinase C (PKC) (Toker, 1998). Já o IP3 liga-se a seus receptores específicos na membrana do retículo endoplasmático, liberando Ca^{2+} desses compartimentos. O Ca^{2+} é outro elemento historicamente classificado como segundo

mensageiro intracelular e o aumento na sua concentração citoplasmática está relacionado a inúmeros processos fisiológicos, como o crescimento de neuritos mediado por ERK em células PC12 (Rusanescu et al, 1995).

A atividade das proteínas da família Gq também pode ser estudada pela utilização de uma toxina bacteriana recentemente descoberta, a YM-254890 (ou YM), que é isolada de culturas de *Chromobacterium* sp. Similarmente ao mecanismo de ação da TP, a YM bloqueia a troca de GDP por GTP em subunidades α da proteína $G_{q/11}$, impedindo que esta seja ativada (Figura 1.3) (Takasaki et al, 2004).

Entretanto, inúmeros GPCRs acoplam-se simultaneamente a mais de uma família de proteínas G em uma mesma célula e a eficácia desse acoplamento varia entre uma família e outra conforme o tipo e contexto celular (Gudermann et al, 1997; Hanke et al, 2006; Wess, 1997). Esse acoplamento de GPCRs a mais de uma família de proteínas G insere, por conseguinte, mais de uma via de sinalização, corroborando para que o estímulo por um único mensageiro modifique largamente a rede de sinalização intracelular. Nesse contexto, estudos de pontos (enzimas a jusante), que integram as vias acionadas por diferentes tipos de proteínas G, podem fornecer informações de maior relevância para o entendimento de uma dada resposta celular. Assim, destaca-se a rota da MAPK ERK como um ponto de convergência de sinais, entre eles, sinais adicionados por famílias de proteínas G. Dessa forma, esse tema será desconstruído no tópico seguinte.

1.3.2 Cascatas das MAPKs

As rotas das proteínas quinases ativadas por mitógenos (MAPKs) certamente estão entre as principais cascatas responsáveis por decodificar inúmeros sinais externos em

respostas celulares, tais como proliferação e diferenciação. Em mamíferos, observa-se as seguintes rotas de MAPKs: rota da ERK1/2 (*Extracellular signal-regulated kinases*); rota da p38; rota da JNK (*c-Jun N-terminal kinases*); e rota da ERK5. As rotas das diferentes MAPKs ocorrem em paralelo entre si, são relativamente bem caracterizadas e estão presentes, provavelmente, em todos os organismos eucariotos, inclusive os unicelulares (Caffrey et al, 1999). As rotas das MAPKs são constituídas por três enzimas que se ativam em sequência. Uma enzima MAPKKK (MAPK *kinase kinase*) ativa uma outra enzima MAPKK (MAPK *kinase*; também conhecida como MEK - *MAP/ERK kinase*) pela fosforilação em resíduos específicos de serina e treonina. A MAPKK, por sua vez, ativa uma MAPK pela fosforilação em resíduos específicos de treonina e tirosina (MAPKKK → MAPKK/MEK → MAPK). Por fim, a MAPK pode fosforilar centenas de substratos importantes para a resposta celular desejada (Caffrey et al, 1999; von Kriegsheim et al, 2009).

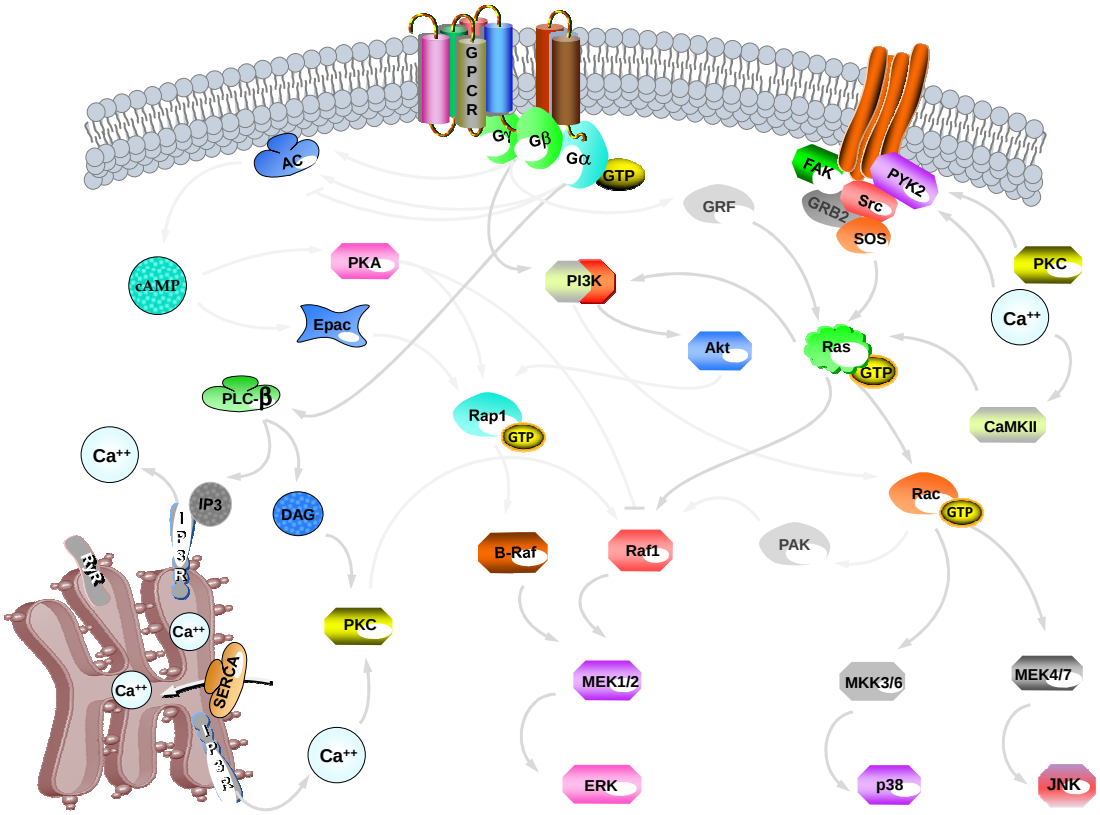


Figura 1.4: Representação esquemática da integração das vias de sinalização acionadas por receptores acoplados as proteínas G nas rotas das MAPKs. Referências, significado das abreviaturas e mais detalhes estão presentes no texto.

Interessantemente, inúmeros mensageiros e diversas vias de sinalização convergem na ativação das cascatas das MAPKs, em especial da ERK. Segundo Murray (1998) e Caffrey (1999), a cascata da ERK parece ser mantida durante a evolução como um ponto integrador, sendo que as formas de ativação e os elementos que a ativam tornaram-se diversificados ao longo do processo, assim como os efetores que a ERK ativada.

Até alguns anos atrás, as rotas das MAPKs eram descrita apenas como acionadas por receptores da família tirosina quinase (RTKs). Resumidamente, a ativação de RTKs resulta, através de proteínas adaptadoras (Src e Grb2), no recrutamento da proteína Sos, que possui atividade GEF de Ras. Ou seja, a proteína Sos catalisa a troca de GDP por GTP de Ras, ativando-a. A Ras-GTP, por sua vez, ativa a rota da MAPK ERK por meio da

ativação de c-Raf1. Esta última é uma enzima MAPKKK que ativa uma MEK1/2. Por conseguinte, MEK1/2 ativa a ERK1/2 (c-Raf1 → MEK1/2 →ERK1/2). Ras também pode resultar na ativação das rotas das MAPKs p38 e JNK ou, até mesmo, da via PI3K/Akt (Revisado por Malumbres e Barbacid (2003) (Figura 1.4).

Atualmente, sabe-se que além dos RTKs, os GPCRs também podem ativar as rotas das MAPKs, observando-se frequentemente a ativação da rota da ERK. Esses receptores podem adicionar essa cascata por intermédio de inúmeras vias, o que depende do tipo e contexto celular, mas, principalmente, da família ou famílias de proteínas G envolvidas, como será descrito a seguir.

Nesse contexto, o aumento nos níveis de AMPc intracelular, resultantes da ativação de ACs pelas proteínas $G_{\alpha s}$ ou $G_{\beta\gamma i}$, pode ativar tanto a proteína quinase ativada por AMPc (PKA) quanto a Epac (*Exchange Protein Directly Activated by cAMP*; uma GEF ativada por AMPc) (Figura 1.4, região superior à esquerda). Essas duas enzimas, por sua vez, induzem a ativação de uma proteína da superfamília das pequenas GTPases relacionadas a Ras, nomeada RAP1 (*Ras-related protein 1*). Em células neurais e endócrinas, a RAP1 ativa a rota da ERK pela ativação de B-Raf, que, assim como c-Raf1, também é uma quinase ativadora de MEK1/2 (B-Raf1 → MEK1/2 → ERK1/2) (Revisado por Impey (1999).

As subunidades βγ da proteína G também podem desencadear outras vias independentes de AMPc que modulam a rota de ERK, incluindo: (1) ativação do GEF de Ras, conhecido como GRF que leva à ativação de ERK (Mattingly & Macara, 1996); (2) ativação de KSR-1 (*protein serine/threonine kinase*-1), que inibe a ativação de Ras e, assim, diminui a ativação de ERK (Bell et al, 1999); e (3) ativação de PI3K, que pode levar à

ativação de ERK por intermédio da Rac(pequena GTPases)/PAK/cRaf1 ou por intermédio de Akt/Rap1/B-Raf (Figura 1.4) (Naor et al, 2000).

Por outro lado, as possibilidades de ativação da rota da ERK por vias acionadas pela família de proteínas Gq são ainda mais amplas. Brevemente, (1) o aumento na concentração de Ca^{2+} livre no citoplasma pode ativar a proteína quinase dependente de cálcio/calmodulina II (CaMKII), que forsforila e inibe a RAS-GAP, deixando Ras ativa e influenciando positivamente na rota da MAPK (Chen et al, 1998); (2) o Ca^{2+} e o DAG podem ativar a GEF Ca^{2+}/DAG que catalisa a ativação de Ras (Farnsworth et al, 1995); (3) a PKC pode fosforilar diretamente a MEKK c-Raf1, levando à ativação da cascata de ERK (Kolch et al, 1993; Marais et al, 1998); (4) o Ca^{2+} e a PKC podem ativar a proteína tirosina quinase PyK2, de grande importância na ativação da rota da ERK em células neurais (Della Rocca et al, 1999) (Figura 1.4 região superior à direita). Nesse último caso, observa-se o envolvimento de integrinas, que consistem em proteínas transmembranares sem atividade tirosina quinase. Dessa forma, as integrinas atuam como proteínas de ancoragem (*scaffold*) para tirosina quinases (não receptoras), tais como a PyK2 e FAK (quinase de adesão focal). Estas últimas, por sua vez, autofosforilam-se e resultam na inserção da rota de ERK por via semelhante à utilizada por RTKs, resultando no recrutamento de Sos, através das proteínas adaptadoras Src e Grb2, e na ativação de Ras (Della Rocca et al, 1999; Pierce et al, 2001). Por outro lado, em alguns tipos celulares, como fibroblastos Rat-1 e células HEK 293, os GPCRs podem acionar a rota de ERK por meio da transativação de RTKs com o receptor de EGF (Della Rocca et al, 1999).

Em suma, inúmeras vias de sinalização, aparentemente redundantes, convergem na ativação da cascata de ERK. Entretanto, estas vias são individualmente importantes,

uma vez que, integrando seus sinais na rota de ERK, elas corroboram para que a mesma seja ativada de forma e topologia específica. A ERK apresenta várias formas e topologias de ativação possíveis, que estão relacionadas com a decodificação das inúmeras informações em respostas celulares (Bouschet et al, 2003; Ebisuya et al, 2005; Santos et al, 2007; Sasagawa et al, 2005).

Nesse contexto, a cinética de ativação de ERK, em conjunto com sua sublocalização celular após ativação, são os pontos chaves da topologia dessa MAPK, que determinam a resposta celular em células PC12.

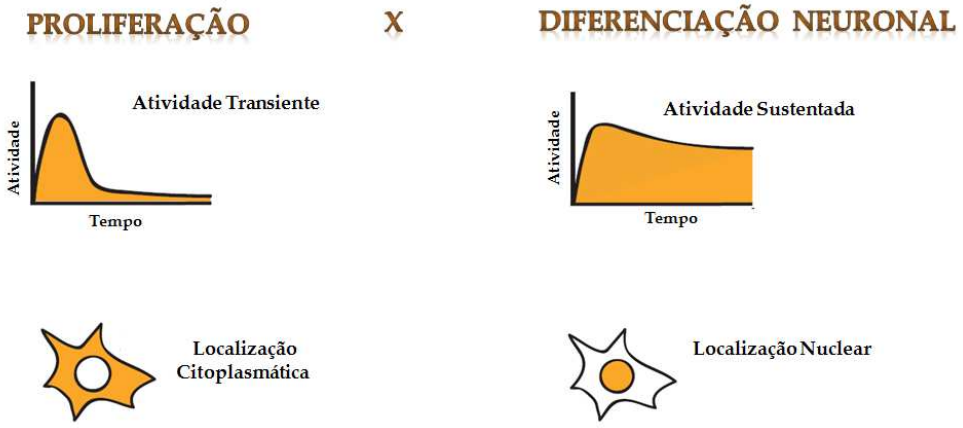


Figura 1.4: Formas de ativação que determinam a especificidade do sinal de ERK. A ativação sustentada e localização nuclear de ERK são formas de ativação dessa MAPK necessárias e em conjunto suficientes para a indução da diferenciação neuronal em células PC12. Modificado de Ebisuya (2005).

A cinética de ativação de ERK está amplamente relacionada com o direcionamento das células PC12 para a proliferação ou para a neurogênese. A ERK induz a proliferação quando é ativada de forma transiente (aproximadamente 5 minutos), como é o caso da ativação por EGF. Por outro lado, ela está envolvida na indução da diferenciação neuronal e supressão da proliferação quando é ativada de forma sustentada (60 min), como é o caso da ativação pelos fatores indutores de neurogênese em PC12, NGF (*Nerve Growth Factor*), PACAP (*Pituitary Adenylate Cyclase-activating Polypeptide*) e BDNF (*Brain-derived*

Neurotrophic Factor) (Figura 1.4) (Bouschet et al, 2003; Ebisuya et al, 2005; Marshall, 1995; Santos et al, 2007; Sasagawa et al, 2005).

A atividade de ERK por período prolongado pode ser desmembrada, como observado através da utilização de inibidores farmacológicos e RNAis, em duas fases: uma rápida e transiente e outra lenta e prolongada (Santos et al, 2007; Sasagawa et al, 2005). A sobreposição destas fases origina o perfil observado na Figura 1.4 (região superior à direita). Estudos iniciais sobre a cinética de ativação de ERK por NGF observaram que a fase inicial e a fase prolongada são mediadas por vias diferentes, mostrando que o receptor de NGF, TrkA, adiciona uma via dependente de Ras, responsável pela indução da fase inicial de ativação de ERK, e outra via dependente de Rap e B-Raf, necessária (mas insuficiente) para indução da segunda fase que mantém a ERK ativa por um período prolongado (Erhardt et al, 1995; Peraldi et al, 1995; York et al, 1998). Além disso, resultados similares foram observados após ligação de PACAP no seu receptor acoplado à proteína G. Esse estímulo induziu a ativação transiente de ERK via Ras e a ativação sustentada foi dependente de Rap1, PKC, PKA e Ca^{2+} e Ras (Bouschet et al, 2003). Nesse contexto, sabe-se que as vias que convergem na ativação de ERK são individualmente necessárias, mas, por outro lado, individualmente insuficientes para atingir a ativação sustentada, que é fundamental para indução da neurogênese em PC12 (Bouschet et al, 2003; von Kriegsheim et al, 2009). É fato, também, que a topologia de ativação de ERK, especialmente no que se refere à segunda fase, não é proveniente apenas de sinais adicionados diretamente pelo receptor, mas provém também de uma rede extremamente complexa, que atua na rota de ERK com diversos mecanismos de

modulações cruzadas com outras vias (*crosstalk*) e de retroalimentações positivas e negativas (Bhalla & Iyengar, 1999; von Kriegsheim et al, 2009).

Outro aspecto da topologia de ativação de ERK que influencia a resposta celular é a sua localização intracelular após ativação. Em células PC12, a ativação da ERK por NGF leva a sua translocação ao núcleo, sendo esta translocação necessária para que a diferenciação ocorra. Em contraposição, a ERK permanece no citoplasma quando ativada por EGF e outros fatores que resultam na proliferação (Impey et al, 1999; von Kriegsheim et al, 2009).

A translocação da ERK para núcleo e, logo, seus efeitos na transcrição gênica, são regulados por outras vias de sinalização (*crosstalk*), como as vias do AMPc/PKA e da PI3K/Akt. Impey e colaboradores (1999) demonstraram que a translocação nuclear de ERK induzida por Ca^{2+} e GF depende da atividade de PKA em células PC12 e neurônios hipocampais. Já a translocação dessa quinase induzida por NGF é regulada por Akt, que fosforila a proteína PEA-15 (von Kriegsheim et al, 2009). Esta última, quando fosforilada por Akt, dissocia-se da MAPK, facilitando sua translocação para o núcleo e, consequentemente, propiciando a diferenciação das células PC12 (von Kriegsheim et al, 2009).

Uma vez compartimentalizada no núcleo, vários fatores de transcrição podem ser regulados pela ERK. Esta última classicamente fosforila a Elk1, que leva à indução da expressão do fator de transcrição c-fos. Além disso, a ERK também ativa as quinases Rsks (*Ribosomal S6 kinases*). Estas são responsáveis pela fosforilação dos fatores de transcrição SRF, c-fos e CREB, em resposta ao aumento de Ca^{2+} intracelular e aos estímulos pelos fatores NGF e BDNF (Ginty et al, 1994; Impey et al, 1999; Xing et al, 1996). Nesse sentido,

Von Kriegsheim (2009) demonstrou, por proteômica quantitativa em células PC12, que a ERK interage com 284 proteínas, mas as interações induzidas especificamente pelo fator neurogênico NGF (60 proteínas) foram, na grande maioria, com proteínas nucleares envolvidas na transcrição e na regulação da expressão de genes.

Adicionalmente, a ativação sustentada e translocação nuclear de ERK foram investigadas no modelo de diferenciação neural de células P19. Estas células são provenientes de carcinoma embrionário murinho e apresentam características de células-tronco pluripotentes. Observou-se que a indução da diferenciação neural clássica pelo fator ácido retinóico induziu a translocação nuclear e a ativação de ERK por tempo prolongado, mais especificamente até a aparência do fenótipo neuronal. Isto sugere a importância da forma/topologia de ativação de ERK em outros modelos de diferenciação, não se restringindo as células PC12 (Reffas & Schlegel, 2000).

1.4 BRADICININA

A bradicinina (BK) é um oligopeptídeo composto por nove aminoácidos (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9), que faz parte do sistema calicreína-cininas. Este sistema inclui as enzimas calicreínas (serino-proteases) tecidual e plasmática, cininogênios de alto e baixo peso molecular (precursores das cininas clivadas pelas calicreínas) e as cininas bradicinina (BK), calidina e seus metabólitos (para revisão ver Moreau (2005). Além disso, o sistema calicreína-cininas é amplamente conhecido e caracterizado pelo seu envolvimento em processos fisiológicos e patológicos, como regulação cardiovascular da pressão sanguínea, inflamação e dor (Leeb-Lundberg et al, 2005; Moreau et al, 2005).

As ações do sistema calicreína-cininas são mediadas por apenas dois receptores, B2 e B1 de cininas (Moreau et al, 2005). A BK e a calidina ligam-se com alta afinidade no receptor B2 de cininas. Este, por sua vez, é um GPCR expresso constitutivamente em uma grande variedade de tecidos, inclusive no sistema nervoso em desenvolvimento, conforme observado em embriões de camundongos por meio da técnica de hibridização *in situ* (Trujillo et al, 2012). As cininas BK e calidina podem ser degradadas pelas enzimas carboxipeptidase M e N, que removem a Arg da extremidade carboxi-terminal, originando a des-Arg^{9}-BK e a Lys-des-Arg^{9}-BK. Estes dois metabólitos podem ligar-se ao segundo receptor do sistema, o receptor B1 de cinina, que também é um GPCR. No entanto, sua expressão é induzida em determinadas situações fisiopatológicas, como por exemplo, em processos inflamatórios (Leeb-Lundberg et al, 2005; Moreau et al, 2005).

A disponibilidade das cininas, para desencadear suas ações, depende das taxas de produção e degradação das mesmas. Na circulação, sabe-se que seus efeitos são detectados em até trinta segundos. Nesse contexto, além da carboxipeptidase M, a BK também pode ser clivada pela enzima conversora de angiotensina I (ECA ou cininase II), que resulta na formação de BK 1-5 e 1-7. Estes últimos peptídeos não ativam o receptor B2 de cininas. Outras enzimas, tais como a endopeptidase neutra (NEP) e a carboxipeptidase N (cininase I) também clivam a BK, mas a importância específica de cada enzima na degradação das cininas depende do tecido investigado (Campbell, 2001; Damas et al, 1992; Madeddu et al, 2007).

Mesmo sendo frequentemente descrito como um prototípico receptor acoplado à proteína G_q, o receptor B2 de cininas também pode catalisar a troca de GDP por GTP nas subunidades α de G_i e G_s (Blaukat et al, 2000; Hall et al, 1993; Liebmann et al, 1996;

Liebmann et al, 1990). Dessa forma, já se conclui que a sinalização intracelular acionada pelo receptor B2 de cininas pode ser bastante complexa e variar grandemente de acordo com o tipo e contexto celular, visto que, como já mencionado anteriormente, a eficácia de acoplamento de determinadas famílias de proteínas G varia com esses aspectos.

Um bom exemplo da mudança de eficácia de acoplamento de proteínas G no receptor B2 de cininas conforme o contexto celular foi demonstrado por Hanke e colaboradores (2006). Eles observaram, através do carregamento com *[α-^{32}P] GTP azidoanilide,* que em células COS-7 na ausência de estímulos mitogênicos, o receptor B2 acopla-se às proteínas G_q e G_i, aumentando a produção de IP3 e AMPc, respectivamente. Entretanto, a exposição das células ao EGF muda a sinalização do receptor B2. Detalhadamente, a ativação do receptor de EGF resulta na ativação da proteína Src, que fosforila o receptor B2 de cininas nos resíduos de tirosina 177 e 347. A fosforilação destes resíduos reduz drasticamente o acoplamento do receptor B2 às proteínas da família G_i, logo, altera a sinalização acionada por esse. Em paralelo, Duchene e colaboradores (2002) demonstraram que, em células mesangiais, a fosfatase SHP2 pode interagir diretamente com o receptor B2 de cininas ligando-se no domínio ITIM deste último. Entretanto, essa interação ocorre apenas sob condições mitogênicas (soro fetal bovino; SFB), produzindo o efeito antiproliferativo dessa cinina. Destaca-se que a SHP2 é conhecida por seu papel crítico na ativação de ERK após ativação de GPCRs, bem como após ativação de receptores de GF, por exemplo, o NGF (Myers et al, 1998; Stofega et al, 2000; Wu et al, 2001).

Assim, dado que o receptor B2 de cininas acopla-se a família de proteínas Gq, inúmeros estudos demonstram que a exposição das células à BK induz um aumento na

concentração de cálcio intracelular livre ($[Ca^{2+}]_i$) (Leeb-Lundberg et al, 2005; Trujillo et al, 2012; Zubakova et al, 2008). Em células PC12, por exemplo, o estímulo com BK induz o aumento rápido e transiente na $[Ca^{2+}]_i$. Este aumento é resultante da mobilização dos estoques intracelulares desse íon e do influxo a partir do meio extracelular, que ocorre por meio da ativação e abertura de canais na membrana plasmática (Appell & Barefoot, 1989). Estes últimos não são canais de Ca^{2+} sensíveis à voltagem em células PC12 (Appell & Barefoot, 1989), mas o são em neurônios da submucosal intestinal (Avemary & Diener, 2010). Adicionalmente, observa-se também o envolvimento de canais TRP (*Transient Receptor Potential)* permeáveis a cátions no influxo de Ca^{2+} induzido por BK. Entretanto, o subtipo específico de canal TRP envolvido varia entre os tipos celulares estudados (Callera et al, 2009; Liu et al, 2006; Suzuki et al, 2011). A jusante ao Ca^{2+}, a via de sinalização acionada pela BK inclui a quinase PyK2. Esta quinase é um ponto chave de ligação entre as vias acionadas pelo receptor B2 de cininas e a rota da MAPK ERK em células PC12 (Dikic et al, 1996).

A Bk induz vias a jusante da proteína G_q em, possivelmente, todos os tipos celulares que o receptor B2 é expresso (Hall et al, 1993). Entretanto, em um grande número de células a interação desse receptor ocorre também com proteínas da família G_s ou $G_{i/0}$ e estas últimas, conectadas ao GTP, podem introduzir diversas vias (Blaukat et al, 2000; Hall et al, 1993; Liebmann et al, 1996; Liebmann et al, 1990). Nesse contexto, observa-se que o estímulo com BK frequentemente resulta na síntese de AMPc intracelular. No entanto, as vias precisas que ativam as ACs variam muito entre os tipos celulares. Dessa forma, pode-se citar como exemplos os casos a seguir. Em células de músculo liso pulmonar, observa-se que a BK resulta na ativação da ACII e aumento da

$[AMPc]_i$ via proteína G_s e PKC (Moughal et al, 1995; Pyne et al, 1994). Por outro lado, em células PC12, a síntese desse clássico segundo mensageiro ocorre por intermédio do complexo Ca^{2+}/calmodulina, que é capaz de ativar as ACs I, III e VIII (Graness et al, 1997). Já em neurônios do corno dorsal da medula espinhal, observa-se a elevação na $[AMPc]_i$ induzida por BK, por intermédio da síntese e estímulo autócrino da prostaglandina E2 (Kohno et al, 2008). Por fim, em outros tipos celulares observa-se o aumento na síntese de AMPc intracelular via subunidades βγ da proteína G_i, que são capazes de ativar as ACs II, IV e VII (Hall et al, 1993; Hanke et al, 2006). Dessa forma, mesmo que o estímulo com BK possa elevar a síntese de AMPc por diversas vias, destaca-se que esse mensageiro intracelular está entre as alvos frequentemente ativados por essa cinina. Isto sugere que o AMPc tem uma importância única na rede de sinalização intracelular acionada por BK.

Ressalta-se que os efeitos das elevações nos níveis de AMPc na rota de ERK são altamente específicos para o tipo celular (Stork & Schmitt, 2002). Em muitos tipos celulares esse clássico segundo mensageiro inibem a rota de ERK via inibição da interação Ras/c-Raf1. Entretanto, em células endócrinas, COS-7 e neurais, como a PC12, níveis elevados de AMPc ativam a rota de ERK via ativação de B-Raf (Crespo et al, 1995; Schmitt & Stork, 2002). Isto ocorre, principalmente, porque esses últimos tipos celulares expressam altos níveis dessa última MAPKKK. Em neurônios do corno dorsal da medula espinhal, por exemplo, a administração intratecal de BK resulta na co-ativação de PKA e PKC, que convergem na ativação de ERK (Kohno et al, 2008).

Nesse contexto, infelizmente a vasta maioria dos estudos que descrevem a ativação da MAPK ERK por BK o fazem com descrições lineares de uma única via sem considerar a rede de sinalização (*networks*). Por exemplo, tem-se demonstrado que a BK

ativa a rota de ERK por intermédio de PKC ou de vias dependentes de Ca^{2+}, envolvendo a quinase PyK2 ou através da transativação do receptor de EGF ou ainda por a via AMPc/PKA/Epac (Dikic et al, 1996; Roscioni et al, 2009). Entretanto, como descrito anteriormente, a ERK integra os sinais de diversas vias e decodifica essas informações em respostas celulares. Dessa forma, estudos pioneiros têm demonstrado que, conforme esperado, a ERK ativada por BK também possui tais características de integrar as informações provenientes de duas ou mais vias, conforme exemplificado a seguir (Adomeit et al, 1999; Blaukat et al, 2000; Velarde et al, 1999).

O estímulo com BK em células HEK293T e HF-15 resulta na ativação de ERK através das proteínas G_q e $G_{i,}$ ativadas pelo receptor B2 de cininas. Estas proteínas integram seus sinais na rota de ERK por intermédio da PKC e Ras, respectivamente, mas ativam essa MAPK de forma independente de PI3K, subunidades βγ e tirosinas quinases, como a Pyk2 (Blaukat et al, 2000). Detalhadamente, o tratamento prévio com a toxina pertussis (TP) influencia fracamente na produção de IP3, após estímulo com BK, indicando apenas uma contribuição fraca de Gi na ativação de PLCβ. Essa influência fraca não é proporcional à grande diminuição de p-ERK e bloqueio de Elk1 observados em células previamente tratadas com TP e expostas à BK. O pré-tratamento com TP resulta na forte inibição da atividade de Ras induzida por BK, a qual se mantém praticamente inalterada com o inibidor de PKC. Entretanto, o tratamento isolado com esse inibidor, similarmente ao observado com TP, resulta em grande diminuição da ativação de ERK por BK. Porém, resulta também no bloqueio total da atividade transcricional de Elk1 induzida por BK. Diante disso, esses dados demonstram que as duas vias são necessárias para a ativação eficiente de ERK. Em outras palavras, essa quinase ativa o fator de transcrição Elk1,

apenas com a integração das duas vias na sua rota (Blaukat et al, 2000). Outro estudo demonstrou, através da expressão exógena do receptor B2 humano em células COS-7, que a ativação eficiente de ERK por BK dá-se pela ativação simultânea e independente de PKC (α e ε) e do receptor de EGF (transativação pela fosforilação de suas tirosinas), demonstrando, novamente, a integração de duas vias acionadas pelo receptor B2 na rota dessa MAPK. A inibição de PI3Kγ não influenciou na ativação de ERK por BK (Adomeit et al, 1999). Entretanto, esses dois estudos não avaliaram a cinética e sublocalização da ERK após integração das vias acionadas pelo receptor B2 de cininas.

Nesse sentido, alguns estudos observam que a BK induz a ativação de ERK de forma rápida e transitória, enquanto outros observam que essa ativação perdura por longo período (Dixon et al, 2002; El-Dahr et al, 1998; Jaffa et al, 1997; Mukhin et al, 2003). Esses resultados distintos, provavelmente, se dão em decorrência dos diferentes contextos e modelos celulares empregados. Destaca-se que estudos que avaliam a cinética de ativação de ERK após estímulo com BK em células mesangiais revelam que sua ativação é mantida por período prolongado e, inclusive, após duas horas da exposição à cinina, observa-se que a MAPK translocou para o núcleo (El-Dahr et al, 1998; Jaffa et al, 1997).

1.4.1 Bradicinina na Diferenciação Neural

Todos os componentes do sistema calicreína-cinina estão presentes no SNC de adultos (Damas et al, 1992; Fujieda et al, 1993) e muitos deles também foram encontrados no cérebro em desenvolvimento de camundongos e ratos (Iwadate et al, 2002);(dados do nosso laboratório ainda não publicados). A BK, conhecidamente, atua estimulando a liberação de neurotransmissores, como a noradrenalina e neuropeptídeo Y

em neurônios simpáticos, células PC12 e cromafins (Appell & Barefoot, 1989; Kansui et al, 2002; Kurz et al, 1997). Níveis extremamente elevados dessa cinina são observados no SNC em condições de dano, como isquemias, acidentes vasculares encefálicos (AVE), traumas e na doença de Alzheimer (Groger et al, 2005; Myagkova et al, 2003).

Nesse sentido, Xia e colaboradores (2004; 2006) observaram a influência da BK em processos pós-isquêmicos na região danificada do cérebro de ratos adultos. Para tal, após 8 horas da oclusão arterial cerebral, adenovírus contendo o gene da calicreína foram administrados por via intravenosa. Observou-se que a inserção de genes da calicreína em ratos recém-isquêmicos promoveu neuroproteção, migração de células gliais ($GFAP^+$), neurogênese na área lesionada e, consequentemente, diminuição dos *scores* de déficits neurológicos. Destaca-se que esses efeitos podem ser atribuídos ao aumento da disponibilidade de cininas (ligam-se no receptor B2), visto que eles foram revertidos pelo tratamento com um antagonista do receptor B2, denominado HOE-140 (princípio ativo do medicamento utilizado nesse estudo, o Icatibant). Na região lesionada, observou-se um aumento no número de células expressando GFAP e as mesmas não expressavam marcadores de proliferação, sugerindo que estas células migraram de outras regiões do cérebro. Além disso, verificou-se também que a BK estimulou a migração de células gliais *in vitro* (cultura primária) de forma dependente da dose. Já a neurogênese foi observada através da administração via intraperitoneal, seguida de imuno-detecção do análogo de timidina BrdU (marcador de proliferação) em conjunto com a imuno-detecção de NeuN, para identificar neurônios. Relembra-se que neurônios recém-originados (provenientes da neurogênese), primeiramente, sofreram proliferação ($BrdU^+$) e, em seguida, tornaram-se neurônios ($NeuN^+$), portanto, eles podem ser identificados pela dupla-marcação $BrdU^+$

NeuN$^+$. Nos estudos citados, a inserção de genes da calicreína aumentou a população de células duplamente marcadas para NeuN e BrdU, na região danificada, em comparação com a mesma região de animais controles, demonstrando que tal intervenção aumentou a neurogênese (Xia et al, 2004; Xia et al, 2006). Nesse sentido, outro trabalho também aponta para os efeitos da BK no favorecimento da neurogênese em animais que sofreram lesão da via nigro-estriatal por 6-hidroxidopamina (modelo animal da doença de Parkinson) (estudo ainda não publicado do lab. Neurosciências IQ-USP). Atribuiu-se a melhora funcional, detectada pelo teste rotacional, e a melhora histológica, detectada pelo aumento da porcentagem de fibras tirosina hidroxilase$^+$ (marcador de neurônio dopaminérgico) à neuroregeneração/neurogênese de CPNs endógenas induzida por BK. Tais melhoras não foram observadas em decorrência do efeito neuroprotetor da BK, visto que a mesma foi adicionada após o estabelecimento completo da lesão. Infelizmente, não há outros estudos nesse sentido. Portanto, as influências da BK na migração e neurogênese de animais adultos podem abranger outras condições fisiopatológicas ainda não investigadas, tornando ainda mais relevante o entendimento de seus mecanismos.

Em paralelo, alguns estudos têm apontado para o envolvimento da BK na diferenciação neural, já em células obtidas de estágios embrionários do desenvolvimento. Inicialmente, Martins e colaboradores (2005) observaram que essa cinina é importante na formação de corpos embrióides de células P19 provenientes de carcinoma embrionário murino, que apresentam características de células-tronco pluripotentes. A BK também apresentou um papel importante na determinação do fenótipo colinérgico de neurônios recém-originados a partir das células P19 e neuroesferas de ratos (Martins et al, 2005; Trujillo et al, 2012). Observou-se que a utilização do antagonista do receptor B2, HOE-

140, durante a diferenciação, resultou em menor expressão e atividade dos receptores muscarínicos de acetilcolina M1, M2 e M3. Posteriormente, as expressões e atividades de todos os componentes do sistema calicreína-cininas foram caracterizadas em neuroesferas, durante a diferenciação. Com isto, foi observado que a BK é secretada para o meio extracelular e que a expressão do receptor B2 mantém-se estável ao longo da diferenciação, ao passo que o receptor B1 não é expresso durante esse processo (Martins et al, 2008; Trujillo et al, 2012).

Um trabalho recente do nosso laboratório evidenciou que a BK participa na migração e determinação do fenótipo neural, tanto na glio- quanto na neurogênese, em diversos modelos de diferenciação (Trujillo et al, 2012). A BK apresentou um papel na determinação do destino neural, favorecendo a neurogênese em detrimento da gliogênese, tanto em neuroesferas de rato e camundongo quanto em células P19 murina e iPS (Induced Pluripotent Stem Cell) humana. O tratamento crônico durante a diferenciação com BK aumentou a porcentagem de células que expressam β3-Tubulina (marcador neuronal), ao passo que diminuiu a população de células que expressam GFAP e S100β (marcadores gliais) no final da diferenciação neural dos modelos acima citados. Além disso, o envolvimento do receptor B2 de cininas foi evidenciado, visto que o tratamento com HOE-140, durante a diferenciação, reduziu a expressão de receptores de neurotransmissores e marcadores neuronais, ao mesmo tempo em que aumentou a população de células gliais. No modelo de diferenciação de neuroesferas, também foi possível observar que a BK potencializou a migração durante a diferenciação neural. No sétimo dia desse processo, verificou-se que o tratamento com essa cinina resultou em um número maior de células que atingem distâncias mais longas, partindo da borda da

neuroesfera, em comparação com as amostras controle. Por outro lado, em neuroesferas obtidas de camundongos *knockout*, para o receptor B2, observou-se uma diminuição das células que migram emergindo da borda da neuroesfera. Adicionalmente, o tratamento com BK, durante a diferenciação de neuroesferas de rato, também originou uma taxa menor de proliferação no sétimo dia de diferenciação, conforme análise pela incorporação de BrdU (Trujillo et al, 2012).

Em suma, essa seção expôs um novo papel da bradicinina (BK) na migração, proliferação e neurogênese que, *in vivo*, são processos responsáveis pela formação do sistema nervoso na fase fetal e manutenção de sua integridade fisiológica na fase adulta. Frente a tais descobertas recentes, faz-se necessário avançar na compreensão dos mecanismos celulares e moleculares, bem como na identificação das vias de sinalização e suas estratégias para decodificar os sinais introduzidos pela BK, que resultam em seus efeitos na migração, proliferação e neurogênese. Nesse sentido, a seção seguinte apresenta os objetivos do presente trabalho.

Objetivos Propostos 2

2.1 Objetivo Geral

Investigar os efeitos da bradicinina na proliferação/auto-renovação de células precursoras neurais (CPNs) indiferenciadas e durante a diferenciação, bem como, buscar uma explicação mecanística-molecular para os efeitos dessa cinina em três processos do desenvolvimento neural estudados *in vitro*: proliferação, migração e diferenciação.

2.2 Objetivos Específicos

1. Caracterizar os efeitos da bradicinina na migração e no destino neural de CPNs obtidas a partir do telencéfalo de embriões de camundongos.
2. Investigar os possíveis efeitos da bradicinina na auto-renovação/proliferação de CPNs indiferenciadas e durante a diferenciação neural, incluindo a investigação das fases do ciclo celular de importância para a manutenção da multipotência e diferenciação.
3. Identificar as vias de transdução de sinais ativadas pela bradicinina em CPNs indiferenciadas, bem como possíveis mecanismos de indução da diferenciação neuronal.
4. Determinar as vias de sinalização da bradicinina envolvidas nos seus efeitos na migração, destino neural e proliferação de CPNs.
5. Avaliar as vias de sinalização e os efeitos da bradicinina na diferenciação de células PC12 e os seus mecanismos de indução do destino neural envolvendo a MAPK ERK.

Materiais e Métodos 3

3.1 Aspectos Éticos

Este trabalho está em conformidade com a Legislação do Comitê de Ética IQ-USP (Certificado número 10/2013 anexado no final desta tese).

3.1.1 Cálculo do tamanho da amostra.

O tamanho da amostra foi calculado com base em estudos prévios conduzidos no laboratório utilizando o mesmo modelo celular (Schwindt et al, 2011). Para um nível de significância de $P<0,05$ o n calculado foi igual a duas fêmeas prenhas a cada mês e um macho, totalizando 24 fêmeas e 12 machos por ano. Tendo ocorrido o acasalamento comprovado pelo plug vaginal, todos os embriões com 13 dias de gestação foram utilizados para isolamento do telencéfalo e posterior obtenção de neuroesferas.

3.1.2 Quanto ao uso de animais.

Foram utilizados para os acasalamentos camundongos machos e fêmeas da linhagem C57BL/6 provenientes do Biotério do Conjunto das Químicas - IQUSP. As fêmeas prenhas no décimo terceiro dia de gestação foram eutanasiadas por overdose de quetamina (200mg/kg do animal) e xilazida (20mg/Kg do animal). A eutanásia foi confirmada pela exposição em câmara de CO_2. Os embriões retirados foram diretamente decapitados e as cabeças foram mantidas em placa de *Petri* contendo meio DMEM (Dulbecco's Modified Eagle Medium, Life Technologies) gelado para posterior isolamento do telencéfalo e formação da cultura primária de CPN. Os animais utilizados neste trabalho foram mantidos no Biotério do Conjunto das Químicas, local que segue todas as normas de segurança exigidas, para tanto, a dieta e a água foram mantidas *ad libitum* a 25°C.

3.1.3 Descarte de materiais

Materiais, carcaças e/ou resíduos que apresentarem qualquer risco biológico, foram descartados em recipientes específicos e devidamente identificados para dejetos com tal classificação e posteriormente encaminhados à incineração.

3.2 OBTENÇÃO E CULTIVO DE CÉLULAS PRECURSORAS NEURAIS

Utilizamos células precursoras neurais, que proliferam em agregados chamados neuroesferas, como modelo da diferenciação neural, extraídas de embriões com 13 dias de gestação (E13) de camundongos C57BL/6 selvagens e *knockout* para o receptor B2 de cininas B2BkR$^{-/-}$ (Bdkrb2^{tm1Jfh}) (The Jackson Laboratory, EUA) (Trujillo et al, 2012). Os animais foram eutanasiados e os telencéfalos dos embriões foram isolados com o auxílio de uma lupa de dissecção e materiais cirúrgicos em condições assépticas. Posteriormente, o tecido foi incubado com tripsina (Invitrogen) por 5 min a 37ºC. Em seguida a digestão enzimática foi interrompida com a adição de soro fetal bovino (SFB - Cultilab, Campinas, Brasil) e as células foram dissociadas mecanicamente para obtenção de células individualizadas. Seguida a filtração por poros de 40 µm, uma alíquota da suspensão celular foi diluída em Trypan Blue (Life Technology, Carlsbad, EUA) para avaliação da viabilidade em câmara de Neubauer, então as células foram cultivadas em suspensão na densidade de 2 x 10^5 células/mL de meio DMEM-F12 (Life Technology), suplementado com 2% B-27 (Life Technology), 20 ng/mL EGF (Sigma-Aldrich), 20 ng/mL FGF2 (Sigma-Aldrich), 100 U/mL de penicilina e 100 µg/mL de estreptomicina (Sigma-Aldrich). No 5° dia de cultura sob condições ideais (37ºC, 5% de CO_2 e 95% de umidade) foi possível observar a formação de neuroesferas (Schwindt et al, 2011).

3.2.1 Diferenciação das neuroesferas

Primeiramente as neuroesferas foram lavadas duas vezes com DMEM para completa remoção dos fatores de crescimento EGF e FGF2 que as mantém proliferando e indiferenciadas. Posteriormente as células foram semeadas em lamínulas, placas ou garrafas tratadas com poli-*L*-lisina (1 mg/mL, Sigma-Aldrich) e laminina (20 µg/mL, Sigma-Aldrich) que forma uma matriz aderente. Para a diferenciação celular foi utilizado o meio de cultura DMEM-F12 (Life Technology), suplementado com 2 % de B-27 e antibióticos na ausência dos fatores de crescimento EGF e FGF2. Após início da diferenciação, o meio de cultura foi trocado a cada dois dias em um total de sete dias de diferenciação. Os inibidores das vias de sinalização foram acrescentados com o meio de cultura, já a BK e HOE-140 foram adicionados todos os dias. Em condições de co-tratamento com inibidores de vias de sinalização na presença de BK, os primeiros sempre eram acrescentados ao meio de cultura 1 hora antes da cinina.

3.3 CULTIVO E DIFERENCIAÇÃO DE CÉLULAS PC12

As células de feocromocitoma PC12 ATCC No. CRL-1721 (Tischler & Greene, 1975) foram mantidas em meio DMEM-*high glucose* (Invitrogen, Carlsbad, CA, USA) suplementado com 10% SFB e 5% de soro de cavalo (SC) (Cultilab, Campinas, Brasil) na presença de 100 IU/mL de penicilina e 100 µg/mL estreptomicina a 37°C em uma atmosfera com 95% de umidade e 5% de CO_2 conforme descrito por Nery e colaboradores (2008). Durante o crescimento celular o meio de cultura foi renovado a cada dois dias até que as células atingissem a confluência de 70% (aproximadamente após quatro dias) necessária para realização dos experimentos ou sub cultivo. Para esse último, o meio de

cultura foi substituído por meio novo e as células foram diretamente dissociadas por ação mecânica, então, as células dissociadas contidas em uma garrafa de cultivo foram transferidas para três garrafas novas.

Para a diferenciação, as garrafas ou placas de cultura foram tratadas com poli-*L*-lisina (1mg/mL) e laminina (10µg/mL) para formar uma matriz aderente. Então as células foram diferenciadas na presença dos fatores de crescimento ou BK em meio DMEM suplementado com 2,5% de SC por dois dias.

3.4 IMUNOCITOQUÍMICA

As células foram fixadas com 4 % paraformaldeído (PFA) em PBS, lavadas em PBS e, posteriormente, incubadas em PBS com 0,1% de Triton X-100 para permeabilizar as membranas e 3 % SFB por 20 min para o bloqueio de sítios inespecíficos. As células foram incubadas com os anticorpos primários (Tabela 1) em 0,1% de Triton X-100, 3% SFB, PBS (*Phosphate Buffered Saline*) por duas horas em temperatura ambiente e então foram removidos por meio de duas lavagens com PBS. As amostras foram incubadas com anticorpos secundários conjugados a fluoróforos (Alexa Fluor 488 e 555 – Tabela 3.1) por 1 hora, seguida de duas lavagens com PBS e incubação durante cinco minutos com DAPI (*4',6-diamidino-2-phenylindole*) (1:10.000 em PBS) para a visualização dos núcleos celulares. As lamínulas foram novamente lavadas e montadas em lâminas utilizando-se o meio de montagem DPX (*1,3-diethyl-8-phenylxanthine*) (Sigma-Aldrich). Os controles negativos foram feitos com a adição dos anticorpos controle de Isotipo dos primários e os secundários conjugados correspondentes. As imagens foram capturadas com a câmera digital Nikon DXM1200F acoplada ao microscópio Axiovert 200 (Zeiss) utilizando-se o

programa NIS-Elements. As imagens foram posteriormente analisadas e sobrepostas através do software ImageJ (National Institutes of Health). Os experimentos foram realizados em triplicatas experimentais e biológicas.

Tabela 3.1– Anticorpos primários utilizados em ensaios de imunocitoquimica (IC), *western blot* (WB) e citometria de fluxo (CF).

Antígeno	Produzido em	Diluição	Empresa
GFAP	Coelho	IC – 1:500 CF – 1:1000	DAKO Systems
β3-tubulina	camundongo	IC – 1:500 CF – 1:1000	Sigma-Aldrich
Nestina	camundongo	IC – 1:500 CF – 1:1000	Chemicon (Millipore)
MAP2	coelho	CF – 1:500	Cell Signaling Technology
BrdU	rato	CF – 1:200	Axyll
β-actina	camundongo	WB -1:1000	Sigma-Aldrich
ERK1/2	camundongo	WB -1:1000	Santa Cruz Biotechnology
p-ERK1/2 (Thr202/Tyr204)	coelho	IC – 1:200 CF – 1:400 WB -1:1000	Cell Signaling Technology
Controle de Isotipo IgG	coelho	IC – 1:200 a 1:1000 CF 1:200 a 1:1000	Cell Signaling Technology
Controle de Isotipo IgG	camundongo	IC – 1:200 a 1:1000 CF 1:200 a 1:1000	Cell Signaling Technology
p-Akt (Thr308)	coelho	IC – 1:100 CF – 1:200	Cell Signaling Technology
p-STAT3 (Tyr705)	coelho	IC – 1:100 CF – 1:200	Cell Signaling Technology
p-JKN (Thr183/Tyr185)	camundongo	IC – 1:200 CF – 1:400	Cell Signaling Technology
Ki67	coelho	CF – 1:500	Chemicon (Millipore)
p-p38 (Thr180/Tyr182)	camundongo	IC – 1:800 CF – 1:400	Cell Signaling Technology
Histona H1	cabra	WB – 1:500	Santa Cruz Biotechnology

Tabela 3.2 – Anticorpos secundário utilizados em ensaios de imunocitoquimica (IC), *Western blot* (WB) e citometria de fluxo (CF).

Antígeno	Fluoróforo	Produzido em	Diluição	Empresa
IgG de rato	**Alexa Fluor 488**	cabra	CF – 1:500	Life Techlonogy
IgG de coelho	**Alexa Fluor 488**	cabra	IC – 1:500 CF – 1:1000	Life Techlonogy
IgG de camundongo	**Alexa Fluor 555**	cabra	IC – 1:500 CF – 1:1000	Life Techlonogy
IgG de coelho	**Alexa Fluor 647**	cabra	WB – 1:1000	Life Techlonogy
IgG de camundongo	**Alexa Fluor 488**	cabra	WB – 1:1000	Life Techlonogy
IgG de cabra	**Alexa Fluor 488**	mouse	WB – 1:1000	Life Techlonogy

3.5 Determinação da produção de AMPc intracelular

As neuroesferas foram cultivadas como descrito anteriormente e 10.000 células foram transferidas para cada poço de placas de 96 poços em meio DMEM/F12 suplementado com 0,5% de B27. As medidas de AMPc intracelular foi realizada utilizando um sistema de imunoensaio, Biotrak (Amersham Biosciences, UK). Após 24h do plaqueamento, as células foram estimuladas com moduladores específicos em tempos e concentrações adequadas. Para a obtenção dos lisados celulares, o meio foi removido e adicionado 200 µL de reagente de lise 1B® (Amersham Biosciences, UK) por poço. Com um pipetador multicanal, as células foram ressuspendidas no reagente e em seguida a placa foi colocada em agitador por 10 min. Os lisados celulares foram imediatamente utilizados para a dosagem de AMPc descrita a seguir.

O *cAMP Biotrakenzymeimmunoassay (EIA) system* (AmershamBiosciences, UK) tem como princípio avaliar, por um determinado número de sítios de ligação dos anticorpos anti-AMPc, a competição entre o AMPc celular e um número fixo de AMPc conjugado com peroxidase. Portanto, a quantidade de AMPc-peroxidase ligada ao

anticorpo é complementar a quantidade de AMPc celular. O complexo anticorpo/AMPc celular e posteriormente o complexo anticorpo/AMPc-peroxidase se ligam aos anticorpos secundários imobilizados nos poço. O AMPc-peroxidase excedente que não se ligou ao secundário, é eliminado nas lavagens. A quantidade de cAMP-peroxidase ligada ao anticorpo primário e secundário é determinada pela adição do substrato da peroxidase, tetrametilbenzidina (TMB), que resulta em um produto colorido após adição de 1M de ácido clorídrico que é quantificável em espectrofotômetro a 450 nm.

O experimento completo consiste nos seguintes paços: equilibrar os reagentes à temperatura ambiente; preparar as soluções de AMPc padrão por diluição seriada nas concentrações de 12,5; 25; 50; 100; 200; 400; 800; 1600; 3200 fmol/poço em reagente de lise 1B®; adicionar 200 µL de reagente de lise 1B® nos poços de ligação não-específica (NSB) e 100 µL nos poços do branco, sempre em duplicata; pipetar 100µL dos padrões e das amostras nos poços correspondentes; pipetar 100µL de antissoro anti-cAMP em todos os poços, exceto no branco e NSB; agitar gentilmente a placa e incubar a 4°C por 2 horas protegida da luz; cuidadosamente adicionar 50µL de AMPc-peroxidase em todos os poços, com exceção dos brancos; agitar gentilmente a placa e incuba-la a 4°C por 1 hora protegido da luz; desprezar o sobrenadante de todos os poços e lava-los 4 vezes com tampão de lavagem fornecido pelo *kit*; adicionar 150µL de TMB (substrato da peroxidase) em todos o poços e colocar a placa em agitador à baixa rotação por 30 minutos à temperatura ambiente; interromper a reação da peroxidase adicionando 100 µL de ácido sulfúrico 1 M em cada poço; fazer a leitura em espectrofotômetro para microplaca (leitor de ELISA) no comprimento de onde de 450nm em no máximo 30 minutos.

Para quantificação da concentração de AMPc intracelular, os valores de densidade óptica (DO) das amostras foram comparados aos valores de DO obtidos dos padrões de AMPc de concentrações conhecidas, usando a seguinte fórmula:

(1) $$\% B/Bo = \frac{(DO\ padrão\ ou\ amostra - DO\ NSB) \times 100}{(DO\ Branco - DO\ NSB)}$$

Onde % B/Bo = porcentagem de AMPc-peroxidase ligado/AMPc-peroxidase livre. Tendo os valores de %B/Bo dos padrões de AMPc, fizemos um gráfico de %B/Bo versus concentração de AMPc a fim de identificarmos na curva os valores de AMPc das amostras.

3.6 VERIFICAÇÃO DE ALTERAÇÕES DA CONCENTRAÇÃO DE CÁLCIO INTRACELULAR LIVRE ($[Ca^{2+}]_i$)

Para avaliar se a cascata de sinalização ativada pela resposta ao receptor B2 de cininas incluía alterações na concentração de cálcio intracelular, adicionamos BK às CPNs na concentração de 1µM. Inicialmente as neuroesferas foram dissociadas e semeadas em placas de 24 poços com superfície aderente. Seis horas depois, as células foram tratadas com 5µM de Fluo-3AM (Molecular Probes) em 0,5% Me_2SO e 0,06% de surfactante não iônico de ácido plurônico F-127 por 30 minutos a 37°C. O meio foi trocado por tampão extracelular (140 mM de NaCl, 3 mM de KCl, 1 mM de $MgCl_2$, 2 mM de $CaCl_2$, 10 mM de Hepes e 10 mM glicose, pH 7.4) imediatamente antes das análises de imageamento de Ca^{2+} realizadas com um microscópio invertido ECLIPSE-TiS (Nikon, Melville, NY) equipado com uma câmera de 14 bit, alta resolução, CCD CoolSNAP HQ2 (Photometrics, Tucson, AZ). O software NIS-Element (Nikon) foi utilizado para as análises das imagens que foram adquiridas a cada 0,5 segundos. O Fluo-3AM foi excitado por uma lâmpada de xenon à

488 ηm e a emissão de fluorescência foi detectada usando-se um filtro *band pass* (515-530 ηm). Quarenta células foram analisadas para cada duplicata em um total de três experimentos independentes. O influxo de cálcio foi estimado pela determinação da variação média entre a intensidade de fluorescência do Fluo-3AM obtida após estímulo (F) e antes desse, que é a fluorescência do estado basal (Fo), normalizado pela Fo ((F-Fo)/Fo).

3.7 CITOMETRIA DE FLUXO

3.7.1 Quantificação de Marcadores de Diferenciação

No sétimo dia de diferenciação, as amostras foram dissociadas pela incubação com tripsina por 3 min a 37ºC. Posteriormente, a digestão enzimática foi interrompida com a adição de SFB, quando então, as células foram dissociadas mecanicamente e filtradas em poros de 40 µm para obtenção de células individualizadas. Depois de lavadas, as células foram fixadas em 4 % de PFA em PBS por 20 minutos a 4°C. Sequencialmente, as amostras foram incubadas em PBS contendo 3% de SFB para bloqueio dos sítios inespecíficos e 0,1% de Triton X-100 para permeabilizar as membranas celulares por 20 minutos. Posteriormente, as células foram incubadas nessa mesma solução com os anticorpos primários anti-Nestina, anti-GFAP, anti-β3-tubulina, anti-MAP-2 nas diluições indicadas na Tabela 1. Após 30 minutos de incubação com anticorpo primário a temperatura ambiente, as células foram lavadas com PBS e incubadas novamente por 30 minutos com os anticorpos secundários conjugados a fluoróforos (Alexa Fluor 488 e 555 – Tabela 2). Por fim, as células foram lavadas e ressuspendidas em 500mL de PBS para

aquisição dos dados no citômetro de fluxo Attune (Life Technology), sendo adquiridos no mínimo 30.000 eventos por amostra.

Gates no *density-plot* do canal de dispersão frontal (FS – *forward scatter* - fornece informações sobre o tamanho celular) versus o canal de dispersão lateral (SS – *side scatter* - fornecem informações sobre a granulosidade/complexidade celular) foram utilizados para excluir células mortas e *doublets* celulares. A amostra negativa consistiu de células expostas ao anticorpo controle de isotipo do anticorpo primário e ao anticorpo secundário. A partir dessa amostra traçaram-se quatro quadrantes onde eventos observados além do quadrante inferior esquerdo (contendo a amostra negativa) foram considerados positivos. Outros dois controles foram utilizados para a compensação dos detectores do citômetro. O primeiro deles consistiu em uma amostra marcada com um anticorpo conjugado com o fluoróforo alexa fluor 488 que é detectado no detector de fluorescência 1 (FL1). Este controle foi utilizados para descontar a interferência do alexa fluor 488 no detector de florescência 2 (FL2). O segundo controle consistiu em uma amostra marcada com anticorpo conjugado com o alexa fluor 555 que é detectado no FL2. Este foi necessário para descontar a interferência do alexa fluor 555 no FL1. A compensação dos detectores permitiu a análise segura de dois marcadores na mesma amostra. As análises foram realizadas no *software* WinMDI2.9.

3.7.2 Quantificação de Quinases Ativas (fosforiladas)

As neuroesferas indiferenciadas foram dissociadas pela incubação com tripsina por 3 minutos a 37ºC. Posteriormente, a digestão enzimática foi interrompida com a adição de SFB, quando então, as células foram dissociadas mecanicamente e filtradas em poros de 40 µm para obtenção de células individualizadas. Após duas lavagens das células

com DMEM/F12 para remoção completa do SFB, as mesmas foram ressuspendidas em meio de cultura DMEM/F12 suplementado com apenas 0,5% de B27 e retornadas para as garrafas com superfícies não aderentes onde foram mantidas em condições ideais de cultivo (5% CO2, 37ºC e 95% de umidade) por 6 horas.

Decorrido este período, procedeu-se o protocolo de citometria de fluxo para fosfo-proteínas conforme previamente caracterizado e utilizado por Chow (2001), Krutzik (2003), Pillat (2009) e Yuan (2011). As células foram tratadas ou não com inibidores das vias de sinalização por 1 hora e/ou com outras drogas por tempos indicados nos experimentos. Atingido o tempo de estímulo desejado, uma solução de PFA a 16% em PBS, pH 8,5, foi imediatamente acrescentada ou meio de cultura de maneira que a concentração final atingida fosse 4%. Após 10 min a temperatura ambiente, as amostras foram centrifugadas e ressuspendidas em etanol 100% a -20°C por no mínimo 12 horas. As células foram então centrifugadas e incubadas em PBS contendo 3% de SFB para bloqueio dos sítios inespecíficos por 20 minutos. Posteriormente, as células foram incubadas nessa mesma solução com os anticorpos primários anti-pERK, anti-pAkt, anti-pSTAT3, anti-pJNK, anti-p38 nas diluições indicadas na Tabela 1. Após 30 minutos de incubação com o anticorpo primário a temperatura ambiente, as células foram lavadas com PBS e incubadas novamente por 30 minutos com os anticorpos secundários conjugados a Alexa Fluor 488 (Tabela 2). Por fim, as células foram lavadas e ressuspendidas em 500mL de PBS para aquisição dos dados no citômetro de fluxo Attune, sendo que no mínimo 30.000 eventos foram adquiridos por amostra.

A quantificação em porcentagem dos eventos positivos bem como o nível de expressão de um determinado antígeno foi obtida através do software Flowjo 7.6.3. Este

software quantifica os eventos positivos da amostra através do tracejado da diferença entre a mesma e o controle negativo (anticorpo controle de isotipo + anticorpo secundário conjugado ao fluoróforo) (Figura 3.1). Já o nível de expressão de um determinado antígeno alvo pode ser avaliado pela intensidade de fluorescência mediana (*Mean fluorescence intensity* - MFI). Este parâmetro fornece medidas arbitrárias que refletem a diferença de expressão do antígeno entre amostras.

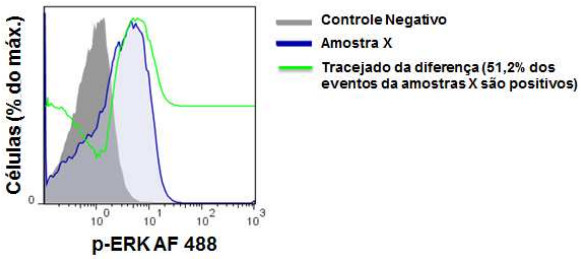


Figura 3.1: Sobreposição de histogramas demonstrando o tracejado da diferença entre o controle negativo e a amostra X a ser analisada. O *software* Flowjo 7.6.3. fornece a diferença numérica em porcentagem entre os dois histogramas usando como estratégia o tracejado da diferença entre eles.

A metodologia para avaliar a fosforilação de quinases em células PC12 é bastante semelhante à metodologia empregada em neuroesferas diferindo apenas em alguns pontos iniciais de preparação das amostras de acordo com o protocolo utilizado por Santos e colaboradores (2007). Primeiramente, 24 horas antes do ensaio, as células PC12 foram mantidas em meio DMEM-*High Glucose*, com 0,5% de SC. Após a adição dos tratamentos e término do período de estímulo, as células foram rapidamente dissociadas mecanicamente e a solução de PFA a 16% foi acrescentada ao meio de cultura para concentração final de 4%. A partir dessa etapa, o protocolo de determinação da expressão das quinases fosforiladas para PC12 segue a mesma metodologia utilizada para neuroesferas, como já descrita à cima.

3.7.3 Ensaio de incorporação de BrdU para análise da proliferação e ciclo celular

A proliferação e o ciclo celular foram avaliados pela incorporação do análogo de timidina, BrdU (5-bromo-2-deoxiuridina; Sigma-Aldrich) ao DNA durante a fase S do ciclo celular e pela marcação do DNA total com iodeto de propídio (PI - *Propidium iodide*) (Invitrogen) conforme previamente publicado (Cappella et al, 2008). Inicialmente, o BrdU foi adicionado ao meio de cultura na concentração final de 50µM e as células foram cultivadas por mais 30 minutos (para análises das fases de ciclo celular) ou 2 horas (para análises apenas da proliferação). Após este período, as neuroesferas foram dissociadas enzimática e mecanicamente e fixadas em etanol 75%. Tara tal fixação, cada *pellet* foi ressuspendido em uma parte de PBS gelado e em seguida foram adicionadas, sob leve agitação, de maneira sequencial, três partes de etanol absoluto a -20°C. As células foram mantidas a 4°C por pelo menos 12 horas. Posteriormente, as células fixadas foram lavadas duas vezes com PBS e incubadas por 30 minutos em uma solução de ácido clorídrico 1,5N contendo 0,5% de Tween 20 para desnaturação do DNA e exposição do BrdU e permeabilização das membranas celulares. As amostras foram lavadas em solução de tetraborato de sódio a 0,1M pH 8,5 e em seguida em solução de PBS. Os sítios inespecíficos de ligação aos anticorpos foram bloqueados após incubação com solução de PBS contendo 3% de SFB e 0,1% Triton X-100 por 20 minutos. Em seguida, as células foram incubadas nessa solução com o anticorpo anti-BrdU (Tabela 3.1) por 1 hora protegidas da luz sob leve agitação. Posteriormente, as células foram lavadas com PBS e incubadas novamente por 1 hora com o anticorpo secundário conjugado a Alexa Fluor 488. Por fim, adicionou-se a solução de marcação de DNA total, PI na concentração de 50 µg/mL, RNAse A (Invitrogen) na concentração de 50 µg/mL e Triton X-100 a 0,1%. As

amostras foram incubadas por 20 minutos a temperatura ambiente protegidas da luz e então avaliadas no citômetro de fluxo. No mínimo 60.000 eventos foram adquiridos.

A análise foi realizada no software Flowjo 7.6.3. A fim de eliminar eventos falsos em G2/M, chamados *doublets* celulares (células individuais que passam juntas no feixe do laser), foi utilizado um *gate* no *density-plot* FL2H versus FL2A , ambos na escala linear detectando a fluorescência do PI (Figura 3.2A). Após exclusão dos *doublets* é possível quantificar com segurança as fases G_0/G_1, S e G_2/M do ciclo celular como mostrado na figura 3.2B.

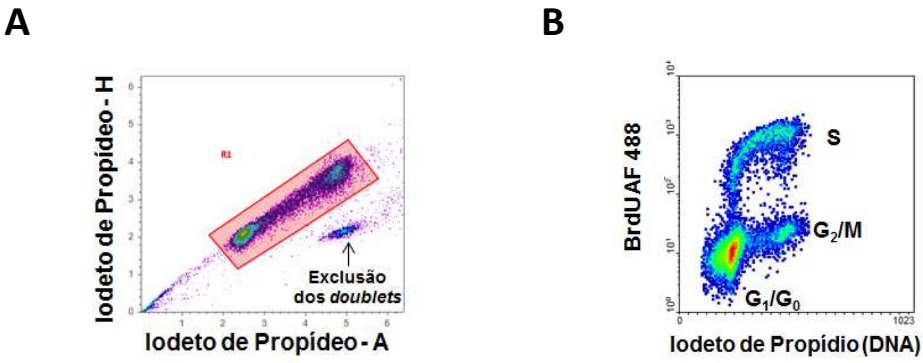


Figura 3.2: análise do ciclo celular pela incorporação de BrdU e marcação do DNA total com iodeto de propídio (PI). **A**) G*ate* (R1) no density-plot de FL2-Altura (H) versos FL2-Área (A) em amostras marcadas com PI. Faz-se inicialmente esse *gate* para exclusão dos *doublets* e *debris* celulares. **B**) Com as células contidas dentro do *gate* R1, faz-se um novo density-plot de PI (FL2A em escala linear) versus BrdU (FL1A em escala logarítmica) onde pode-se observar claramente três populações em fases distintas do ciclo celular, G_1/G_0 (população inferior esquerda), S (população $BrdU^+$) e G_2/M (população na região inferior direita).

3.7.4 Ensaio de marcação com Ki67 e iodeto de propídio para quantificação das células na fase G_0 do ciclo celular

O protocolo de marcação com Ki67 e PI empregado neste trabalho foi identificado por Williams (1990) como o melhor método para tal ensaio. Primeiramente as amostras são dissociadas enzimática e mecanicamente e posteriormente são fixadas com 4% de PFA por 10 minutos, seguido pela utilização de etanol 75%. A fixação prévia com PFA previne que a estrutura da proteína Ki67, incluindo seu epítopo, seja desnaturada pelo

etanol e este último, por sua vez, é o melhor agente de fixação para que o propídeo intercale ao DNA, fornecendo o menor coeficiente de variação (CV) dos picos do ciclo celular. As amostras foram então mantidas em etanol 75% a -20°C por no mínimo 12 horas. Sequencialmente, as amostras foram lavadas e incubadas com o anticorpo anti-ki67 (Tabela 3.1) em solução de PBS contendo 3% de SFB e 0,1% Triton X-100 por 30 minutos. Posteriormente, as células foram lavadas com PBS e incubadas novamente por 30 minutos com o anticorpo secundário conjugado a Alexa Fluor 488. Por fim, os pellets foram resuspendidos em uma solução de PI como anteriormente descrito e as amostras adquiridas no citômetro de fluxo.

3.8 Ensaio de co-marcação de EdU e marcadores de diferenciação

O ensaio de incorporação de EdU (5-etinil-2-deoxiuridina) apresenta algumas vantagens perante o ensaio de incorporação de BrdU (Cappella et al, 2008). Ambos são análogos da timidina utilizados para avaliar a proliferação e ciclo celular, no entanto, somente o último é detectado por anticorpo específico e, dessa forma, para que o anticorpo tenha acesso ao BrdU, o DNA precisam ser desnaturado. A utilização de 2M de ácido clorídrico é eficiente na desnaturação do DNA, no entanto, também altera a estrutura das proteínas, incluindo a estrutura dos epítopo. Isso inviabiliza a detecção simultânea do BrdU e antígenos protéicos, como por exemplo, os marcadores de diferenciação. Já o EdU é um análogo de timidina que apresenta um grupo etinil na posição 5 do anel pirimidina. Esse grupo etinil do EdU reage, na presença de cobre, com azida conjugada a fluoróforo (reação química nomeada de click) (Rostovtsev et al, 2002)

e, portanto, pode ser facilmente detectado sem que seja necessária a desnaturação do DNA com HCl, permitindo assim, a dupla marcação com antígenos protéicos.

Dessa forma, a proliferação de neurônios, astrócitos e células precursoras neurais foi avaliada pela incorporação do EdU utilizando o Click-iT EdU Cytometry Assay Kits (Molecular Probes). Inicialmente, o EdU foi adicionado ao meio de cultura na concentração final de 50µM e as células foram cultivadas por mais 16 horas. Após este período, as células foram lavadas com PBS e fixadas em 2 % de PFA por 15 min a temperatura ambiente. Posteriormente, as células foram lavadas duas vezes com 1% de BSA em PBS e incubadas por 15 minutos em uma solução contendo saponina (Componente E do kit Click-iT®) para permeabilização das membranas celulares. Em seguida o coquetel para a reação Click-iT® foi preparado segundo tabela a baixo:

Tabela 3.3: reação Click-iT®

Componentes da Reação	**Número de Reações**			
	1	2	10	50
PBS	219 µL	438µL	2,19mL	10,95mL
$CuSO_4$	5 µL	10 µL	50 µL	250 µL
Azida Conjugada com fluoróforo	1,25 µL	2,5 µL	125 µL	625 µL
Tampões Aditivos da Reação	25 µL	50 µL	250 µL	1,25mL
Volume Total da Reação	250 µL	500 µL	2,5mL	12,5mL

As células foram então incubadas com o coquetel por 30 minutos a temperatura ambiente protegidas da luz. Em seguida, as amostras foram lavadas com solução de saponina e incubadas com os anticorpos primários anti-β3-Tubulina, anti-Nestina e anti-GFAP. A partir dessa etapa, o protocolo segue a mesma metodologia já descrita no tópico imunofluorescência.

3.9 EXTRAÇÃO DE RNA TOTAL E SÍNTESE DE cDNA

Os RNAs das CPNs diferenciadas (dia 7) foram isolados com o reagente TRizol (Invitrogen) de acordo com o protocolo fornecido pelo fabricante. A quantificação das amostras de RNA foi realizada em espectrofotômetro (NanoDrop ND-1000 Spectrophotometer) utilizando os comprimentos de onda (λ) 260 e 280 ηm. A concentração de RNA total foi obtida a partir da absorbância no λ 260 ηm. A razão entre os valores de absorbâncias no λ 260 e 280 ηm foi utilizada para avaliar a pureza dos RNAs de cada amostra, dado que quanto mais próximo de 2 mais pura ela está e considerando valores acima de 1,8 bom grau de pureza. A integridade das amostras de RNA foi observada através da análise dos RNAs ribossômicos (18s e 28s) por eletroforese em gel de agarose 1% corado com 0,5 µg/mL de brometo de etídio (Huggett et al, 2005). As imagens das bandas correspondentes aos RNAs ribossômicos foram obtidas através do transiluminador com luz UV.

Após a extração do RNA total, possíveis contaminações desse com DNA foram removidas das amostras pela adição da DNAse I (1 U/µg de RNA, Ambion) em tampão de reação (40 mM Tris-HCl, 6 mM $MgCl_2$ e 20 mM de EDTA) a 37°C por 15 minutos. Após este período, a DNAse I foi inativada (10 minutos a 65 °C) e o RNA total foi então utilizado na transcrição reversa (Lameu et al, 2012).

A síntese do DNA complementar (cDNA) foi realizada a partir de 3 µg de RNA total, utilizando-se a enzima U MMLV-RT (RevertAid H *minus Moloney murine leukemia virus-reverse transcriptase*, Fermentas) segundo o protocolo fornecido pelo fabricante, sendo utilizado: 40 U de inibidor de RNAse; 0,2 mM de dNTPs; 50 ηg de hexameros randômicos; 50 ηg de oligodT18; 50 mM de $MgCl_2$; tampão da enzima em um volume final de 20 µL. As

reações foram realizadas em termociclador Eppendorf Mastercycler Gradient nas condições: 20ºC por 10 min, 42ºC por 45 min (período de atividade da enzima) e 95ºC por 5 min (inativação da enzima) (Lameu et al, 2012).

3.10 Reação em Cadeia da Polimerase em Tempo Real

A análise quantitativa da expressão dos RNAs mensageiros (RNAm) de GFAP, β3-tubulina e MAP2 em células diferenciadas foi realizada pela técnica de reação em cadeia da polimerase (PCR – *Polymerase Chain Reaction*) em tempo real. O cDNA foi amplificado usando o *StepOne RealTime PCR System* (Applied Biosystems, Foster City, Ca, USA) como sistema de detecção. Essa técnica consiste na marcação da dupla fita do cDNA específico com uma sonda, como por exemplo o SYBR Green (Applied Biosystems), sendo que a intensidade da fluorescência emitida por essa sonda é proporcional ao número de moléculas amplificadas, permitindo, dessa forma, a detecção quantitativa do produto da PCR.

As reações foram realizadas em triplicata em um volume final de 25µL. O cDNA foi utilizado a partir de 3 µg de RNA, 12,5 µL de SYBR-Green e 200 ηM de cada *primer* (Tabela 3.4). A temperatura das reações foi de 95°C por 10 min, 40 ciclos de desnaturação 95°C por 15 segundos e 60°C por 1 min. O nível de expressão gênica de GAPDH (*Glyceraldehyde 3-phosphate dehydrogenase*) foi utilizado como normalizador e controle endógeno da reação (Lameu et al, 2012).

Os resultados são apresentados por quantificação relativa a partir do grupo controle sem tratamento, utilizando o método $2^{-\Delta\Delta Ct}$. A análise das expressões relativas de cada transcrito foi conduzida conforme Ueno (2002) e Trujillo e colaboradores (2012).

Tabela 3.4 - Sequências de *primers*.

cDNA	*Primer Forward* (5'-3')	*Primer Reverse* (5'-3')
GFAP	TGACCGCTTTGCTAGCTACATC	GCGCCTTGTTTTGCTGTTC
MAP2	TCCTCCAAAGTCCCCAGCTA	CCGGCAGTGGTTGGTTAATA
β 3-tubulina	GAGACCTACTGCATCGACAATGAAG	GCTCATGGTAGCAGACACAAGG
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC

3.11 Extração das Frações Protéicas Nucleares e Citoplasmáticas e Dosagem de Proteínas

O fracionamento de proteínas presentes no núcleo e no citoplasma foi realizado através do kit *NE-PER® Nuclear and Cytoplasmic Extraction Reagents* (Thermo Scientific) de acordo com as instruções do fabricante. Brevemente, para cada condição experimental $1x10^7$ células PC12 foram ressuspendidas e lavadas com PBS gelado. O *pellet* celular foi então ressuspendido em 500 µL da solução CER I® suplementada com *cocktail* de inibidores de proteases (Thermo-Scientific) e com *cocktail* de inibidores de fosfatases em mini *tablets* (Thermo-Scientific). A amostra foi então submetida à agitação vigorosa em *vortex* e incubação no gelo por 10 minutos. Posteriormente, 1000 µL de CER II® foram adicionados à amostra que foi novamente agitada e incubada no gelo por 1 minuto. Após nova agitação, a amostra foi centrifugada por 5 minutos a 16.000 *x g*. Em seguida, o sobrenadante contendo as proteínas citoplasmáticas foi transferido para um novo tubo e armazenado a -80°C. O *pellet* contendo as proteínas nucleares foi ressuspendido na solução NER® suplementada com os inibidores de proteases e fosfatases descritos a cima. Essa amostra foi então submetida à agitação vigorosa em *vortex* por 15 segundos, a cada 10 minutos, para um total de 40 minutos, sempre mantida a 4°C. Posteriormente, o tubo

contendo a amostra foi novamente centrifugado a 16.000 *x g* por 10 minutos. O sobrenadante contendo a fração de proteínas nucleares foi então transferido para um novo tubo e armazenado a -80°C até ser utilizado.

A concentração de proteínas contidas na fração citoplasmática e nuclear das amostras foi determinada pelo método azul de *coomassie* (Bradford, 1976) tendo a albumina do soro bovino (*bovine serum albumin*; BSA) como padrão. As leituras das absorbâncias foram realizadas em espectrofotômetro acoplado ao leitor de placas Flexstation utilizando-se o comprimento de onda de 595 ηm.

3.12 *WESTERN BLOT*

Para o ensaio de *Western Blot*, 30µg de proteínas do extrato nuclear e citoplasmático foram desnaturadas a 95°C por 5 minutos e separadas em condições redutoras em gel SDS-PAGE (eletroforese em gel de poliacrilamida contendo dodecil sulfato de sódio) com concentração de 10% a uma voltagem constante de 140 V. Em sequência, as proteínas foram transferidas para uma membrana de nitrocelulose (Thermo-Scientific) a amperagem constante de 400 mÅ por 1 hora. Para bloqueio de ligações inespecífica dos anticorpos, um tampão TBS-T (*Tris Buffered Saline* - Tween 0,1%) com 5% de BSA foi adicionado sobre a membrana e incubado por 30 minutos à temperatura ambiente com agitação constante. A membrana foi então incubada com os anticorpos primários anti- pERK (Thr202/Tyr204) (Cell Signaling Technology), ERK (Santa Cruz Biotechnology), β-actina (Sigma-Aldrich) e Histona H1 (Santa Cruz Biotechnology) nas concentrações especificadas na tabela 3.1 por 16 horas a 4°C. Decorrido este período, a membrana foi lavada seis vezes e incubada novamente com anticorpo secundário

apropriado conjugado com fluoróforo por 1 hora a temperatura ambiente com agitação constante e protegido da luz. Os anticorpos primários e secundários foram diluídos em TBS-T com 1% de BSA. Por fim, a membrana foi novamente lavada por seis vezes com TBS-T e suas imagens adquiridas no equipamento Typhoon™ (GE Healthcare Life Sciences). Para a análise de densitometria das bandas, o *software* ImageJ foi utilizado, sendo que os valores de densitometria foram calculados pela razão da intensidade de cada banda subtraindo-se o ruído da imagem (Cappellari et al, 2012).

3.13 Inibidores e ativadores enzimáticos

- U0126 (Cell Signaling Technology): inibidor da MEK. Concentração: 10µM (Mukhin et al, 2003).
- PD98059 (PD) (Sigma-Aldrich): inibidor da MEK. Concentração: 20µM (Kumar et al, 2008).
- Ester de forbol (PMA-*phorbol 12-myristate 13-acetate*) (Tocris Bioscience): análogo do diacilglicerol, ativador de PKC. Concentração: 1µM (Moughal et al, 1995).
- LY294002 (Ly) (Tocris Bioscience): inibidor da PI3K. (Kumar et al, 2008).
- SB203580 (SB) (Cell Signaling Technology): inibidor da p38. Concentração: 10µM (Wang et al, 2006).
- U73122 (Sigma-Aldrich): inibidor da PLC. Concentração: 10µM (Mukhin et al, 2003).
- Gö6973 (Go) (Tocris Bioscience): inibidor das PKCs clássicas (cPKCs; ativadas por Ca^{2+}, DAG e fosfatidilserina)(Martiny-Baron et al, 1993)
- YM254890 (YM) (Astellas): inibidor proteína Gq. (Takasaki et al, 2004)

- Toxina Pertussis (TP): Inibidor proteína Gi. Concentração: 200ng/mL (Mukhin et al, 2003)
- Toxina Cólera (TC) (Sigma-Aldrich): Inibidor da atividade GTPase da proteína Gs, deixando-a sempre ativa. Concentração: 0,5µg/mL (Forti et al, 2002)
- Forscolina (Calbiochem): ativador de todas as classes de adenilato ciclases. Concentração: 50µM (Santos & Araujo, 2001)
- Tapsigargina (Tocris Bioscience): inibidor da SERCA (*sarco/endoplasmic reticulum Ca2+-ATPases*), depletando os estoques intracelulares de cálcio. (Tykocki et al, 2013)

3.14 Análise Estatística

Os experimentos foram realizados em triplicata e estão mostrados em todas as figuras como média ± SEM. A análise foi realizada com o auxílio do *software* GraphPad Prism5 e os dados foram submetidos a estatística utilizando o teste T e ANOVA (*Analysis of Variance*) com a comparação múltipla através do teste *post hoc* Tukey, com nível de significância fixado em $P < 0,05$. Quando não identificado nas figuras a quem se refere à diferença estatisticamente significativa de uma amostra, esta se refere ao controle.

4 Resultados

Desde que os efeitos da bradicinina na diferenciação neural passaram a ser um dos principais focos de nosso laboratório, a análise profunda de sua influência sobre a proliferação e transição através das fases do ciclo celular, bem como de suas vias de sinalização e mecanismos responsáveis por seus efeitos na migração, neurogênese e proliferação tornaram-se imprescindíveis.

4.1 PARTICIPAÇÃO DA BRADICININA NA MIGRAÇÃO E DIFERENCIAÇÃO DE NEUROESFERAS MURINAS

A migração das células para regiões distantes da sua origem e a determinação do fenótipo celular são dois processos que fazem parte do desenvolvimento neural. Um estudo em especial, no qual fui colaboradora com resultados em neuroesferas murinas, fundamentou esse trabalho. Esse demonstrou, entre outros achados, que a BK potencializa a migração neural e neurogênese em detrimento da gliogênese em diversos modelos de diferenciação (Trujillo et al, 2012). Portanto, inicialmente demonstraremos a influência da BK na migração e diferenciação de CPNs obtidas de embriões de camundongos.

Para as CPNs serem capazes de migrar e diferenciar, as neuroesferas são postas em superfície aderente em meio desprovido dos fatores de crescimento (GF) EGF e FGF2 por sete dias. A Figura 4.1 apresenta imagens representativas da migração no primeiro e sétimo dia de diferenciação neural, onde a região delimitada entre as linhas pontilhadas compreende aproximadamente 95% das células migradas. Na presença de BK na

concentração de 1µM, tanto no dia 1 quanto no dia 7 de diferenciação, observa-se um maior número de células que atingem distâncias mais longas partindo da borda da neuroesfera em relação aos respectivos controles.

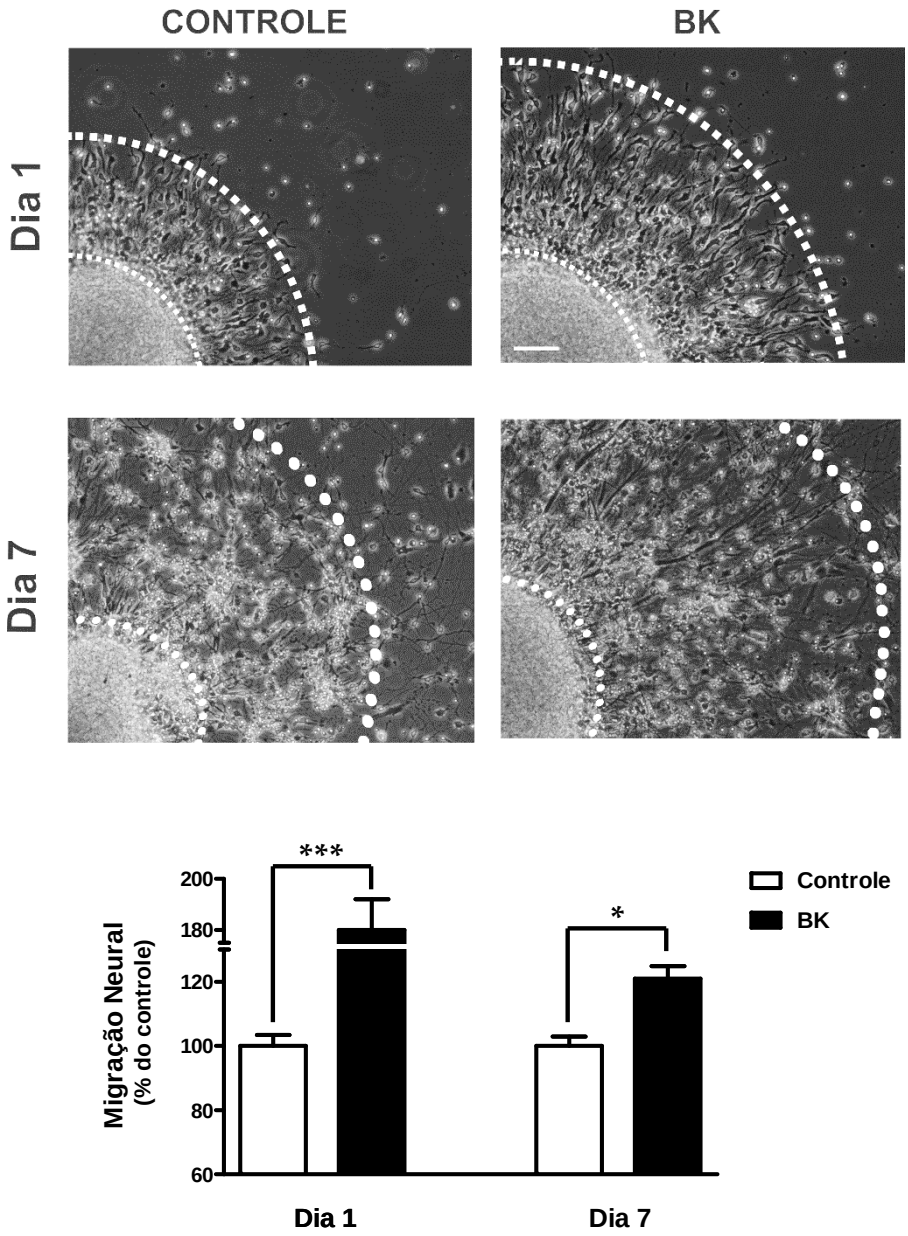


Figura 4.1 – Migração e diferenciação neural na presença de bradicinina (BK). Imagens de contraste de fase representativas do padrão de migração radial após 1 dia e 7 dias de diferenciação neural sob condições controle e na presença de 1 µM de BK. A região delimitada entre as linhas pontilhadas compreende aproximadamente 95% das células que migraram. Escala de 100 µm (*$P < 0,05$; ***$P < 0,001$).

Investigou-se, pela técnica de PCR em tempo real, o destino das CPNs murinas pela análise da expressão dos RNAms dos marcadores neuronais (MAP2 e β 3-tubulina) e gliais (GFAP) após a diferenciação. O papel do receptor B2 de cininas na neuro-gliogênese foi evidenciado pela utilização do seu antagonista HOE-140 e agonista BK comparando a expressão dos marcadores acima citados nessas amostras em relação à amostra controle (Figura 4.2). Como esperado, o tratamento durante a diferenciação na presença de 1µM de HOE-140 diminui a expressão dos marcadores neuronais MAP2 e β3-tubulina, ao passo que a do marcador GFAP é consideravelmente aumentada. Em contrapartida, o tratamento crônico com 1µM de BK resulta no inverso, ou seja, reduz a expressão gênica do marcador glial GFAP e aumenta a expressão do marcador neuronal MAP2.

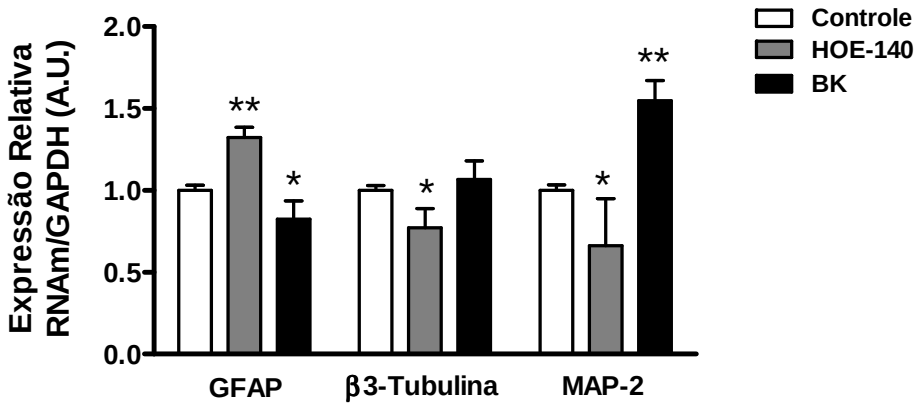


Figura 4.2 - Expressão relativa dos RNAm dos marcadores neuronais (MAP2 e β 3-tubulina) e gliais (GFAP) após diferenciação de CPNs na presença de antagonista (HOE-140) e agonista (bradicinina; BK) do receptor B2 de cininas. As análises quantitativas da expressão relativa dos marcadores MAP2, β 3-tubulina e GFAP, no sétimo dia de diferenciação, foram realizadas por PCR em tempo real tendo como referência a expressão gênica de GAPDH (*housekeeping gene*), visto que sua expressão não se altera nas condições experimentais. Os dados são representativos de quatro experimentos independentes conduzidos em triplicatas (*$P < 0,05$ e **$P < 0,01$).

Sequencialmente, a expressão dos marcadores de diferenciação neuronal e glial foi avaliada a nível protéico. Para tal, inicialmente, ensaios de imunocitoquímica foram realizados no dia 7 de diferenciação em condições controle (CTL) e na presença de BK

(1µM) com os marcadores GFAP, β 3-Tubulina e DAPI. Observa-se na figura 4.3, um aparente deslocamento da diferenciação para a neurogênese em detrimento da gliogênese sob influência da BK. Em seguida, foram realizados experimentos quantitativos em nível de expressão proteica utilizando a técnica de citometria de fluxo. Similarmente ao observado por PCR real time e imunocitoquímica, a análise por citometria de fluxo apresentada na figura 4.4 mostra que a população positiva para expressão de GFAP diminui após tratamento crônico com BK durante a diferenciação e, por outro lado, o tratamento com esta cinina aumenta consideravelmente a população positiva para MAP2 e β 3-Tubulina.

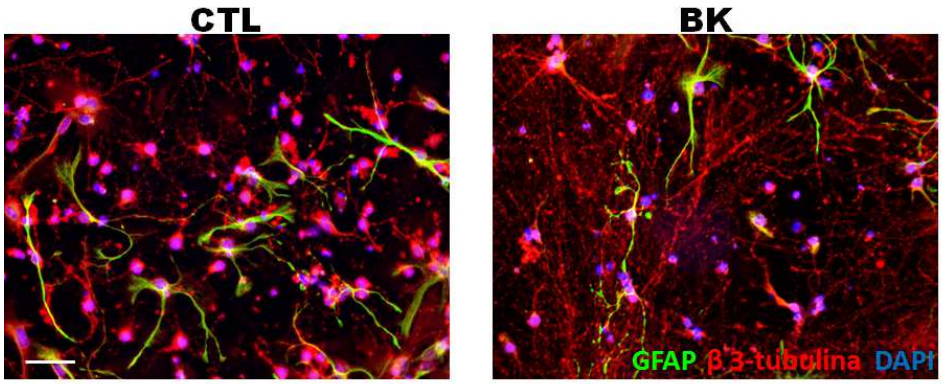


Figura 4.3: Análise qualitativa por imunocitoquímica da população neuronal e glial após diferenciação sob condições controle ou na presença de bradicinina. As neuroesferas foram diferenciadas por sete dias na ausência (Controle; CTL) ou presença de 1µM de bradicinina (BK). Marcação para GFAP Alexa Fluor (AF) 488; β 3-Tubulina com AF 555; marcação do núcleo com DAPI. As imagens são representativas de 5 experimentos independentes e a escala é de 100µm.

A população que expressa Nestina, ou seja, CPNs, não apresentou variação significativa alguma após processo de diferenciação na presença de BK assim como já demonstrado em neuroesferas de ratos (Trujillo et al, 2012). Como pode ser observado nos *density-plots* de citometria de fluxo na figura 4.4, as CNPs co-expressam Nestina e GFAP ($Nestina^{+}GFAP^{+}$), provavelmente, por tratar-se de glias radiais, um tipo de precursor neural. Em contrapartida, as células gliais não expressam Nestina ($GFAP^{+}Nestina^{-}$). Desta

maneira, cabe esclarecer que quando nos referimos a expressão de GFAP sem mencionar a expressão de Nestina em resultados de citometria de fluxo, estamos nos referindo a população GFAP^{+}Nestina^{-}, ou seja, a população de células gliais.

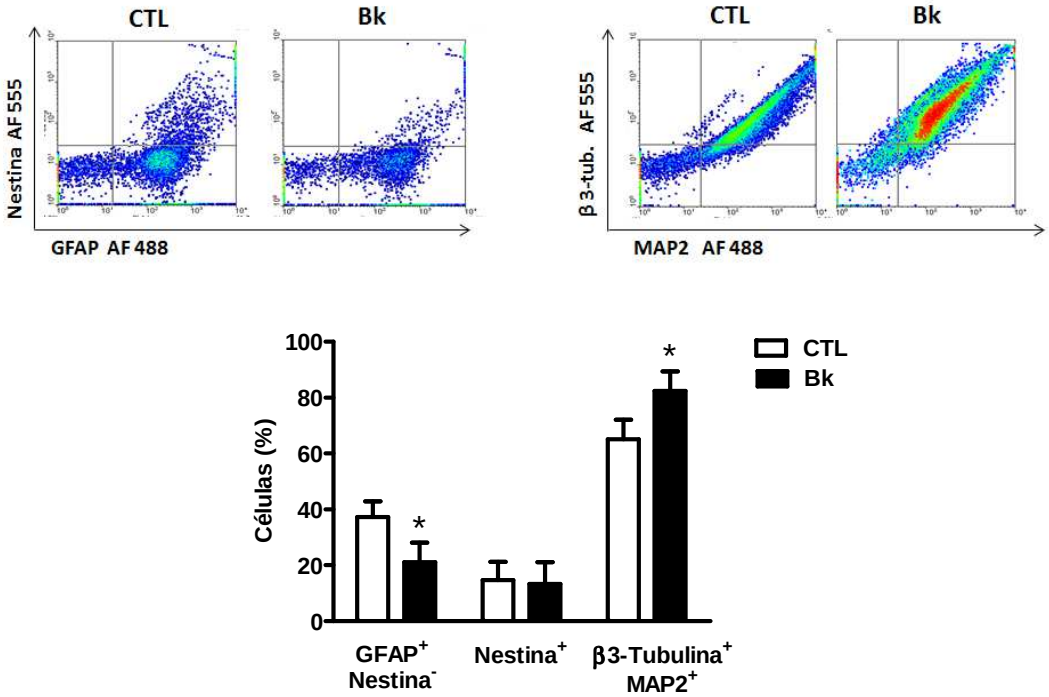


Figura 4.4: Porcentagem de células positivas para GFAP, Nestina, β 3-Tubulina e MAP2, após tratamento com bradicinina durante a diferenciação. As neuroesferas foram diferenciadas na ausência de tratamento (CTL) ou na presença de 1µM de bradicinina (BK) adicionada ao meio de cultura todos os dias. No sétimo dia de diferenciação as amostras foram fixadas e marcadas com os anticorpos anti-GFAP, Nestina, MAP2 e β 3-Tubulina. Posteriormente, as amostras foram marcadas com os anticorpos secundários Alexa Fluor (AF) 488 e AF 555, seguindo o protocolo de citometria de fluxo. Os dados são representativos de cinco experimentos independentes (*$P < 0,05$).

Estes resultados mostram a importante participação da BK no enriquecimento da população neuronal em detrimento da população glial durante a diferenciação de CPNs murinas assim como já demonstrados em outros modelos de diferenciação (Trujillo et al, 2012), sugerindo que esta cinina favorece a neurogênese em detrimento da gliogênese.

4.2 PARTICIPAÇÃO DA BRADICININA NO CICLO CELULAR E PROLIFERAÇÃO DURANTE A DIFERENCIAÇÃO

Trujillo e colaboradores (2012) demonstraram que neuroesferas obtidas de embriões de ratos, após se diferenciarem em neurônios e células da linhagem glial por sete dias na presença de BK, não sofrem nenhuma alteração na viabilidade celular mas proliferam significativamente menos que o observado em condições controle. Simultaneamente, observou-se um aumento na população de neurônios em cultura em detrimento da população glial.

Visto que o tratamento com BK não altera a viabilidade celular, mas altera simultaneamente a proliferação e enriquecimento neuronal no dia 7 de diferenciação, ainda fazia-se necessário avaliar se (1) a baixa taxa de proliferação observada após tratamento com BK seria em decorrência da maior proporção de neurônios em cultura estimulada por esta cinina, visto que neurônios não proliferam ou, ao contrário, (2) a alteração da razão populacional em cultura (maior proporção de neurônios) poderia estar sendo observada em decorrência da baixa taxa de proliferação de CPNs e astrócitos estimulada por BK.

Na busca de responder as questões acima, inicialmente avaliamos o perfil do ciclo celular durante todo o processo de diferenciação neural. A proporção de células nas fases G_1/G_0, S e G_2/M foi identificada través da análise de incorporação de BrdU e marcação com iodeto de propídio (PI) utilizando amostras no segundo, quarto e sétimo dias de diferenciação tratadas ou não com BK. Os resultados adquiridos em citômetro de fluxo, cujos *density-plots* representativos estão na figura 4.5A e as análises estão na figura 4.5B, demonstram que a exposição das células a BK exógena ao longo da diferenciação reduz o

número de células nas fases S e G_2/M do ciclo celular a partir do dia 4 de diferenciação. Evidentemente, observa-se também um maior número de células nas fases G_1/G_0 quando as amostras são tratadas com BK em comparado as amostras controle. A figura 4.5C sumariza todas as fases do ciclo celular durante a diferenciação nessas duas condições experimentais.

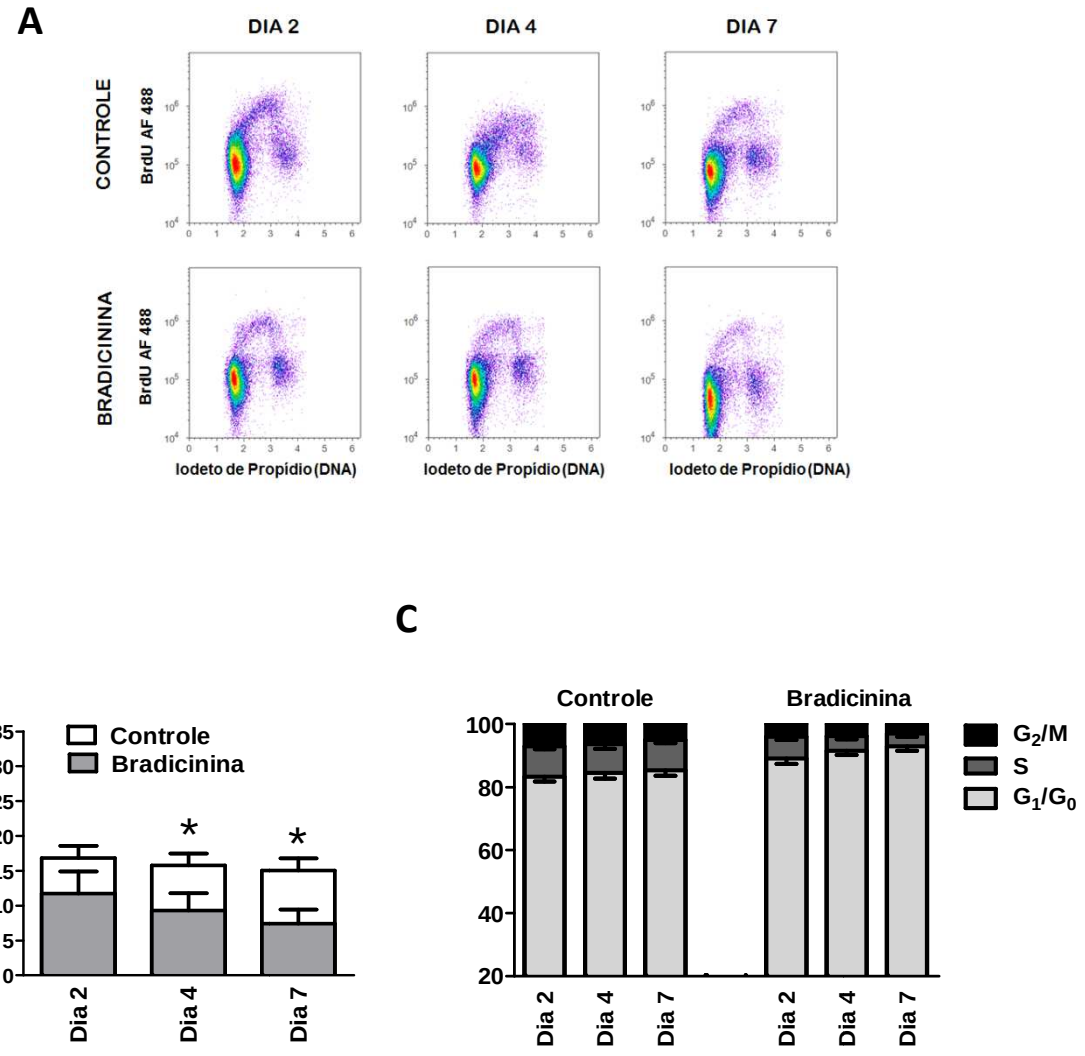


Figura 4.5: Análise do ciclo celular durante a diferenciação em condições controle e na presença de bradicinina. As amostras não foram tratadas (Controle) ou foram tratadas com 1µM de BK todos os dias durante os sete dias de diferenciação neural. Nos dias 2, 4 e 7 da diferenciação, as amostras foram tratadas com BrdU por 30 minutos pra a incorporação deste no DNA de células na fase S do ciclo celular. Posteriormente, as células foram dissociadas, fixadas e marcadas com anti-BrdU e Alexa Fluor 488 e Iodeto de Propídio (marcação do DNA total) e analisadas no citômetro de fluxo conforme descrito na metodologia.A) *Density-plots* representativos. B) Porcentagem de células em S+G_2/M. C) Porcentagem de células em todas as fases do ciclo celular. Os dados mostrados são representativos de cinco experimentos independentes. (**P* < 0,05).

É sabido que neurônios não proliferam, permanecendo sempre em estado quiescente. Diz-se nesses casos, que a célula saiu do ciclo celular e encontra-se na fase denominada G_0. Levando em consideração essa característica dos neurônios, as fases G_0 e G_1 foram analisadas separadamente através da marcação com PI e Ki67. Essa última proteína é expressa apenas em células que estão no ciclo celular, ou seja, o Ki67 está ausente ($Ki67^-$) apenas em células quiescentes (G_0) (Tsurusawa & Fujimoto, 1995). Dessa forma, no pico G_1/G_0 de fluorescência do PI, separa-se as células na fase G_0, que são negativas para Ki67, e as células na fase G_1, que são positivas para Ki67. Observamos que o tratamento com BK ao longo da diferenciação aumentou a população de células na fase G_0 no dia 7 de diferenciação em comparação à condição controle (Figura 4.6). Os *density-plots* representativos são mostrados na figura 4.6A, e as análises das células em G_0, G_1 e todas as fases do ciclo juntas são mostradas nas letras B e C, respectivamente. Esses dados corroboram a hipótese de que o favorecimento da neurogênese pela BK esteja influenciando indiretamente nas taxas de proliferação observadas no dia 7 de diferenciação. No entanto, estes resultados não são conclusivos acerca dessa hipótese, já que células precursoras de neurônios, os chamados neuroblastos, ainda proliferam e que astrócitos também podem entrar em G_0, apesar disso não ser comum durante o desenvolvimento neural.

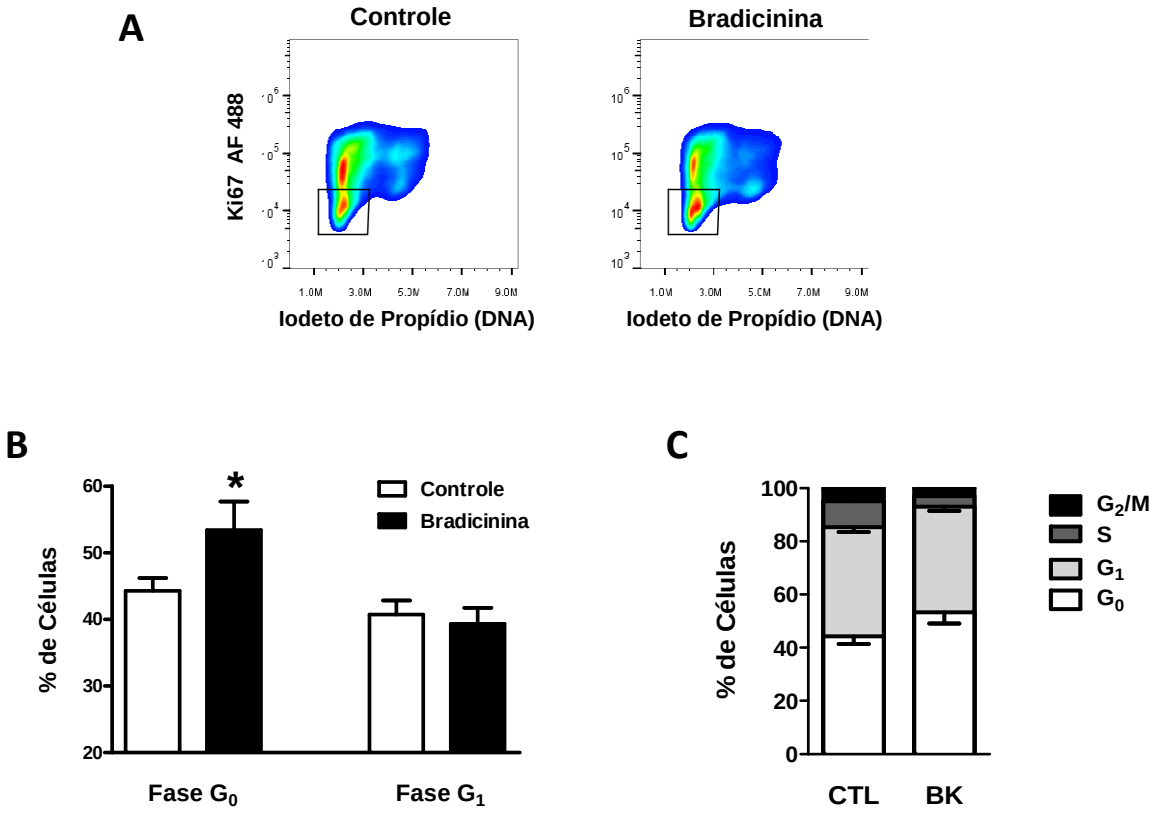


Figura 4.6: Análise das fases G_0 e G_1 do ciclo celular em células diferenciadas em condições controle ou na presença de bradicinina (BK). As CPNs diferenciaram por sete dias sob condições controle (CTE) ou na presença de bradicinina (BK, 1µM). Nos final da diferenciação, as amostras foram fixadas, marcadas com Ki67 e PI (marcação do DNA total) e avaliadas no citômetro de fluxo conforme descrito na metodologia. A) *Density-plots* representativos. Dentro do *gate* estão as células em G_0 ($Ki67^-PI^{low}$), acima do *gate* estão as células em G_1 ($Ki67^+PI^{low}$). B) Porcentagem de células em G_0 e G_1. C) Porcentagem de células em todas as fases do ciclo celular, incluindo G_0. Os resultados foram analisados no software Flowjo V10 e são representativos de 3 experimentos independentes. (*$P < 0,05$).

Por fim, um ensaio de identificação simultânea de células proliferando e CPNs, glias ou neurônios foi realizado no dia 7 de diferenciação em condições controle e sob tratamento crônico com BK (Figura 4.7). Para tanto, o EdU foi acrescentado ao meio de cultura por 16 horas e em seguida as células foram fixadas e marcadas para esse nucleotídeo modificado, GFAP (expresso em células da glia), Nestina (expressa em CPNs) e β 3-Tubulina (expressa em neurônios). Nestas condições em amostras controle observou-se que aproximadamente 29,7 ± 8,9% das células da glia, 18,2 ± 5,0% das CPNs e somente 4,6 ± 2,2 dos neurônios foram positivos para EdU, ou seja, proliferaram.

Entretanto, interessantemente, a BK não diminuiu a incorporação de EdU em nenhum tipo celular específico, ou seja, a BK não suprimiu a proliferação de células da glia, CPNs ou neurônios avaliados isoladamente. Esses dados sugerem que a baixa taxa de proliferação observada no dia 7 de diferenciação na presença de BK deve-se a maior proporção de neurônios em cultura.

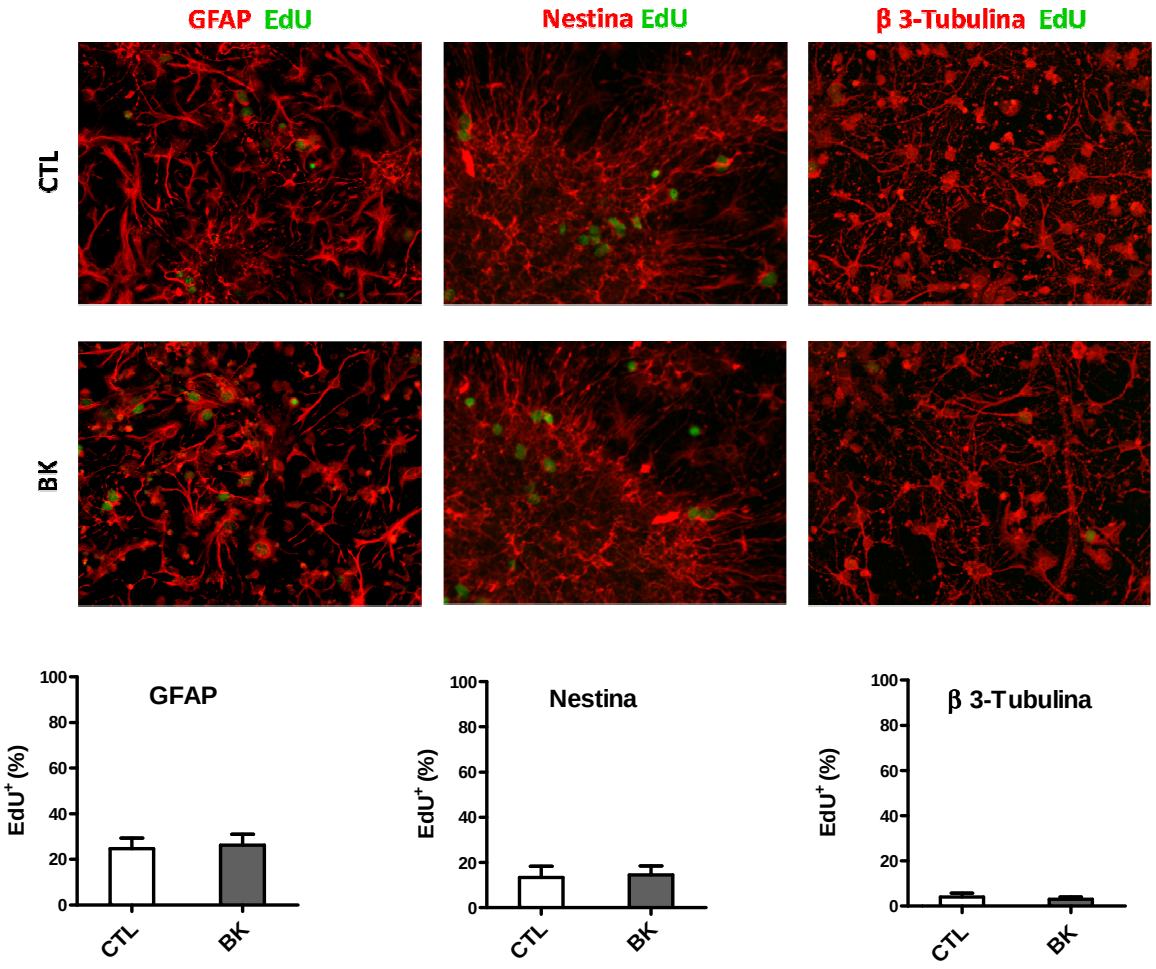


Figura 4.7: Análise da proliferação de células da glia, precursoras neurais e neurônios no dia 7 de diferenciação sob condições controle (CTL) ou na presença de bradicinina (BK). As amostras não foram tratadas (Controle) ou foram tratadas com 1µM de BK todos os dias durante os 7 dias de diferenciação neural. Nos dia 6 da diferenciação, 50µM de EdU foi adicionado ao meio de cultura por 16 horas. Então as células foram fixadas e marcadas para esse nucleotídeo, Nestina (presente em CPNs), GFAP (presente em células da glia) e β3-Tubulina (presente em neurônios) conforme descrito na metodologia. As imagens são representativas de 3 experimentos independentes e foram adquiridas com o aumentado de 200 vezes.

Assim, visto que o tratamento com BK não modifica a proliferação de nenhum tipo celular durante a diferenciação, concluímos que a sinalização induzida pela Bk é essencial para a determinação do destino neural.

4.3 EFEITOS E MECANISMOS IMPOSTOS POR BRADICININA NA PROLIFERAÇÃO E CICLO CELULAR DE CPNS INDIFERENCIADAS

Demonstramos, até o momento, que a BK não teve efeito direto na proliferação no dia sete de diferenciação sob cultivo na ausência de fatores de crescimento, contudo, esta cinina poderia ainda suprimir a proliferação de CPNs indiferenciadas que estão sob condições proliferativas devido ao intenso estímulo dos fatores (GF – *growth factors*) EGF e FGF2. Assim, também avaliaremos a influência da BK sobre a proliferação e ciclo celular em células nesse estágio. A análise desses parâmetros antes da diferenciação é mais simples, pois se tem basicamente uma única população, as células precursoras neurais (CPNs) que são Nestina^{+}.

Segundo Reynolds & Weiss (1992; 1996), que introduziu as neuroesferas como modelo de diferenciação, os GFs EGF e FGF2 atuam induzindo a auto-renovação de CPNs mantendo-as indiferenciadas. Tal fenômeno foi também observados aqui pela dupla marcação de Nestina e EdU (adicionado ao meio de cultivo 16 horas antes da fixação). Verificou-se uma massiva proliferação das CPNs sob estímulo desses GFs, visto que 77,2 ±6,7% das células Nestina^{+} incorporaram EdU (Figura 4.8).

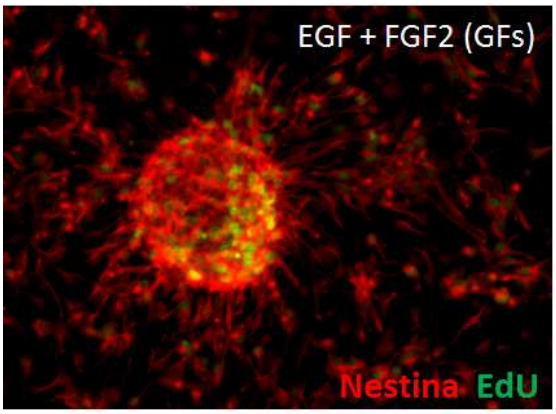


Figura 4.8: Análise da proliferação de CPNs indiferenciadas na presença de EGF e FGF2. A neuroesfera é formada a partir de uma única célula precursora neural que prolifera na presença de EGF e FGF2 por cinco dias. No quarto dia o EdU foi adicionado ao meio de cultura e as células proliferando o incorporaram. Após 16 horas, as amostras foram fixadas e marcadas para esse nucleotídeo e Nestina (presente em CPNs) conforme descrito na metodologia. A imagem é representativa de três experimentos independentes e foi adquirida com o aumentado de 100 vezes.

Em seguida avaliou-se o efeito da BK sobre a proliferação de CPNs indiferenciadas estimuladas com os GFs EGF e FGF2 (ambos na concentração de 20ng/mL). Os GFs foram adicionados ao meio de cultura 1 hora antes da adição de BK (1µM), e então permaneceram estimulando as células por 24 horas. A Bk resultou em significativa redução da proliferação em comparação a amostras tratadas somente com os GFs, visto que, a incorporação de BrdU nas últimas 2hs de cultivo foi expressivamente menor, de 29,6 ± 1,5% para 18,5 ± 4,2% das células (Figura 4.9). Observa-se também que as células não tratadas (controle; CTL) e tratadas somente com BK proliferaram pouco e não houve diferenças significativas entre elas.

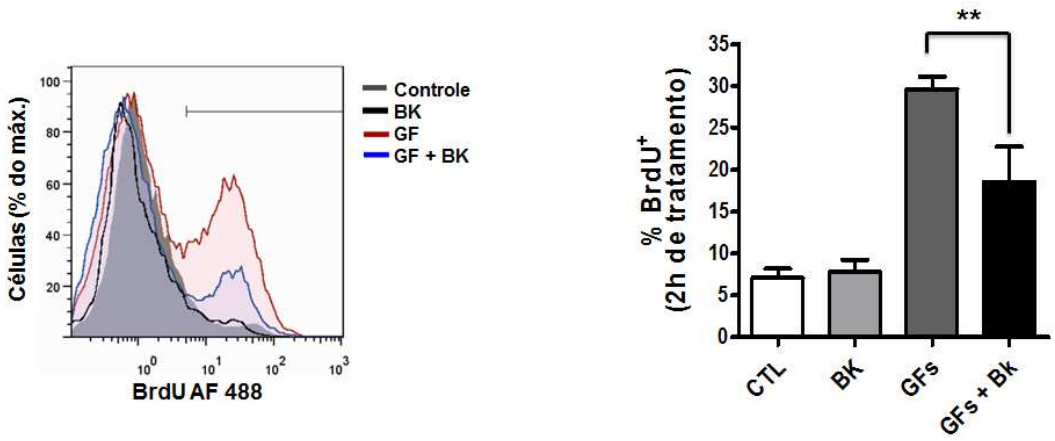


Figura 4.9: Análise da proliferação de CPNs indiferenciadas na ausência de tratamento, na presença de BK, GFs e GFs+BK. Os fatores foram adicionados ao meio de cultura onde permaneceram por 24 horas. Duas horas antes de completar 24 horas o BrdU foi também adicionado. As células foram então fixadas e marcadas para esse nucleotídeo conforme descrito na metodologia. Os resultados foram analisados no *software* Flowjo V10 e são representativos de cinco experimentos independentes (**P* < 0,05; ***P* < 0,01).

Avaliamos também os efeitos de BK sobre o ciclo celular de CPNs indiferenciadas. As neuroesferas foram expostas aos fatores e a BK por 24 horas e foram fixadas após 30 min de tratamento com BrdU para marcação da fase S. Observa-se que o tratamento com BK em células estimuladas com GFs diminuiu a proporção de células nas fases S e G_2/M do ciclo celular conforme os dados apresentados na forma de *density-plots* na Figura 4.10A e as análises estatísticas na Figura 4.10B. Vê-se também, uma maior proporção de células nas fases G_1/G_0 quando as amostras são tratadas com GFs + BK em comparação ao tratamento somente com GFs conforme sumarizado na figura 4.10C.

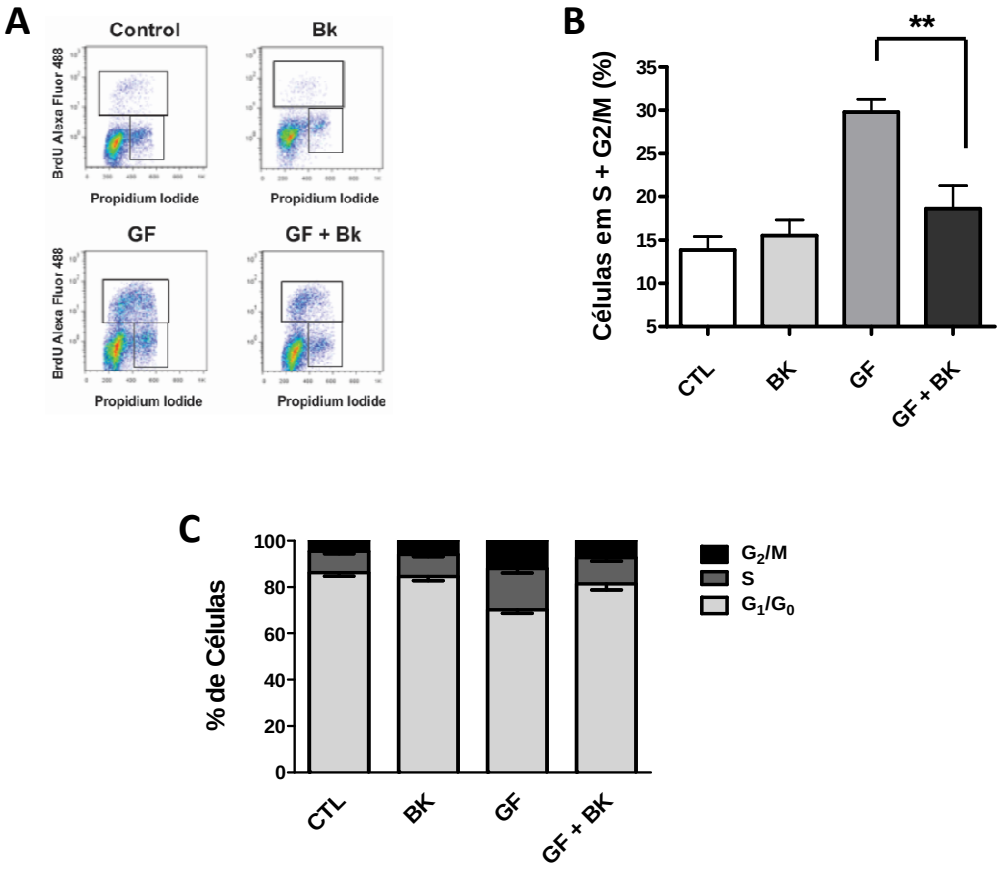


Figura 4.10: Análise do ciclo celular de células indiferenciadas na ausência de tratamento, na presença de BK, GF e GF+BK. Após 24 horas do tratamento com os fatores, o BrdU foi adicionado ao meio de cultura por 30 minutos para a incorporação no DNA de células na fase S do ciclo celular. Posteriormente, as células foram dissociadas, fixadas e marcadas com anti-BrdU conjugado a Alexa Fluor 488 e iodeto de propídio (marcação do DNA total) e analisadas no citômetro de fluxo conforme descrito na metodologia.
Os resultados foram analisados no software WinMDI 2.9 e são representativos de 5 experimentos independentes (*$P < 0,05$; **$P < 0,01$).

Cabe relembrar que, conforme abordado na subseção 1.1, CPNs que fazem divisão proliferativa possuem um ciclo celular mais curto (devido à fase G_1 abreviada) que o ciclo das CPNs que fazer divisão para diferenciação (Calegari et al, 2005; Orford & Scadden, 2008). Interessantemente, a diferenciação neuronal dessas células foi induzida pelo prolongamento da fase G_1 do ciclo celular (Calegari & Huttner, 2003; Neganova et al, 2009), suportam a hipótese que essa modificação no ciclo trata-se de um mecanismo indutor da neurogênese (Lako et al, 2009).

Dessa forma, procuramos identificar em qual fase do ciclo celular, G_0 ou G_1, as CPNs estimuladas com BK na presença de GFs encontram-se prioritariamente. Para essa investigação, as células foram tratadas como descrito anteriormente e marcadas com PI e Ki67. Observou-se que a adição de BK as células previamente estimuladas com esses fatores, responsáveis por manter a multipotência dessas células, resultou em um acúmulo de células na fase G_1 do ciclo celular em comparação as amostras tratadas somente com os GFs. (Figura 4.11B). As análises de todas as fases do ciclo juntas são mostradas na Figura 4.11C.

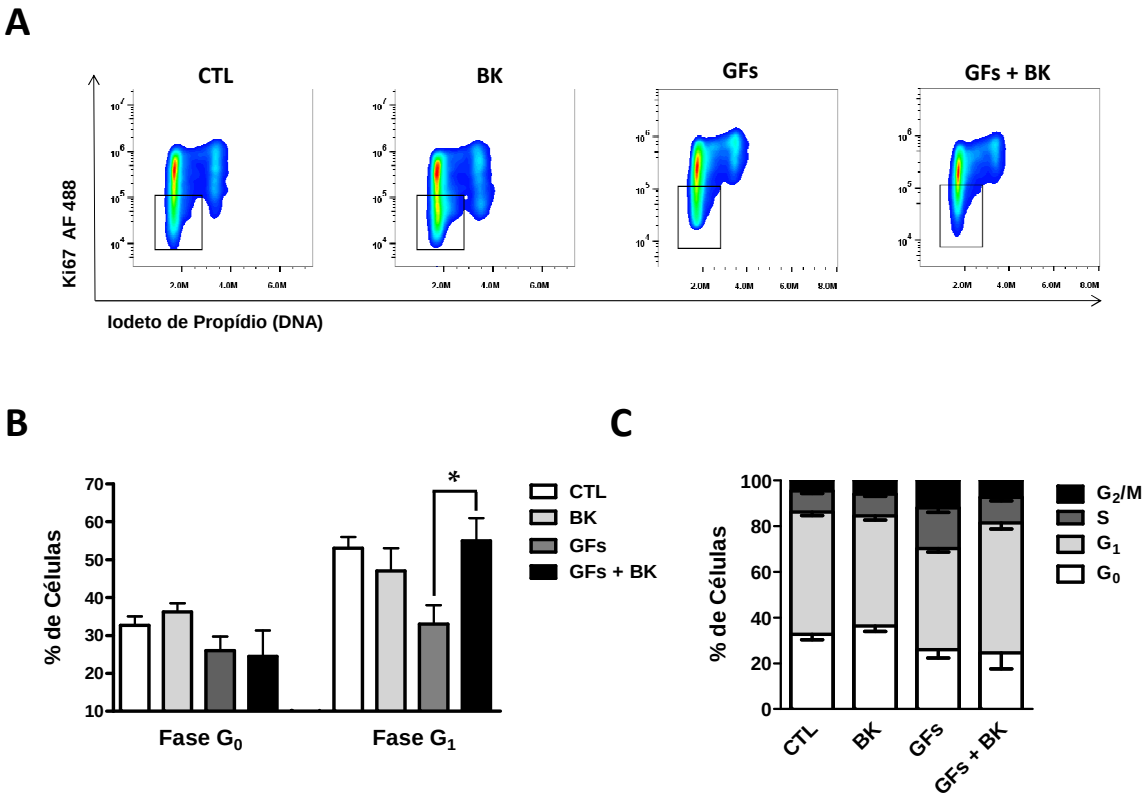


Figura 4.11: Análise das fases G_0 e G_1 do ciclo celular em CPNs indiferenciadas. As amostras foram tratadas ou não por 24 horas, dissociadas, fixadas, marcadas com Ki67 e PI (marcação do DNA total) e avaliadas no citômetro de fluxo conforme descrito na metodologia. Dentro dos *gates* estão as células em G_0 ($KI67^{-}PI^{low}$), acima dos *gates* estão as células em G_1 ($KI67^{+}PI^{low}$). Os dados foram analisados no software Flowjo V10 e são representativos de três experimentos independentes. (*$P < 0,05$).

Em suma, esses resultados são relevantes e vão de encontro com os efeitos da BK no favorecendo a diferenciação neuronal. O aumento da proporção de células na fase

G_1 do ciclo celular pode tratar-se de um mecanismo indutor da neurogênese ou um pré-requisito para tal processo utilizado pela BK na diferenciação de CPNs.

4.4 VIAS DE SINALIZAÇÃO ACIONADAS POR BRADICININA EM CPNS

Tendo em vista as recentes descobertas quanto aos efeitos da BK na diferenciação neural a identificação de sua via, ou possíveis vias, de transdução de sinais em CPNs bem como, os mecanismos de indução de seus efeitos tornaram-se imprescindíveis.

Inicialmente, o clássico segundo mensageiro AMPc foi avaliado em CPNs. Para tal, a cinética de produção de AMPc intracelular na presença de BK foi avaliada pelo kit *cAMP Biotrak enzymeimmunoassay system***.** Observa-se uma estimulação rápida na produção de AMPc, dentro de 1 e 2 minutos, com posterior retorno da concentração desse segundo mensageiro aos níveis basais (figura 4.12). Estes dados demonstram que a BK resulta na ativação da enzima adenilato ciclase (AC) que produz AMPc.

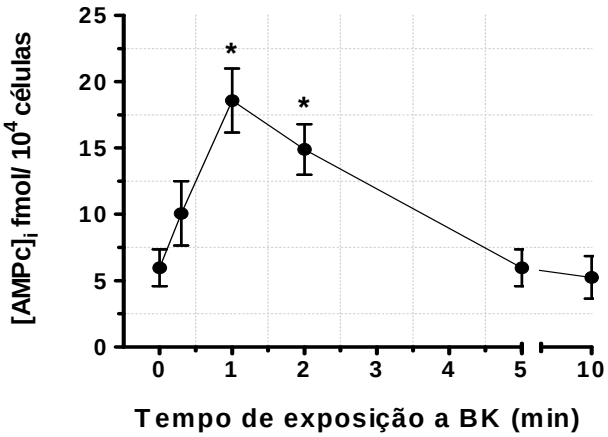
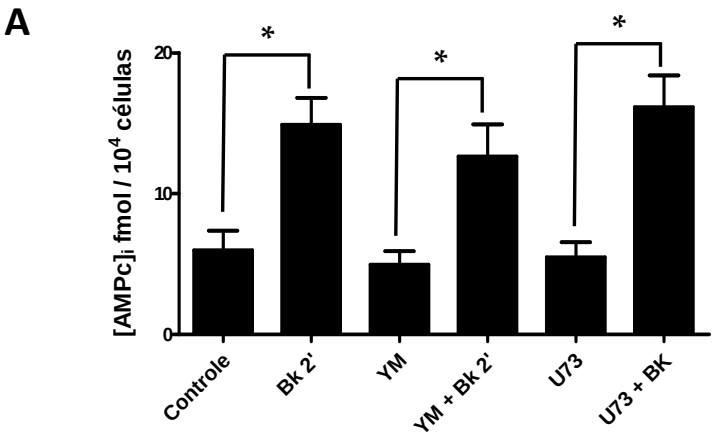


Figura 4.12: Cinética de produção de AMPc na presença de BK em CPNs. 1 x 10^4 células foram transferidas para placas de 96 poços em meio DMEM/F12 suplementado com 0,5% de B-27 um dia antes das medidas de AMPc intracelular. No dia seguinte, as células foram estimuladas com 1 µM de BK por 30 segundos, 1, 2, 5 e 10 minutos e foram imediatamente lisadas seguindo o protocolo de imunoensaio, Biotrak. Os dados são representativos de três experimentos independentes conduzidos em duplicata (*P < 0,05).

Posteriormente, investigamos se a estimulação da adenilato ciclase, após estímulo com BK, dá-se pela ação de proteínas G e qual família dessas. Inicialmente avaliamos a influência da proteína Gq e sua via a jusante que passa pela PLCβ. Cabe ressaltar que mesmo que a proteína Gq não tenha ação direta sobre a adenilato ciclase (AC), muitas proteínas a sua jusante, como a PKC e a calmodulina, sabidamente são capazes de modular a atividade de vários subtipos dessa enzima (Pyne et al, 1994). Assim, as células foram tratadas com o inibidor da proteína Gq YM-254890 (YM) na concentração de 10µM e o inibidor da PLC U73122 (U73) na concentração de 10 µM por 15 e 30 minutos, respectivamente (Figura 4.13A). Em seguida, algumas amostras foram estimuladas com BK (1µM) por 2 minutos e a concentração de AMPc intracelular ($[AMPc]_i$) foi determinada. Observa-se que o tratamento prévio com YM e U73 não impediu o aumento, induzido por BK, na concentração desse segundo mensageiro, sugerindo que a proteína Gq e sua via a jusante não mediam a estimulação da adenilato ciclase induzida por BK.



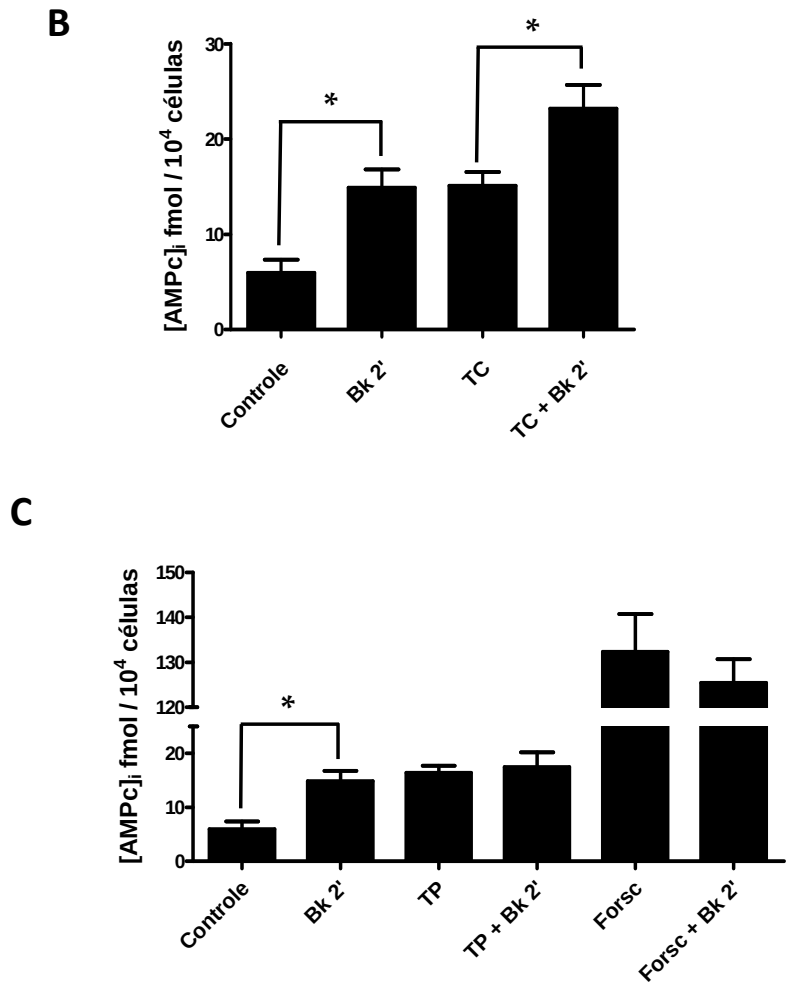


Figura 4.13: Produção de AMPc estimulada por BK na presença de moléculas que interferem na atividade das proteínas G. A) Análise da influência da proteína Gq e sua via a jusante na produção de AMPc estimulada por BK. Quando especificado, as células foram tratadas com o inibidor da proteína Gq YM-254890 (YM) na concentração de 10µM por 15 min e o inibidor da PLC U73122 (U73) na concentração de 10 µM por 30 minutos**.** Em seguida algumas amostras foram estimuladas com 1µM de BK por 2 minutos e foram imediatamente lisadas para detecção da $[AMPc]_i$**.** B) Análise se o receptor B2 de cininas acopla-se a proteína Gs induzindo a produção de AMPc. As células foram tratadas com a toxina cólera (TC) na concentração de 0,5µg/mL por 16 horas e com BK por 2 minutos. C) Estudo se o receptor B2 de cininas induz ativação da adenilato ciclase via subunidade βγ da proteína Gi. Quando especificado, as células foram tratadas com a toxina pertussis (TP) na concentração de 200ng/mL por 16 horas ou com forscolina na concentração de 50µM por 2 horas e/ou com BK por 2 minutos. As células foram então imediatamente lisadas para detecção da $[AMPc]_i$ seguimento o protocolo de imunoensaio, Biotrak, descrito na metodologia. Os dados são representativos de três experimentos independentes conduzidos em duplicata (*$P < 0,05$).

Em sequência, investigamos se o receptor B2 de cininas poderia estar acoplado à família de proteínas Gs em CPNs, classicamente conhecidas por ativar muitos tipos de adenilato ciclase. Para tanto, as células foram tratadas por 16 horas com a toxina cólera (TC) (0,5µg/mL) que impede a atividade GTPase das proteínas Gs e portanto, as mantem

na forma ativa ($G_{\alpha S}GTP$). Após 16 horas de exposição das células a TC espera-se que todas as proteínas Gs estejam na forma ativa e, portanto, estímulos que induzem a produção de AMPc via Gs, passaram a não alterar mais os níveis de AMPc. Por outro lado, estímulos que ativam as ACs de maneira independente da proteína Gs, permaneceram aumentando os níveis de AMPc em comparação ao tratamento somente com TC. Assim, as amostras foram então estimuladas ou não com BK (1µM) por 2 minutos e a $[AMPc]_i$ foi determinada. Observa-se na letra B da figura 4.13 que o tratamento prévio com TC não impediu o aumento, induzido por BK, na concentração desse segundo mensageiro, sugerindo que o receptor B2 de cininas não está acoplado à proteína Gs em CPNs.

Por fim, investigamos se a estimulação da AC, após estímulo com BK, dá-se pela ação das subunidades βγ da proteína Gi. Estas subunidades podem ativar a AC II, IV e VII e, assim como a subunidade $G\alpha_i$, são sensíveis a toxina pertussis (TP) (Chen et al, 1995). Dessa forma, as células foram tratadas com TP na concentração de 200ng/mL e forscolina na concentração de 50µM por 16 e 2 horas, respectivamente (Figura 4.13C). A forscolina ativa todos os tipos de ACs. Em seguida algumas amostras foram estimuladas com BK (1µM) por 2 minutos e a $[AMPc]_i$. Observa-se que o tratamento prévio com TP impediu o aumento da $[AMPc]_i$ estimulado por BK. Estes dados indicam que o receptor B2 de cininas está acoplado a proteína Gi e que provavelmente suas subunidades βγ induzem a ativação da AC e a produção de AMPc. O tratamento com forscolina, como esperado, aumentou a concentração desse segundo mensageiro e foi usado como controle positivo. A adição de BK às células previamente tratadas com forscolina não alterou significativamente a $[AMPc]_i$ em comparação ao tratamento com apenas forscolina.

Estudos do nosso laboratório, já demonstraram que a Bk induz elevações na concentração de cálcio intracelular livre ($[Ca^{2+}]_i$) através da ativação do seu receptor B2 de cininas em dois modelos de diferenciação neural, neuroesferas de rato e P19 (Martins et al, 2005; Trujillo et al, 2012). Sendo assim, aqui também avaliamos a $\Delta[Ca^{2+}]_i$ após estímulo com BK em CNPs murinas através da utilização do indicador fluorescente da concentração de Ca^{2+} intracelular livre, Fluo 3-AM.

As CPNs foram dissociadas e transferidas para placas de 24 poços para que as mesmas pudessem aderir e para que a análise de células individualizadas pudesse ser realizada. Após 6 horas em meio DMEM-F12 com somente 0,5% de B27, o Fluo 3-AM foi adicionado ao meio de cultura seguindo o protocolo descrito na metodologia. As células foram então estimuladas com BK na concentração de 1µM (a seta na Figura 5.14 indica o momento da aplicação de BK). Os resultados mostram que essa cinina produz um aumento na $[Ca^{2+}]_i$ demostrando que esta também é uma via de sinalização ativada por BK em CNPs.

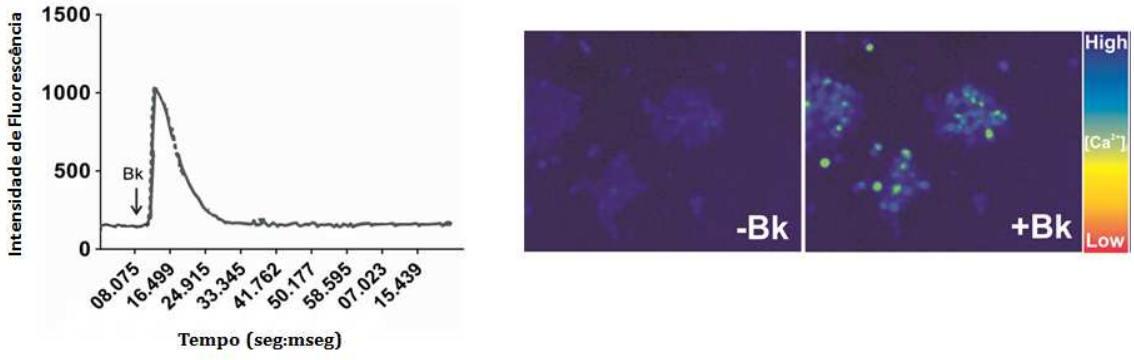


Figura 4.14: Elevação transiente na $[Ca^{2+}]_i$ induzida por BK em CPNs indiferenciadas. As células foram carregadas com o indicador sensível a Ca^{2+}, Fluo-3AM, por 30 minutos. Posteriormente elas foram expostas a BK (1µM) sob monitoramento usando imageamento de $[Ca^{2+}]_i$ através do microscópio ECLIPSE-TiS (Nikon, Melville, NY). A seta indica o momento da aplicação da BK. Os dados mostrados são os valores de fluorescência ao longo do tempo de um experimento representativo. Imagem demonstrando fluorescência no estado basal (esquerda) e depois do estímulo com BK (direita) de um experimento representativo.

A fim de delinear a cascata de sinalização que leva o aumento na $[Ca^{2+}]_i$ pela BK, utilizamos inibidores da proteína Gq e Gi e avaliamos a resposta a BK nessas condições. Em outras palavras, as CPNs foram pré-incubadas com: YM-254890 (YM; 10µM); toxina pertussis (TP; 200ng/mL); YM + TP (Figura 4.15). Surpreendentemente, os tratamentos somente com YM ou somente com TP inibiram parcialmente e significativamente a resposta a BK, como pode ser observado no gráfico (médias ± SEM) e nos traçados representativos. Por outro lado, o tratamento conjunto com os inibidores de Gq (YM) e Gi (TP) bloqueou o aumento na $[Ca^{2+}]_i$ pela BK, sugerindo novamente que ambas as proteínas G estão acopladas ao receptor B2 de cininas e que são mediadoras do aumento na $[Ca^{2+}]_i$.

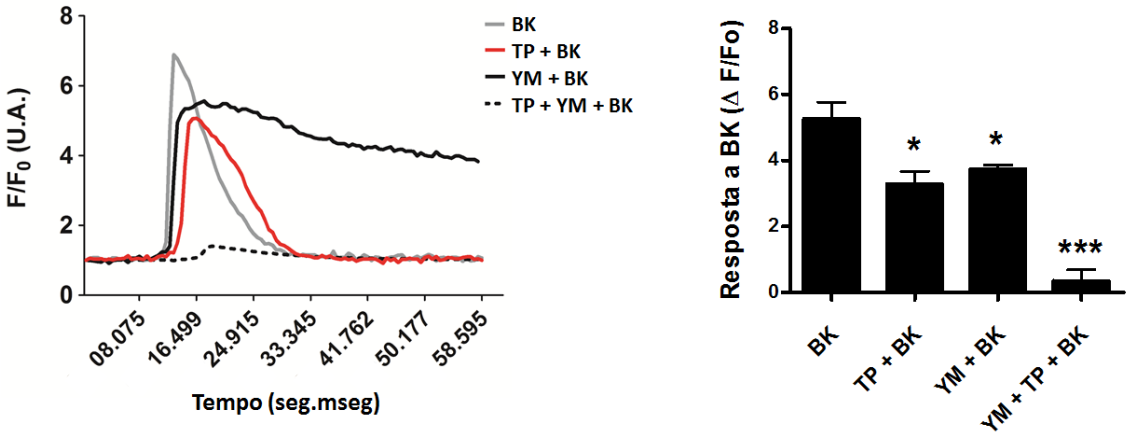


Figura 4.15: Elevação na $[Ca^{2+}]_i$ induzida pela BK na presença de moléculas que interferem na atividade das proteínas Gq e Gi em CPNs. As células foram carregadas com o indicador sensível a Ca^{2+}, Fluo-3AM, por 30 minutos. Quando especificado, as células foram previamente tratadas com o inibidor da proteína Gq YM-254890 (YM) (10µM) por 15 min ou com a toxina pertussis (TP) (200ng/mL) por 2 horas ou com ambos, YM + TP. Posteriormente elas foram expostas a BK (1µM) sob monitoramento usando imageamento da $[Ca^{2+}]_i$ através do microscópio ECLIPSE-TiS (Nikon, Melville, NY). Os dados mostrados a esquerda são os valores de fluorescência ao longo do tempo de um experimento representativo, cada traçado indica um tratamento diferente. A direita valores médios ± SEM são mostrados de 3 experimentos independentes, conduzidos em duplicata e avaliando-se aproximadamente 40 células para cada duplicata. (*P < 0,05; **P < 0,01; ***P < 0,001).

Observamos que o tratamento com YM inibiu o retorno da $[Ca^{2+}]_i$ aos níveis basais após estímulo com BK. No entanto, no presente trabalho não temos a pretensão de

avaliar detalhadamente a influência da proteína Gq ou do efeito inespecífico da droga YM no retorno a homeostase de $[Ca^{2+}]_i$ após tratamento com BK.

Dando sequência ao estudo da via que leva ao aumento na $[Ca^{2+}]_i$ induzido por BK, investigamos se esse aumento dá-se pela mobilização dos estoques de cálcio intracelulares ou pela ativação de canais de cálcio na membrana plasmática ou por ambos. Para tal, utilizamos a tapsigargina, um inibidor da bomba Ca^{+2}-ATPase SERCA (*sarcoplasmic/endoplasmic reticulum Ca2+-ATPase*) localizada na membrana dos retículos sarcoendoplasmáticos (RS). Essa bomba é responsável por manter essas organelas com altos níveis de cálcio no seu interior e, portanto, a inibição da SERCA pela tapsigargina impede o estoque desse íon no RS. Sabendo disso, as CPNs foram tratadas com tapsigargina na concentração de 1µM por uma hora. Após tal período, a BK (1µM) foi adicionada sob monitoramento usando imageamento de $[Ca^{2+}]_i$ através do microscópio ECLIPSE-TiS. Na figura 4.16 podemos observar que o tratamento prévio com tapsigargina bloqueou completamente o aumento na $[Ca^{2+}]_i$ induzida por BK, demonstrando que esse aumento ocorre pela mobilização dos estoques de cálcio intracelulares.

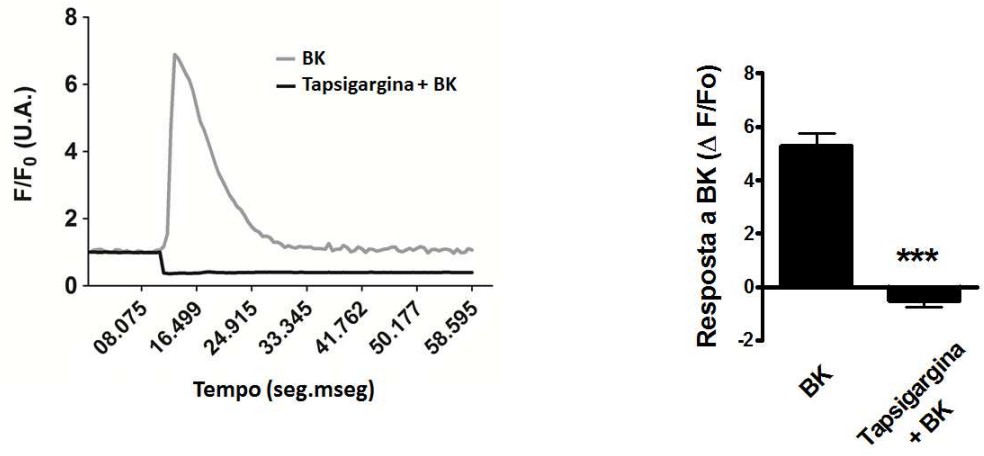


Figura 4.16: A Bk induz mobilização dos estoques de Ca^{2+} dos retículos sarcoplasmático em CPNs. As células foram carregadas com o indicador sensível a Ca^{2+}, Fluo-3AM, por 30 minutos. Quando especificado, as células foram previamente tratadas com tapsigargina na concentração de 1µM por 1 hora. Posteriormente elas foram expostas a BK (1µM) sob monitoramento usando imageamento da $[Ca^{2+}]_i$ através

do microscópio ECLIPSE-TiS (Nikon, Melville, NY). A esquerda observa-se os valores de fluorescência ao longo do tempo de um experimento representativo, cada traçado indica um tratamento diferente. Os dados mostrados são os valores da $\Delta[Ca^{2+}]_i$ médias ± SEM de 3 experimentos independentes (*P < 0,05; **P < 0,01; ***P < 0,001).

Além dos segundos mensageiros AMPc e Ca^{2+}, investigamos também algumas vias canônicas de transdução de sinais possivelmente ativadas pela BK a nível de moléculas efetoras (quinases). Para isso, as células foram dissociadas como descrito na metodologia e mantidas em meio de cultura com 0,5% do suplemento B-27 por 6 horas. Após esse período, as células não foram estimuladas (controle) ou foram estimuladas com 1µM de BK por 10 minutos. As amostras foram então imediatamente fixadas e marcadas com os anticorpos anti- p-STAT3, p-JNK, p-Akt e p-p38 (Tabela 3.1) como descrito na metodologia. Sob as condições experimentais descritas a cima, as amostras tratadas com BK não apresentaram diferenças significativas na expressão das enzimas fosforiladas JNK e STAT3 quando comparadas com as amostras não tratadas (Figura 4.17). Em contraposição, observou-se um maior nível de p-p38 e p-Akt após estímulo com esta cinina, demonstrando que estas são vias de transdução de sinal ativadas pela BK em CPNs (Figura 4.17). As porcentagens de células que são ou se tornaram positivas para as fosfo-quinases em comparação as amostras negativas (marcadas com anticorpo controle de isotipo e secundário) são mostradas na figura 4.17B. Já as intensidades de fluorescência mediana (MFI - *Median Fluorescence Intensity*), que fornece informações a respeito da expressão mediana das fosfo-enzimas em toda população celular, são mostradas na letra C. Em ambas as análises, observa-se que os resultados se assemelham muito.

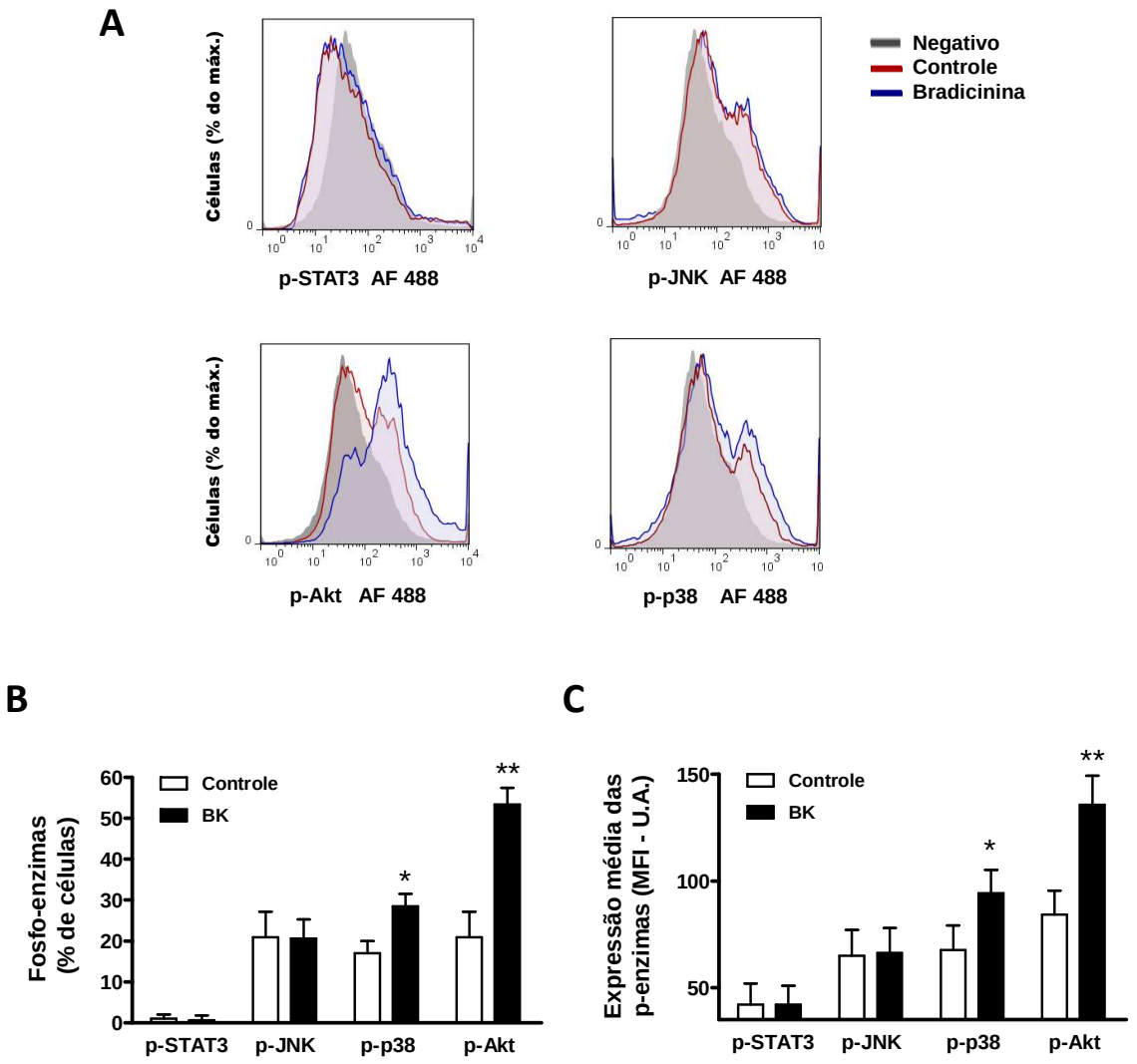


Figura 4.17: Bradicinina não induz ativação da STAT3 e JNK, mas induz ativação de p38 e Akt em CPNs. Algumas amostras não receberam tratamento (controle) enquanto outras foram tratadas com 1µM de bradicinina (BK) por 10 minutos. As células foram imediatamente fixadas e marcadas com o anticorpo controle de Isotipo, p-STAT3, p-JNK, p-p38 ou p-Akt. Posteriormente, as amostras foram marcadas com anticorpo secundário Alexa Fluor 488 seguindo o protocolo de citometria de fluxo. A quantificação dos eventos positivos para fosfo-quinases foi determinada pelo tracejado da diferença entre as amostras e o controle de isotipo (B), já o nível de expressão dessas enzimas fosforiladas foi estimado pela intensidade de fluorescência mediada (MFI) das amostras (C). Estes dados foram analisados no *software* Flowjo V10 (**P* < 0,05; ***P* < 0,01).

Até o momento, verificamos que a BK ativa um conjunto de vias de sinalização em CPNs indiferenciadas. Nesse contexto, é sabido que varias vias de sinalização bem conhecidas, como a PI3K, PKC, Ca^{2+}, p38, AMPc podem regular a ativação de MEK/ERK ou ainda, integrar seus sinais nessa cascata. Segundo Murray (1998), a cascata da MAPK

parece ser mantida durante a evolução como um ponto integrador, sendo que as formas de ativação e os componentes ativados foram sendo diversificados bem como os efetores ativados por ela.

Com base nas informações apresentados no parágrafo acima e sabendo que a ERK pode controlar processos como proliferação e diferenciação neuronal, avaliamos a influência da BK sobre essa MAPK de forma mais aprofundada. Inicialmente, utilizou-se o ester de forbol PMA (*phorbol 12-myristate 13-acetate*) como controle positivo e os inibidores da MEK U0126 e PD98059 como controles negativos dos ensaios. Verificou-se que estes últimos estão efetivamente inibindo a via MEK/ERK, já que foram observados níveis extremamente baixos de ERK ativada. Como apresentado na Figura 4.18, verificou-se que a BK ativa a cascata da ERK após tratamento com 1 µM dessa por 10 minutos. Este tratamento faz com que a grande maioria das células torne-se positiva para p-ERK (4.18B) e o nível de expressão médio dessa fosfo-quinase aumente (4.18C) em relação ao controle.

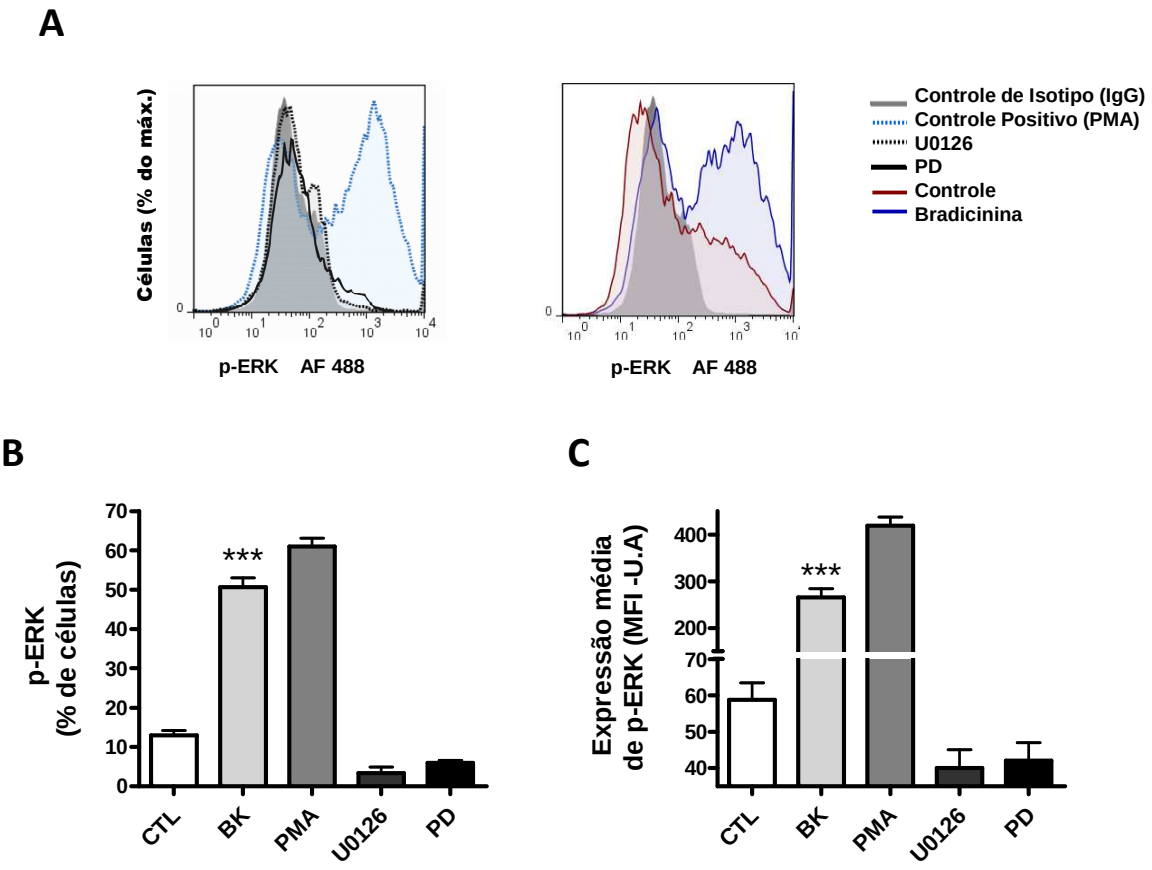


Figura 4.18: **Bradicinina induz ativação da MAPK ERK em neuroesferas.** Algumas amostras não receberam tratamento (controle; CTL) enquanto outras foram tratadas com 1µM de bradicinina (BK), 1µM de PMA (controle positivo / phorbol 12-myristate 13-acetate) por 10 minutos, 10µM de U0126 (controle negativo / inibidor MEK/ERK) por 1 hora e 20µM de PD98059 (inibidor MEK/ERK) por 1 hora, . As células foram imediatamente fixadas e marcadas com o anticorpo controle de isotipo (IgG coelho) ou anti- p-ERK. Posteriormente as amostras foram marcadas com o anticorpo secundário Alexa Fluor 488 seguindo o protocolo de citometria de fluxo. A quantificação dos eventos positivos para p-ERK foi determinada pelo tracejado da diferença entre as amostras e o controle de isotipo (B), já o nível de expressão médio dessas amostras foi estimado pela identificação de seus respectivos MFI (C) fornecidos pelo *software* Flowjo (**P* < 0,05; ***P* < 0,01; ****P* < 0,001).

Para avaliarmos se os efeitos de BK dão-se de forma específica através da sua ligação no receptor B2 de cininas, realizamos o ensaio de fosforilação de ERK com neuroesferas obtidas de camundongos *Knockout* para esse receptor. Observamos que a BK não apresentou efeito algum sobre a fosforilação dessa quinase em comparação ao controle não tratado. O PMA (controle positivo), por outro lado, que é um análogo sintético do diacilglicerol capaz de atravessa livremente a membrana plasmática,

aumentou os níveis observados de p-ERK. Esses dados demonstrando que a atividade de BK se dá através da ligação do receptor B2 de cininas.

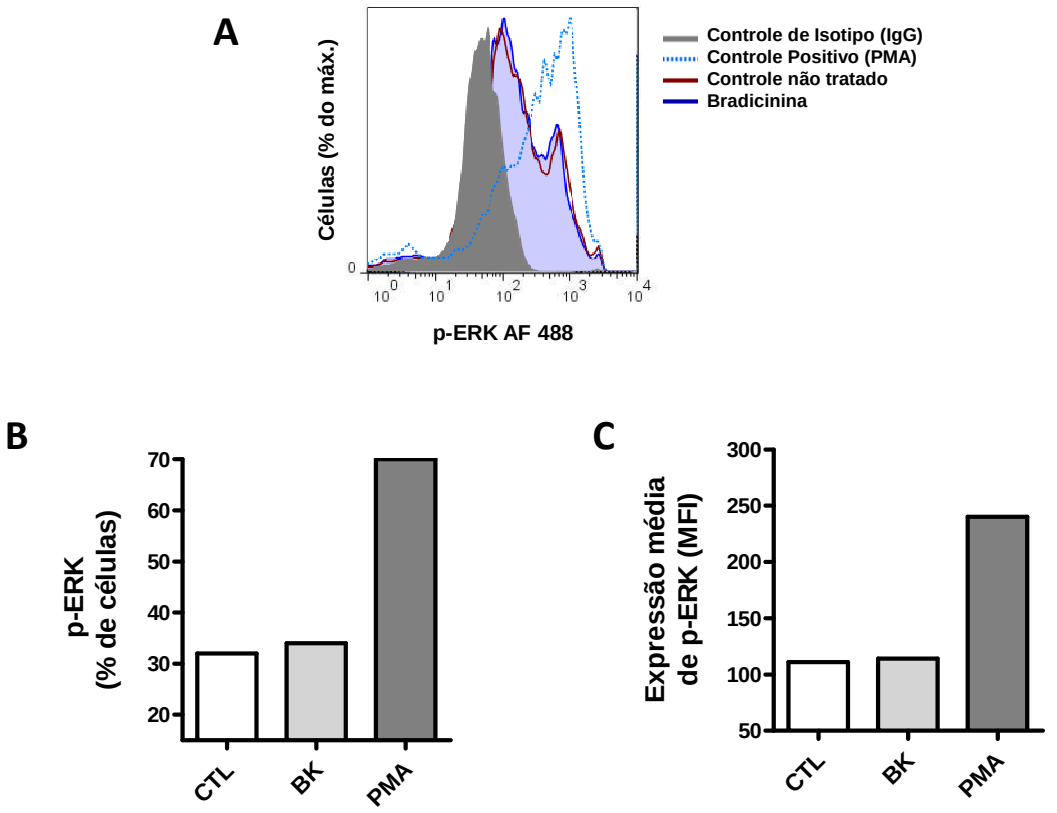


Figura 4.19: **Bradicinina não induz ativação da MAPK ERK em neuroesferas B2BkR knockout. A)** As amostras não receberam tratamento (controle) ou foram tratadas com 1µM de bradicinina ou 1µM de PMA (controle positivo / phorbol 12-myristate 13-acetate) por 10 minutos. As células foram imediatamente fixadas e marcadas com o anticorpo controle de isotipo (IgG coelho) ou anti-p-ERK. Posteriormente as amostras foram marcadas com o anticorpo secundário Alexa Fluor (AF) 488 seguindo o protocolo de citometria de fluxo. A quantificação dos eventos positivos para p-ERK foi determinada pelo tracejado da diferença entre as amostras e o controle de isotipo (B), já o nível de expressão médio ERK nessas amostras foi estimado pelos valores de MFI (C). Os dados foram analisados no *software* Flowjo.

Vários trabalhos abordam o papel duplo da ERK na proliferação *versus* diferenciação (Santos et al, 2007; Sasagawa et al, 2005). Conforme abordado na subseção 1.3.2, a ativação da MAPK por uma via ou pela integração de várias vias resulta em diferentes cinéticas e localização celular que, por sua vez, originam respostas celulares

diferentes. Em outras palavras, a duração da atividade de ERK e a sua localização celular, são pontos chaves que influenciam no efeito celular observado.

Sabe-se que esta quinase induz a proliferação quando é ativada de forma transiente (aproximadamente 5 a 10 min), e por outro lado, a ERK induz a diferenciação neuronal e supressão da proliferação quando a mesma permanece ativada por um tempo prolongado (60 min), como é o caso da ativação por NGF em células PC12 (Santos et al, 2007; Sasagawa et al, 2005).

Da mesma forma como realizado anteriormente, amostras controles e tratadas com 1µM de BK por 2, 5, 10, 15, 30 e 60 min foram fixadas e marcadas com anticorpo controle de isotipo (IgG de coelho) ou anticorpo anti-p-ERK segundo protocolo de citometria de fluxo. Interessantemente, observou-se que a BK ativa a ERK por um período longo (no mínimo 60 minutos; Figura 4.20), fornecendo forte indicativo de que os efeitos desta cinina na diferenciação e supressão da proliferação dão-se através dessa cinética.

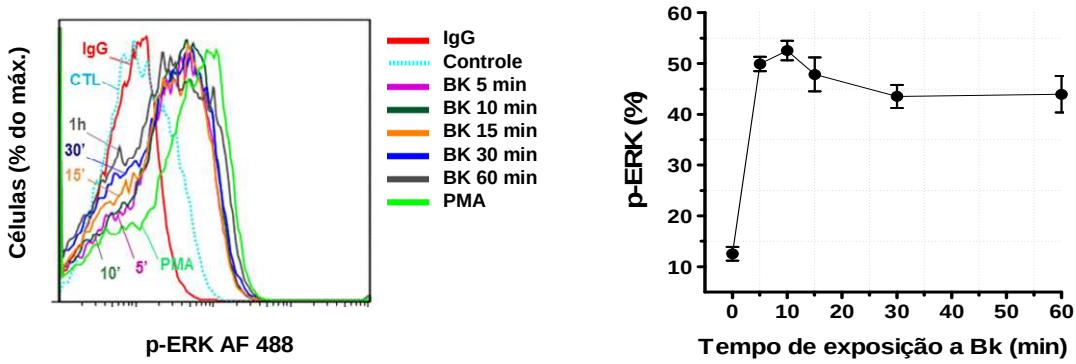


Figura 4.20: Bradicinina ativa ERK por tempo prolongado. Amostras de neuroesferas foram tratadas com 1µM de bradicinina (BK) de 2 a 60 minutos ou com 1µM de PMA (controle positivo / phorbol 12-myristate 13-acetate) por 10 minutos, e então, foram fixadas imediatamente. Em seguida as amostras foram marcadas com o controle de isotipo (IgG de coelho) ou com p-ERK e posteriormente com Alexa Fluor 488 seguindo o protocolo de citometria de fluxo. A quantificação dos eventos positivos para p-ERK foi determinada pelo *plot* da diferença entre as amostras e o controle de Isotipo. Os resultados foram analisados no software Flowjo.

Outro aspecto da topologia de ativação da ERK que determinam a resposta mitogênica ou neurogênica em células PC12 é a sua sublocalização intracelular. A ativação da MAPK por NGF leva a sua translocação nuclear, sendo esta translocação necessária para que a diferenciação ocorra, visto que regula vários fatores de transcrição importantes para tal processo. Em contraposição, a ERK permanece no citoplasma quando é ativada por EGF e outros fatore que resultam na proliferação de células indiferenciadas (Impey et al, 1999; von Kriegsheim et al, 2009).

Dessa forma, incialmente avaliamos se o estímulo com BK em CPNs aumenta a pERK nuclear por imunocitoquímica. Para tanto, as células foram dissociadas e plaqueadas em superfície aderente na ausência de fatores de crescimento e na presença de 2% de B27. Seis horas antes do experimento as células foram mantidas com apenas 0,5% de B27. Em sequência, as células não foram tratadas (controle; CTL) ou foram tratadas com BK (1µM) por 15 minutos quando foram imediatamente fixadas e marcadas para p-ERK, Nestina e DAPI. O merge (sobreposição) das imagens com as marcações p-ERK + Nestina e p-ERK + DAPI permitiu a visualização da MAPK nuclear e citoplasmática, respectivamente (Figura 4.21). Dez imagens foram obtidas e mil células foram analisadas para cada uma das duas condições experimentais. Como observado nas imagens ilustrativas e nos gráficos da Figura 4.21, após exposição à BK, a proporção de células expressando níveis detectáveis de p-ERK no núcleo aumentou significativamente em comparação as amostras controle.

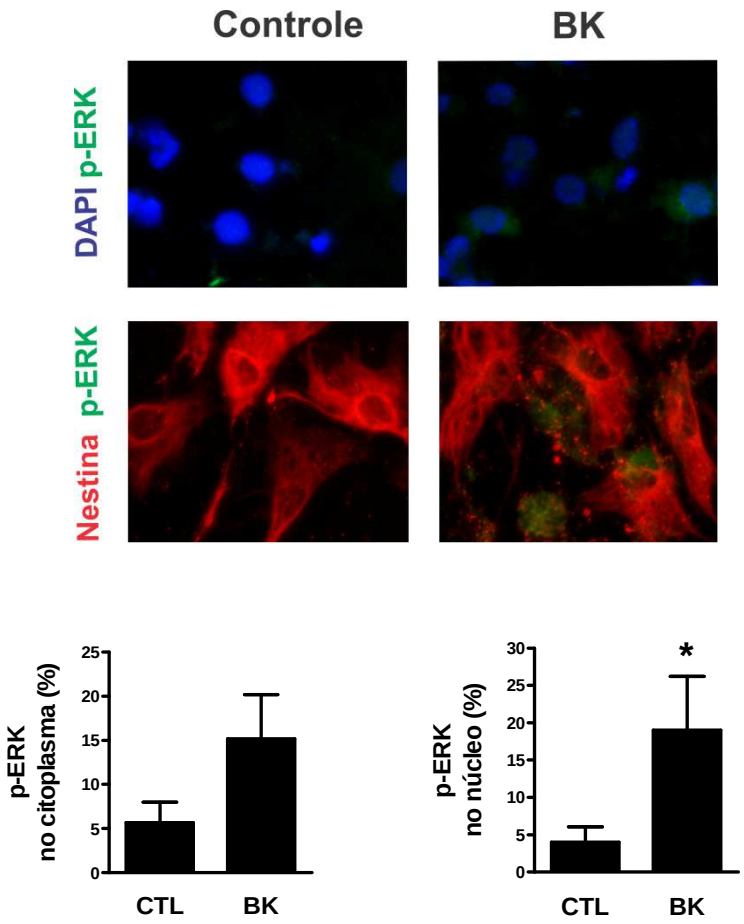


Figura 4.21: p-ERK citosólica e nuclear em CPNs não tratadas e tratadas com bradicinina. As CPNs não foram tratadas ou foram tratadas com bradicinina (BK) na concentração de 1µM por 15 minutos. Posteriormente, as amostras foram imediatamente fixadas conforme protocolo de imunocitoquímica descrito na metodologia. A proporção de células expressando níveis detectáveis de p-ERK no citoplasma e no nuclear foi estimada pela dupla-marcação de p-ERK e Nestina ou DAPI, para visualização da p-ERK nuclear e citoplasmática, respectivamente. Dez imagens foram obtidas e mil células foram analisadas para cada condição experimental com auxilio do *software* ImageJ. As imagens são representativas de 3 experimentos independentes conduzidos em duplicata e foram adquiridas com o aumentado de 200 vezes (*$P < 0,05$).

Experimentos adicionais de cinética de ativação de ERK após estímulo com BK e sua sublocalização, por fracionamento de proteínas citosólicas e nucleares, foram realizados em células PC12 e são apresentados na subseção 4.7. No entanto, até o momento, já é possível observar que em CPNs a BK induz a mesma topologia (localização e cinética) de ERK amplamente caracterizada por ser necessária e suficiente para induzia a neurogênese em células PC12.

4.5 VIAS DE SINALIZAÇÃO ACIONADAS POR BRADICININA QUE MODULAM A MIGRAÇÃO E DESTINO NEURAL DE CPNS

Identificamos que a atividade de BK em CPNs se dá através da sua ligação ao receptor B2 de cininas, que é um receptor acoplado a proteína G. Esse receptor parece acoplar-se tanto a proteína Gq quando a proteína Gi ativando a produção/mobilização dos segundos mensageiros AMPc e Ca^{2+}. Esses, por sua vez, diretamente ativam quinases que, historicamente, são denominadas moléculas efetoras. No nível de quinases, identificamos que a BK induz a ativação de p38, ERK e PI3K/Akt e não induz a ativação de JNK e STAT3. Dessa forma, optamos por avaliar a influência somente dos efetores p38, ERK e PI3K na diferenciação neural.

A p38 e a ERK são MAPK e, portanto, suas rotas dão-se em paralelo e relativamente independentes. A PI3K, no entanto, a depender do estímulo, pode localizar-se a montante de ERK. Dessa forma, avaliamos se a BK leva a ativação de ERK por intermédio de PI3K. A fosforilação dos resíduos de Thr202/Tyr204 de ERK foi avaliada na presença de um inibidor específico de PI3K, Ly294002 (Ly). As CPNs foram pré-incubadas por 30 minutos com Ly na concentração de 20µM e posteriormente foram estimuladas por 10 minutos com BK (1µM). Nenhuma diferença na ativação de ERK foi observada entre as amostras tratadas somente com BK e Ly+BK, demonstrando que a ativação da ERK pela BK ocorre de uma forma independente da atividade de PI3K (Figura 4.22A). Sabendo disso, foi possível avaliar os efeitos da BK nos processos do desenvolvimento neural que se dão via PI3K/Akt, sem influenciar na ativação de ERK.

Com os resultados da Figura 4.22A, observa-se também que o inibidor Ly não tem efeito inespecífico sobre a atividade de ERK. Já na Figura 4.22B observa-se a

funcionalidade deste inibidor visto que o mesmo bloqueou a ativação de Akt pela BK. Esse dado demonstra também que a ativação de Akt pela BK dá-se via PI3K.

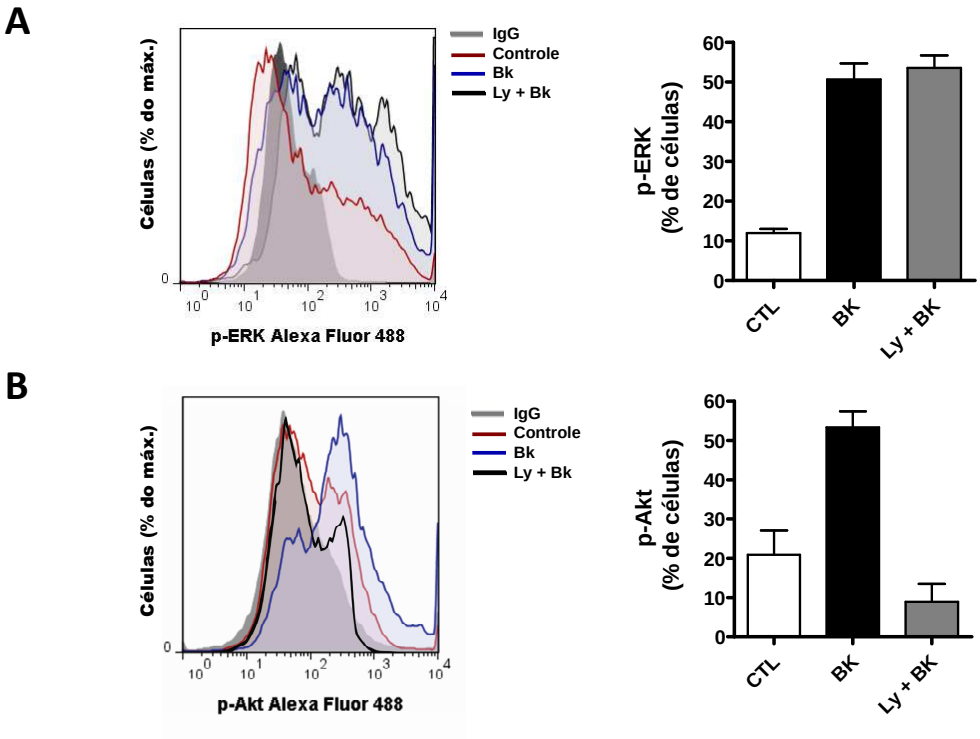


Figura 4.22: Bradicinina induz a ativação de ERK independentemente de PI3K, já a ativação de Akt é via PI3K. As amostras controle não receberam tratamento enquanto outras foram tratadas somente com 1µM de BK por 10 minutos ou com 20µM do inibidor da PI3K Ly294002 (Ly) por 30 minutos + BK. As células foram imediatamente fixadas e marcadas com o controle de isotipo (IgG rabbit), p-ERK ou p-Akt e com anticorpo secundário Alexa Fluor (AF) 488 para analise no citômetro de fluxo. A quantificação dos eventos positivos foi determinada pelo tracejado da diferença entre as amostras e o controle de Isotipo. A) Marcação para p-ERK. B) Marcação para p-AKt. Os resultados foram analisados no *software* Flowjo V10 a partir de 3 experimentos independente.

Antes de utilizarmos os inibidores farmacológicos das vias de sinalização nos ensaios de proliferação, migração e diferenciação, realizamos ensaios de viabilidade celular na presença desses. Visto que as etapas finais da apoptose são caracterizadas pela intensa degradação de DNA, realizamos o ensaio de marcação desse ácido nucléico com iodeto de propídio (PI). Este ensaio permite detectar as células com DNA fragmentado já

que essas possuem marcação para PI inferior as células com 2n (pico G_1/G_0) e, portanto, reflete a taxa de morte celular na cultura.

A análise por citometria de fluxo revelou que neuroesferas diferenciadas (controle) apresentam 23,3 ± 3,0% (média ± SD) de células com DNA fragmentado/eventos sub-G_1/G_0 (região em destaque nos histogramas) e valores semelhantes foram observados em amostras tratadas com os inibidores que foram utilizados neste trabalho U0126 (10µM), PD (20µM), Ly (20µM) e SB (10µM) conforme demonstrado na Figura 4.23. Portanto, os inibidores não induziram efeito citotóxico capaz de refletir na quantidade de células viáveis comparados aos experimentos controle. Os tratamentos com essas drogas também não resultaram em nenhuma alteração morfológica celular significativa conforme pode ser visto nas imagens de contraste de fase e imunocitoquímica no decorrer deste trabalho.

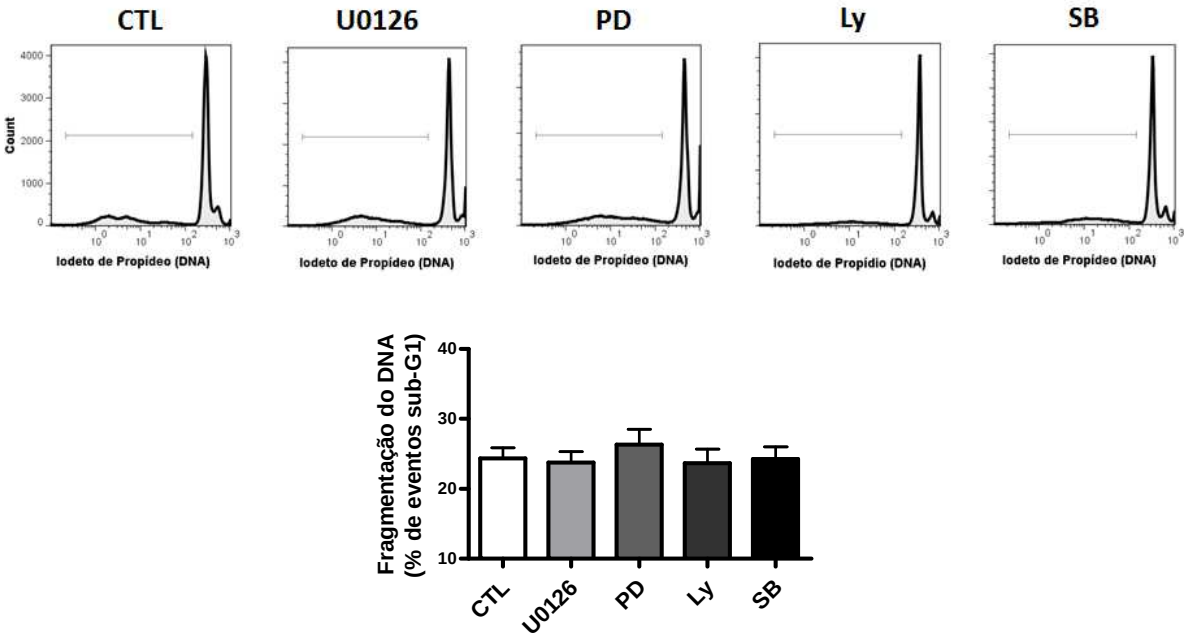


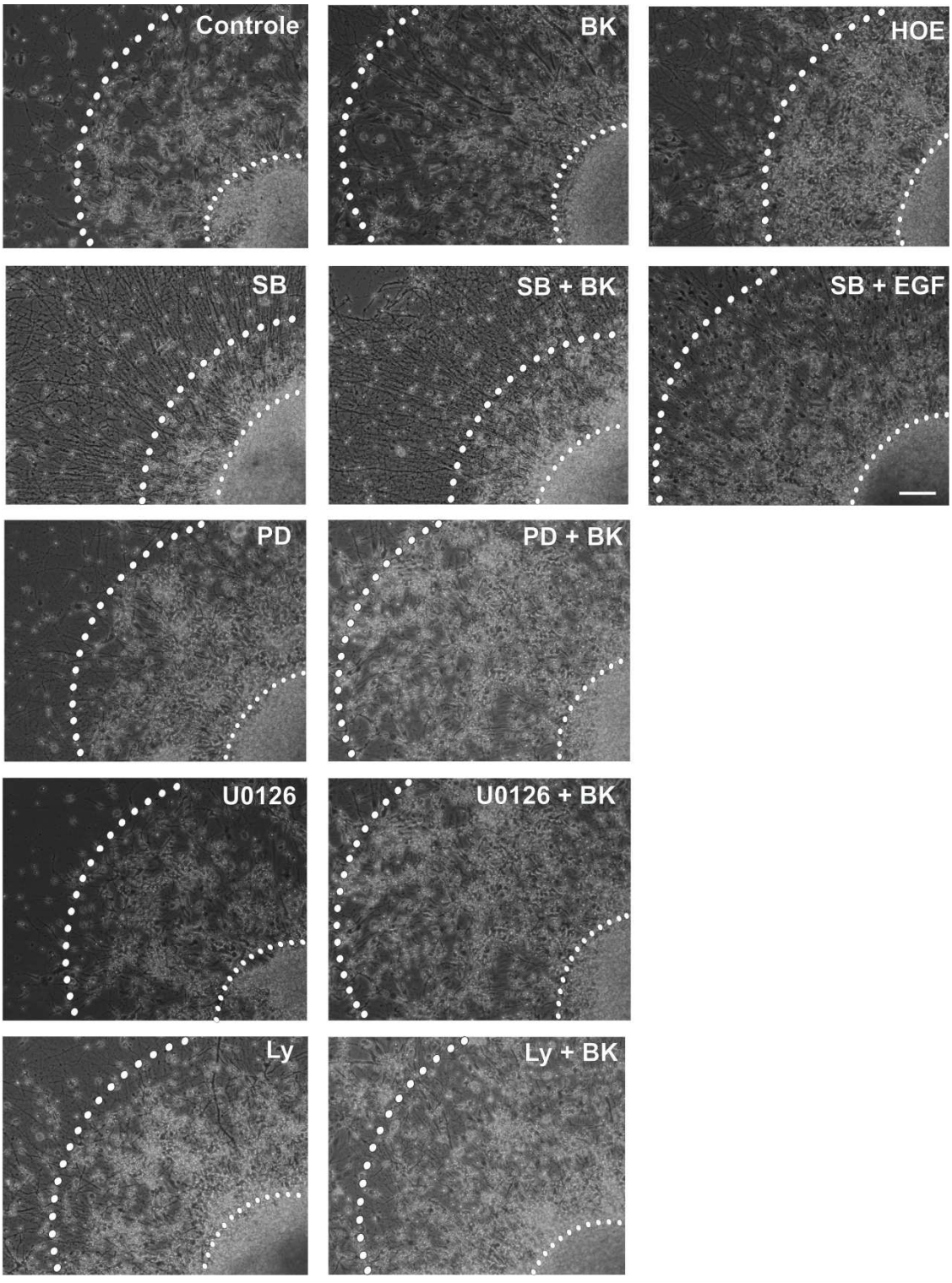
Figura 4.23: Viabilidade celular na presença de inibidores da MER/ERK (U0126 e PD) PI3K (Ly) e p38 (SB). As neuroesferas foram diferenciadas na presença de U0126 (10µM), PD (PD98059; 20µM), Ly (Ly294002; 20µM) e SB (SB203580; 10µM). Os tratamentos foram adicionados em conjunto com o meio que foi trocado a cada dois dias. Os histogramas em escala logarítmica mostram a viabilidade celular pelo ensaio de marcação com iodeto de propídeo após sete dias de diferenciação. Na escala logarítmica pode-se observar com mais clareza células mortas que possuem DNA fragmentado, ou seja, quantidades de DNA abaixo de

2n. Foram adquiridas no mínimo 30.000 células por amostra em um total de três experimentos independentes. Estes dados foram analisados no *software* Flowjo V10. Os dados mostrados são os valores médios ± SEM.

Sabendo-se que as vias, ERK, p38 e PI3K, ativam-se em paralelo após estímulo com BK e que os seus inibidores específicos não alteram a viabilidade celular durante a diferenciação, avaliamos a participação dessas vias da BK no seu efeito favorecendo a migração neural. Inicialmente, o envolvimento do receptor B2 de cininas foi novamente evidenciado, visto que o tratamento com o antagonista HOE-140 durante a diferenciação resultou no efeito oposto ao tratamento com BK, ou seja, a redução da migração neural.

Sabendo-se que as vias, ERK, p38 e PI3K, ativam-se em paralelo após estímulo com BK e que os seus inibidores específicos não alteram a viabilidade celular durante a diferenciação, avaliamos a participação dessas vias da BK no seu efeito na migração neural. Inicialmente, o favorecimento da migração induzido pelo tratamento com BK e o envolvimento do receptor B2 de cininas foram novamente evidenciado. Como pode ser visto na Figura 4.24, o tratamento com esta cinina durante a diferenciação favoreceu a migração celular a partir da borda da neuroesfera, ao passo que o tratamento com o antagonista do seu receptor, HOE-140, resultou no efeito oposto, ou seja, na redução dos níveis de migração.

Adicionalmente, na Figura 4.24 pode-se observar que a adição de BK em culturas tratadas previamente com PD (20µM) e U0126 (10µM) resulta em um maior número de células que migram maiores distâncias a partir da borda da neuroesfera em comparação ao tratamento somente com os inibidores da MEK/ERK (PD e U0126). Esses últimos não influenciam na migração das células. Com estes dados, portanto, pode-se concluir que a BK exerce seu efeito na migração de células neurais durante a diferenciação de maneira independente de ERK.


Controle
BK
HOE
SB
SB + BK
SB + EGF
PD
PD + BK
U0126
U0126 + BK
Ly
Ly + BK

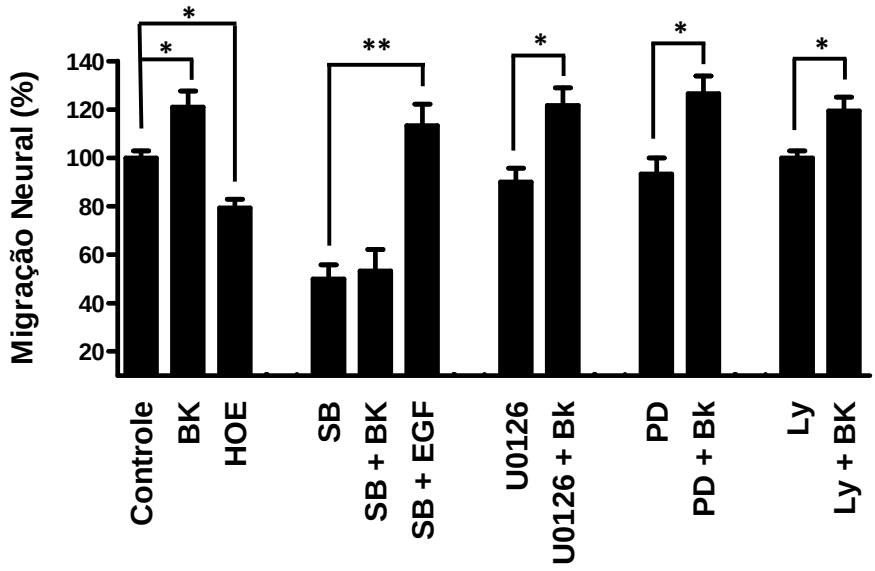


Figura 4.24 – Migração e diferenciação neural na presença de bradicinina, HOE-140, inibidores da ERK (U0126 e PD), PI3K (Ly) e p38 (SB). Imagens de contraste de fase representativas do padrão de migração radial após sete dias de diferenciação neural na presença de 10µM de U0126, 20µM de PD (PD98059), 20µM de Ly (Ly294002), 10µM de SB (SB203580), 1 µM de HOE (HOE-140) e 1 µM de bradicinina (BK). A região delimitada entre as linhas pontilhadas compreende aproximadamente 95% das células que migraram. Escala de 100 µm. Os dados mostrados são os valores médios ± SEM de no mínimo três experimentos independentes, consistindo de aproximadamente 10 imagens para cada experimento (*P < 0,05; **P < 0,01).

Resultados similares foram obtidos com o a inibição da PI3K. O inibidor Ly (20µM) não influencia na migração e permite que a BK exerça seu efeito potencializando a migração. Novamente, estes dados sugerem que a BK estimula a migração independentemente da sua via PI3K/Akt.

Contudo, na inibição da MAPK p38 pelo tratamento com SB (10µM) observa-se um número substancialmente menor de células migratórias. Interessantemente, a BK não tem efeito algum sobre a migração quando a p38 é inibida pelo tratamento com SB (Figura 4.24). Adicionalmente, como controle positivo do experimento, as células foram tratadas com SB + EGF (20ng/mL). O EGF potencializou a migração celular mesmo na presença do inibidor da p38 demonstrando que as células são capazes de migrar com a p38 inibida. Estes dados sugerem em que a Bk aumenta a migração celular durante a diferenciação por mecanismos dependentes de sua via MAPK p38.

Visto que, conforme apresentado anteriormente, a BK favorece a neurogênese em detrimento da gliogênese, fazia-se necessário ainda investigação das vias ativadas por essa cinina que determinam tal destino celular. Para este fim, as vias ERK, PI3K e p38 foram inibidas durante o processo de diferenciação.

Inicialmente a MAPK ERK foi inibida durante o processo de diferenciação. Utilizamos os inibidores farmacológicos U0126 (10µM) e PD (20µM) que foram adicionados ao meio de cultura. Relembrando, os resultados de diferenciação neural apresentados na subseção 4.1 demonstram que o tratamento crônico com BK diminui a marcação para GFAP e aumenta a marcação de β 3-Tubulina. Surpreendentemente, observa-se aqui que a inibição da rota MEK/ERK resulta no inverso, ou seja, aumenta a expressão de GFAP enquanto diminui a marcação para β 3-Tubulina (Figura 4.25). A adição de BK durante a diferenciação não reverte o efeito observado com os tratamentos U0126 e PD. Observa-se que esta cinina perde totalmente seu efeito na diferenciação neural quando a MAPK é bloqueada, demonstrando que ERK está implicada nos mecanismos efetores da glio- neurogênese impostos por BK.

Como descrito na subseção 4.1, o tratamento com BK durante a diferenciação não resulta em variação significativa na população de CPNs (Nestina^{+}). Esta ausência de efeitos da BK na população Nestina^{+} permaneceu em todas as condições avaliadas neste trabalho (dados não mostrados).

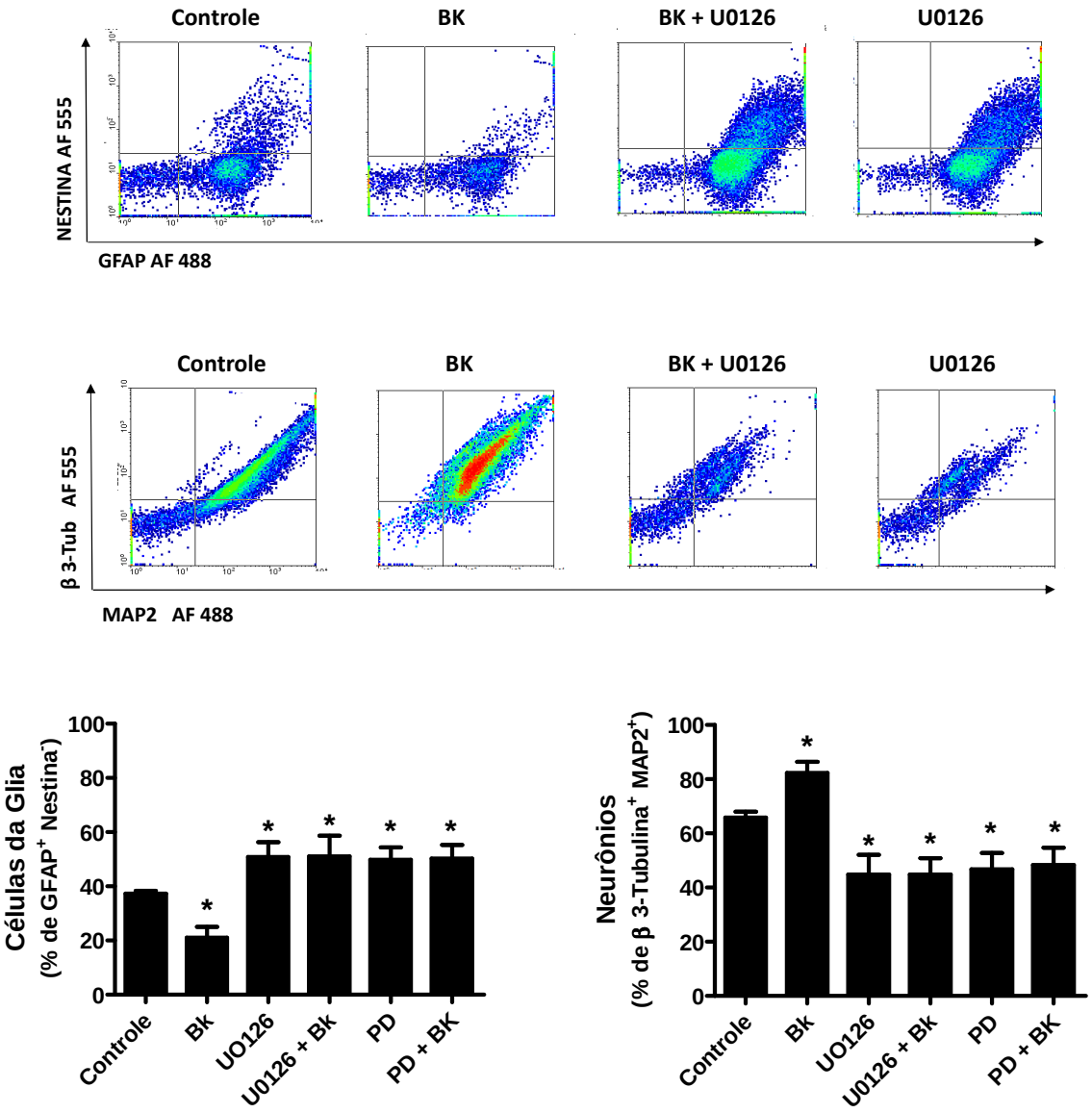


Figura 4.25: Porcentagem de neurônios e células gliais após a diferenciação na presença de bradicinina (BK)e inibidores da ERK (U0126 e PD). As neuroesferas foram diferenciadas sob condições controle; na presença de 1µM BK; 10 µM de U0126; 20µM de PD; U0126+BK; PD+BK. As drogas foram adicionadas ao meio de cultura a cada dois dias com exceção da BK que foi adicionada todos os dias. No sétimo dia de diferenciação as amostras foram fixadas e marcadas com os anticorpos anti-GFAP, anti-Nestina, anti-β3-Tubulina e anti-MAP2. Posteriormente, as amostras foram marcadas com os anticorpos secundários conjugados aos fluoróforos Alexa Fluor 488 e 555, seguindo o protocolo de citometria de fluxo. Foram adquiridas 30.000 células por amostra em um total de cinco experimentos independentes. Estes dados foram analisados no *software* WinMDI2.9. Os dados mostrados são os valores médios ± SEM (**P* < 0,05).

Ainda que a citometria de fluxo ofereça informações quantitativas a respeito das populações presentes após diferenciação neural, empregamos também a técnica de imunocitoquímica que fornece informações visuais qualitativas importantes a respeito da

distribuição dos marcadores específicos de neurônios e células da glia. As imagens de imunocitoquímica confirmaram as diferenças de distribuição dos marcadores entre as amostras vistas por citometria de fluxo. Em amostras tratadas com somente BK durante a diferenciação, observa-se uma maior e menor presença de β 3-Tubulina e GFAP, respectivamente. Já em todas as condições em que a ERK fora inibida (U0126; U0126 + BK; PD; PD + BK) claramente observa-se um aumento da população de células gliais em detrimento da população neuronal (Figura 4.26).

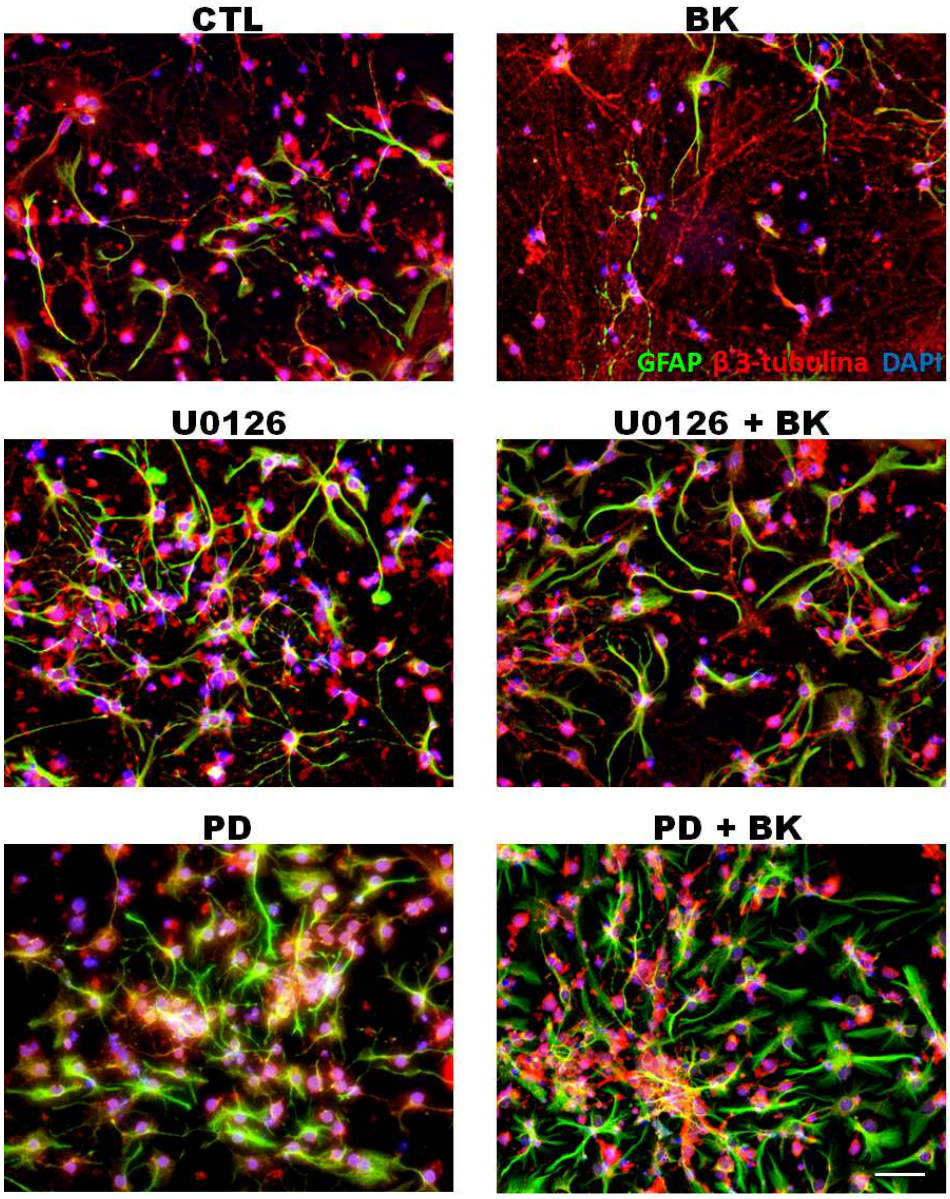


Figura 4.26: Expressão de GFAP e β 3-Tubulina por imunocitoquímica após diferenciação na presença de bradicinina e inibidores de ERK. As neuroesferas foram diferenciadas sob condições controle ou na presença de bradicinina (BK; 1µM), U0126 (10µM), U0126+BK, PD (20µM) e PD+BK. Marcação para β3-Tubulina com anticorpo conjugado ao fluoróforo Alexa Fluor 555 (vermelho) e para GFAP com anticorpo conjugado ao fluoróforo Alexa Fluor 488 (verde). Os dados mostrados são representativos de pelo menos quatro experimentos independentes. Marcação do núcleo com DAPI. Escala de 20µm.

Em conjunto investigamos se a MAPK p38 também poderia media os efeitos da BK na neuro- gliogênese. Para isso, tratamos as neuroesferas com SB na concentração de 10µM durante a diferenciação neural. Interessantemente, a inibição desta quinase aumentou a população de células expressando β 3-Tubulina no sétimo dia de

diferenciação e diminui a população expressando GFAP em comparação com as amostras controle (Figura 4.27). Assim, a inibição da p38 resultou em efeitos similares aos efeitos observados com o tratamento de BK durante a diferenciação, ou seja, a p38 ativa provavelmente possui efeitos antagônicos à BK na neuro- gliogênese. Estes resultados, *per si*, sugerem que a BK não induz seus efeitos no destino neural via p38.

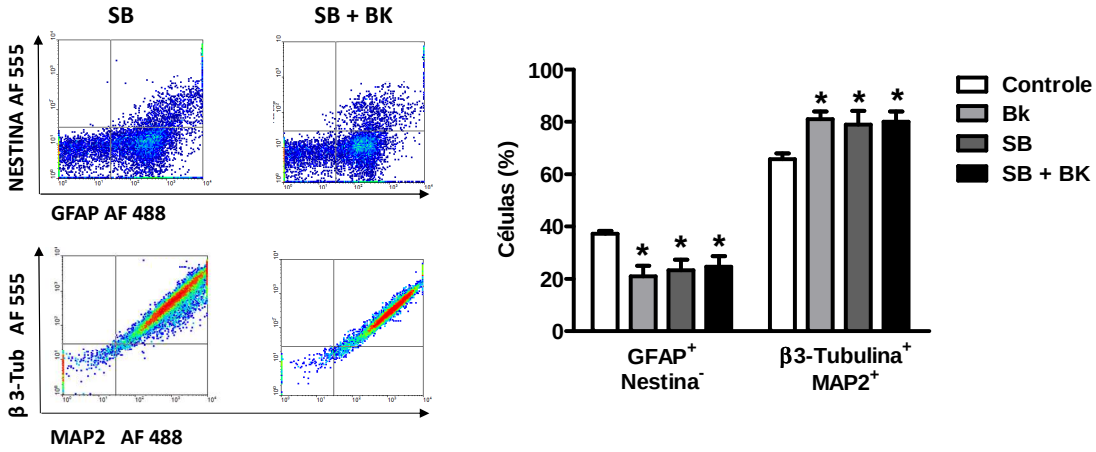


Figura 4.27: Porcentagem de neurônios (β 3-Tubulina^{+} MAP2^{+}) e células gliais (GFAP^{+} Nestina^{-}) após diferenciação na presença de bradicinina (BK) e inibidor da p38 (SB203580). As neuroesferas foram diferenciadas sob condições controle; na presença de 1µM BK; 10 µM de SB203580 (SB); SB + BK. O meio de cultura foi trocado a cada dois dias em conjunto com o inibidor SB, já a BK foi adicionada ao meio todos os dias e no sétimo dia de diferenciação as amostras foram fixadas e marcadas com os anticorpos anti-GFAP, anti-Nestina, anti-β3-Tubulina e anti-MAP2. Posteriormente, as amostras foram marcadas com os anticorpos secundários conjugados aos fluoróforos Alexa Fluor 488 e 555, seguindo o protocolo de citometria de fluxo. Foram adquiridas 30.000 células por amostra em um total de três experimentos independentes. Estes dados foram analisados no *software* WinMDI 2.9 e os valores médios ± SEM são mostrados (*$P < 0,05$).

Ensaios de Imunocitoquímica também foram realizados no dia sete de diferenciação em condições controle, na presença de BK, SB e SB + BK com os marcadores GFAP, β 3-Tubulina e DAPI. Dado que o bloqueio da MAPK p38 pelo inibidor SB reduz significativamente a migração, a expressão dos marcadores pode ser observada apenas em imagens obtidas com maior aumento mostrando as neuroesferas (Figura 4.28). Assim, em concordância com os resultados de citometria de fluxo, as imagens representativas de

imunocitoquímica sugerem um deslocamento da diferenciação para a neurogênese em detrimento da gliogênese quando a p38 está inibida durante a diferenciação.

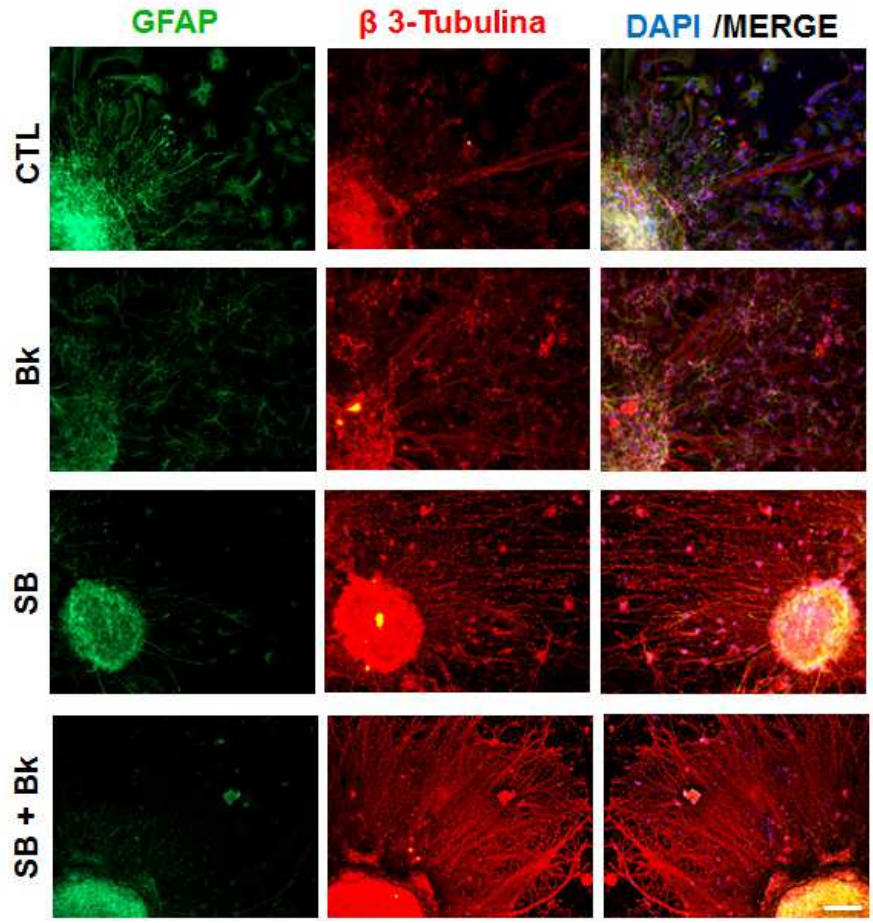


Figura 4.28: Expressão de GFAP e β 3-Tubulina por imunocitoquímica após diferenciação na presença de bradicinina (BK) e inibidor da MAPK p38 (SB). As neuroesferas foram diferenciadas sob condições controle ou na presença de BK (1µM), SB203580 (SB) (10µM), SB+BK. A marcação para β3-Tubulina e GFAP foi realizada com anticorpos conjugados aos fluoróforos Alexa Fluor 555 (vermelho) e 488 (verde), respectivamente, e a marcação do núcleo foi obtida com DAPI. Os dados mostrados são representativos de quatro experimentos independentes. Escala de 100µm.

Também avaliamos se a BK exerce seus efeitos na modulação da diferenciação por intermédio de sua via PI3K/Akt. Para tanto, utilizamos o inibido da PI3K Ly294002 (Ly) na concentração de 20µM durante a diferenciação. Como pode ser observado na Figura 4.29, a BK, mesmo na presença do inibidor Ly, permaneceu diminuindo a porcentagem de células que expressão GFAP e aumentando a população que expressa β 3-Tubulina.

Assim, esses dados sugerem que a BK atua na modulação da diferenciação de CPNs de maneira independente de sua via PI3K/Akt.

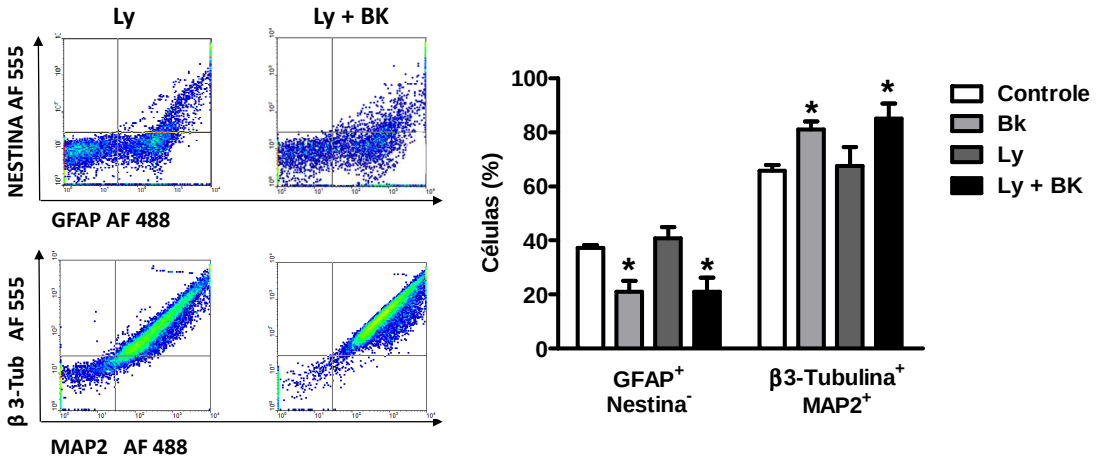


Figura 4.29: Porcentagem de neurônios (β 3-Tubulina^{+} MAP2^{+}) e células gliais (GFAP^{+} Nestina^{-}) após diferenciação na presença de bradicinina (BK) e inibidor de PI3K (Ly). As neuroesferas foram diferenciadas sob condições controle; na presença de 1µM BK; 20 µM de Ly294002 (Ly); Ly + BK. No sétimo dia de diferenciação as amostras foram dissociadas, fixadas e marcadas com os anticorpos anti-GFAP, Nestina, MAP2 e β 3-Tubulina. Posteriormente, as amostras foram marcadas com os anticorpos secundários Alexa Fluor (AF) 488 e AF 555, seguindo o protocolo de citometria de fluxo. Os dados são representativos de quatro experimentos independentes (*P < 0,05).

Além dos resultados de citometria de fluxo, ensaios de imunocitoquímica no sétimo dia de diferenciação foram realizados. Para tanto, novamente o inibido Ly na concentração de 20µM foi utilizado para inibir a PI3K durante a diferenciação. As imagens desses ensaios corroboram com os dados anteriores, apontando para um aumento na população expressando β 3-Tubulina em detrimento da população expressando GFAP (Figura 4.30).

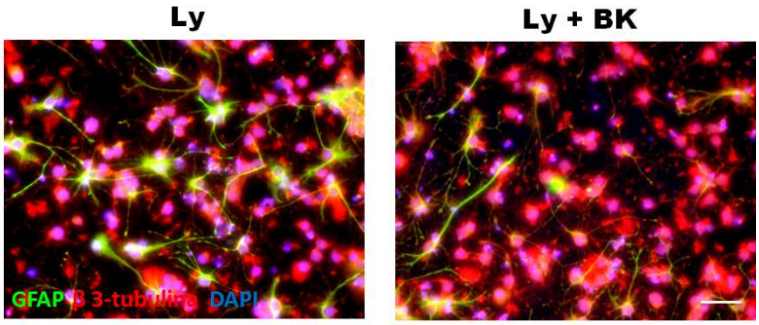


Figura 4.30: Análise por imunocitoquímica da população de neurônios (β 3-Tubulina$^+$) e células gliais (GFAP$^+$) após diferenciação influenciada por BK exógena e bloqueio de sua via PI3K/Akt. As neuroesferas foram diferenciadas na presença do inibidor da PI3K Ly294002 (Ly) na concentração de 20µM ou na presença desta + BK. No sétimo dia de diferenciação as amostras foram fixadas e marcadas com os anticorpos anti-GFAP e β 3-Tubulina conjugados com os fluoróforo Alexa Fluor 488 e 555, respectivamente. A marcação do núcleo foi obtida com DAPI. Os dados mostrados são representativos de três experimentos independentes. Escala de 100µm.

4.6 VIAS DE SINALIZAÇÃO DA BRADICININA QUE MODULAM A PROLIFERAÇÃO DE CPNS

Na subseção 4.3 compreendemos melhor as influências da BK na proliferação e ciclo celular de células precursoras neurais indiferenciadas. Observamos que esta cinina suprimiu a proliferação induzida pelos fatores de crescimento (GF) EGF e FGF2, os quais atuam estimulando a auto-renovação e mantendo-as multipotentes. A ação antiproliferativa de BK deu-se em decorrência do acumulo de células na fase G_1 do ciclo celular, fase essa, conhecida por tornar-se prolongada/estendida em células que deixaram a divisão proliferativa (auto-renovação) e se comprometeram com a neurogênese.

Nesta subseção, ainda estamos em busca de avanços rumo a uma explicação mecanística-molecular para o efeito antiproliferativo disparados por BK em CPNs. Assim, voltamos nossa atenção para identificarmos quais as vias de sinalização acionadas por BK estão implicadas nos mecanismos efetores da supressão da proliferação.

Utilizamos a mesma estratégia de Dixon e colaboradores (2002) para a análise das vias ativadas por BK envolvidas na inibição da proliferação estimulada por GFs.

Inicialmente, as neuroesferas foram pré-tratadas com os GFs por uma hora, em seguida, elas foram tratadas com os inibidores das vias por 30 minutos e, por fim, a BK foi acrescentada ao meio de cultura. A fixação das amostras foi realizada após 24 horas. As concentrações utilizadas foram: 20ng/mL de EGF; 20ng/mL de FGF2; 10µM de U0126; 20µM de PD; 10µM de SB; 20µM de Ly ; 1µM de BK.

As MAPKs p38 e ERK estão implicadas nos mecanismos efetores da supressão da proliferação impostos por BK. Isto pode ser concluído, pois as condições experimentais GFs+inibidores das MAPKs+BK não apresentaram diferença significativa alguma em comparação as condições com apena GFs+inibidores das MAPKs. Em outras palavras, a inibição farmacológica dessas rotas com as drogas U0126 (inibidor da MEK/ERK), PD (inibidor da MEK/ERK) e SB (inibidor da p38) bloquearam totalmente o efeito dessa cinina na proliferação (Figura 4.31).

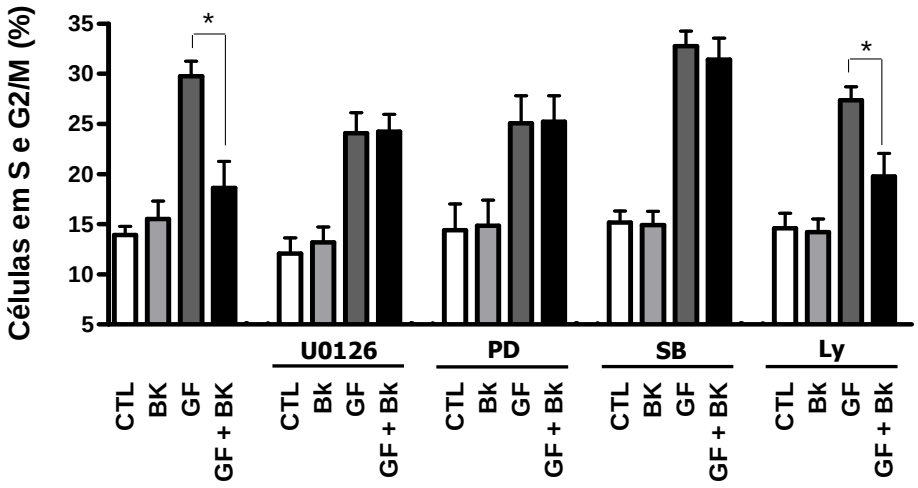


Figura 4.31: Envolvimento das vias de sinalização acionadas por bradicinina no seu efeito antiproliferativo em CPNs indiferenciadas. Inicialmente as CPNs não foram tratadas (CTL) ou foram tratadas com fatores de crescimento (GFs; *growth factor*) (EGF - 20ng/mL e FGF2 - 20ng/mL). Após uma hora de estímulo, os inibidores da ERK (U0126 - 10µM e PD98059 - 20µM), p38 (SB203580 - 10µM) e PI3K (Ly294002 - 20µM) foram acrescentados. Por fim, decorridos mais 30 minutos, a bradicinina (BK; 1µM) foi ou não acrescentada ao meio de cultura conforme condições experimentais descritas na figura. Então as células foram mantidas em cultura por mais 24 horas. No dia seguinte as amostras foram tratadas com BrdU por 30 minutos pra a incorporação deste no DNA. Posteriormente, as células foram dissociadas, fixadas e marcadas com anti-BrdU, Alexa Fluor 488 e iodeto de propídio e analisadas no citômetro de fluxo conforme descrito na

metodologia. Os dados mostrados são os valores médios ± SEM de quatro experimentos independente (*$P < 0,05$).

Também avaliamos a influência da via PI3K/Akt no efeito antimitogênico da BK. Para inibição de tal via utilizamos o inibidor Ly294002 (Ly) na concentração de 20µM. Os resultados mostram que o boqueio dessa via não impediu o efeito antiproliferativo da cinina. (Figura 4.31). Portanto, PI3K/Akt não estão implicadas nos mecanismos efetores da supressão da proliferação impostos por BK.

A fim de melhor compararmos o grau de influência das rotas ativadas por BK no efeito supressor da proliferação dessa cinina, calculamos a porcentagem de supressão da proliferação induzida por essa cinina em condições controle e quando as vias são bloqueadas (Figura 4.32). Observa-se que os inibidores da MEK/ERK, (U0126 e PD) e da p38 (SB) bloqueiam totalmente a atividade antiproliferative da cinina (Figura 4.32). Por outro lado, a inibição de PI3K não influencia significativamente nessa ação da BK. Esses resultados sugerem que a BK exerce seu efeito antiproliferativo em CPNs por intermédio da ativação das cascatas da MAPK ERK e p38 e, por outro lado, independe da via de PI3K/Akt.

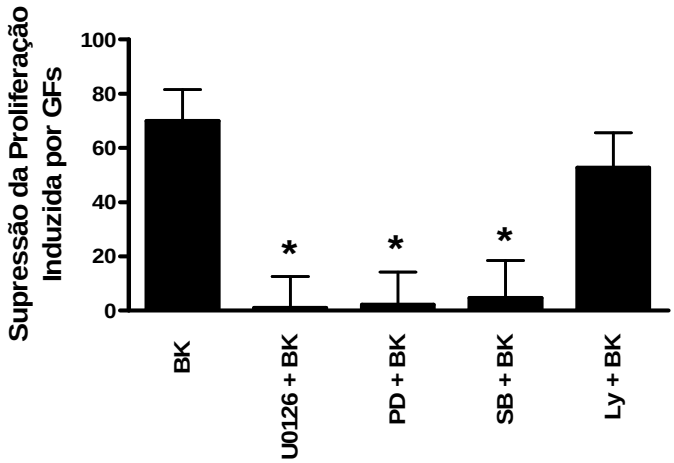


Figura 4.32: Análise da participação das vias de sinalização de ERK, p38 e PI3K/Akt sobre o efeito antiproliferativo de bradicinina. A partir dos dados da figura anterior calculou-se a porcentagem de

supressão da proliferação induzida por bradicinina (BK) na ausência ou na presença de inibidores enzimáticos das vias de sinalização. Inicialmente, calculou-se a proliferação máxima para cada inibidor ([% de células em S+G_2/M estimulada por GFs + inibidor] – [% de células em S+G_2/M tratadas somente com o inibidor]), e a proliferação verificada por GFs + inibidor + BK ([% de células em S+G_2/M estimulada por GFs + inibidor + BK] – [% de células em S+G_2/M tratadas somente com o inibidor]). Com base nesses resultados, calculou-se a porcentagem de supressão da proliferação induzida por BK na presença dos inibidores enzimáticos. Os dados mostrados são os valores médios ± SEM de quatro experimentos independente (*P < 0,05).

4.7 DIFERENCIAÇÃO E VIAS DE SINALIZAÇÃO ACIONADAS POR BRADICININA EM CÉLULAS PC12: UM ENFOQUE NA CINÉTICA E SUBLOCALIZAÇÃO DE p-ERK

As neuroesferas, principal modelo de diferenciação neural dessa tese, abrangem boa parte dos processos complexos que ocorrem nos estágios iniciais do desenvolvimento neural, tais como, proliferação de CPNs, migração, neurogênese e gliogênese. Entretanto, em investigações mecanísticas-moleculares da diferenciação, a complexidade do modelo experimental torna-se uma desvantagem e uma abordagem reducionista com modelos mais simplificados são necessários. Nesse sentido, inúmeros estudos optam por utilizar as células P12, visto que esse modelo trata-se de uma linhagem celular imortalizada que permite avaliar os mecanismos da neurogênese com poucas variações e interferentes. Adicionalmente, a utilização de células PC12 nessa tese fornece uma base de comparação com trabalhos previamente publicados.

Dessa forma, inicialmente verificamos se em células PC12 a BK induz a ativação das mesmas vias de sinalização já observadas em CPNs. Esses experimentos foram realizados segundo o protocolo descrito por Santos e colaboradores (2007). Um dia antes dos experimentos, deixamos as células em meio de cultura suplementado apenas com 0,5% de SC e então, as tratamos com 1µM de BK por 10 minutos. Pouco tempo antes de completar os 10 minutos, obtivemos células individualizadas em suspenção por ação mecânica visto que as mesmas apresentam um poder de adesão fraco. As células foram

então imediatamente fixadas e seguimos o protocolo de marcação de quinases fosforiladas como já descrito na metodologia (item 3.7.2).

Observamos que o tratamento com BK na concentração de 1µM por 10 minutos resulta na ativação de Akt e p38 em PC12. A exposição dessas células a BK induziu um aumento de 0,6 (de 53587 para 85256) e 0,4 (de 3368 para 4718) vezes na expressão média (MFI) de p-Akt e p-38, respectivamente, em comparação as amostras não tratadas (Figura 4.33).

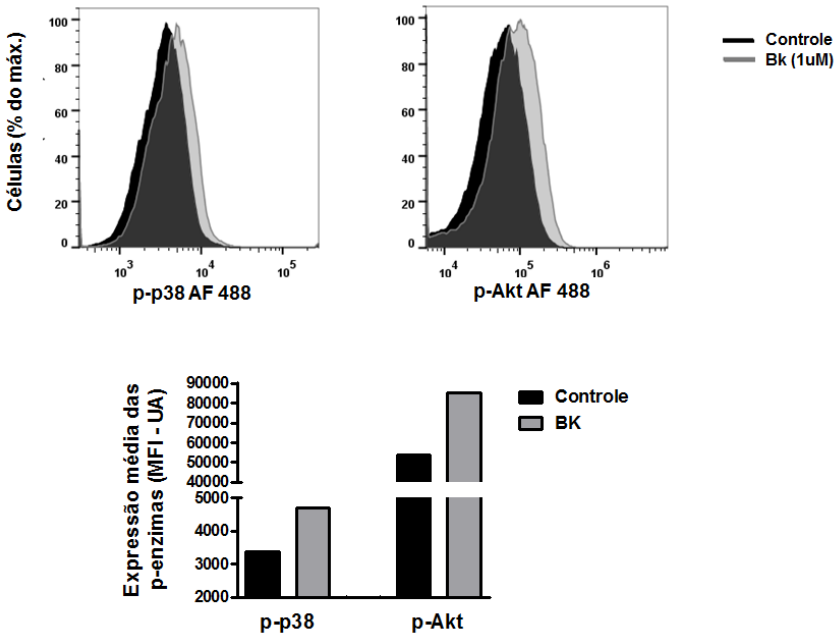


Figura 4.33: Expressão de p-Akt e p-p38 em células PC12 após tratamento com bradicinina. Algumas amostras não receberam tratamento (controle) enquanto outras foram tratadas com bradicinina (BK; 1µM) por 10 minutos. As células foram imediatamente fixadas e marcadas com o anticorpo controle de Isotipo, p-p38 ou p-Akt. Posteriormente, as amostras foram marcadas com anticorpo secundário Alexa Fluor (AF) 488 seguindo o protocolo de citometria de fluxo. O nível de expressão dessas enzimas fosforiladas foi estimado pela intensidade de fluorescência mediada (MFI) das amostras. Os resultados foram analisados no software Flowjo V10.

Como já mencionado, o tempo de duração da atividade de ERK é um dos mecanismos que regula sua especificidade para a resposta celular resultante. Considerando que a maioria dos estudos que comprovam tal mecanismo foram realizados em células PC12 (Marshall, 1995; Santos et al, 2007; Sasagawa et al, 2005), nós

investigamos a cinética de ativação da ERK por BK também nesse modelo de diferenciação. Inicialmente, repetimos os dados já publicados da cinética de ativação da MAPK por EGF e NGF, sabendo que o primeiro é um fator mitogênico e o segundo é um fator neurogênico. Como esperado, observou-se que o EGF ativou a ERK de forma transiente, ou seja, inicialmente observou-se uma intensa ativação desta MAPK seguida por uma rápida desativação da via com retorno aos níveis basais em menos de 30 minutos (Figura 4.34 superior). Por outro lado, a cinética de ativação da ERK por NGF, como esperado, resultou em um perfil bastante diferente. Esse fator induziu a ativação sustentada dessa quinase que permaneceu em níveis elevados mesmo quando analisada após 60 minutos do estímulo (Figura 4.34 região central). Por fim, avaliamos a cinética de ativação da ERK pela BK nesse modelo celular. Interessantemente, verificamos que, assim como já observado em CPNs, o tratamento com esta cinina induz uma ativação sustentada de ERK em células PC12. Esses dados demonstram que, em termos cinéticos, a BK ativa a ERK da mesma forma que o fator neurogênico NGF.

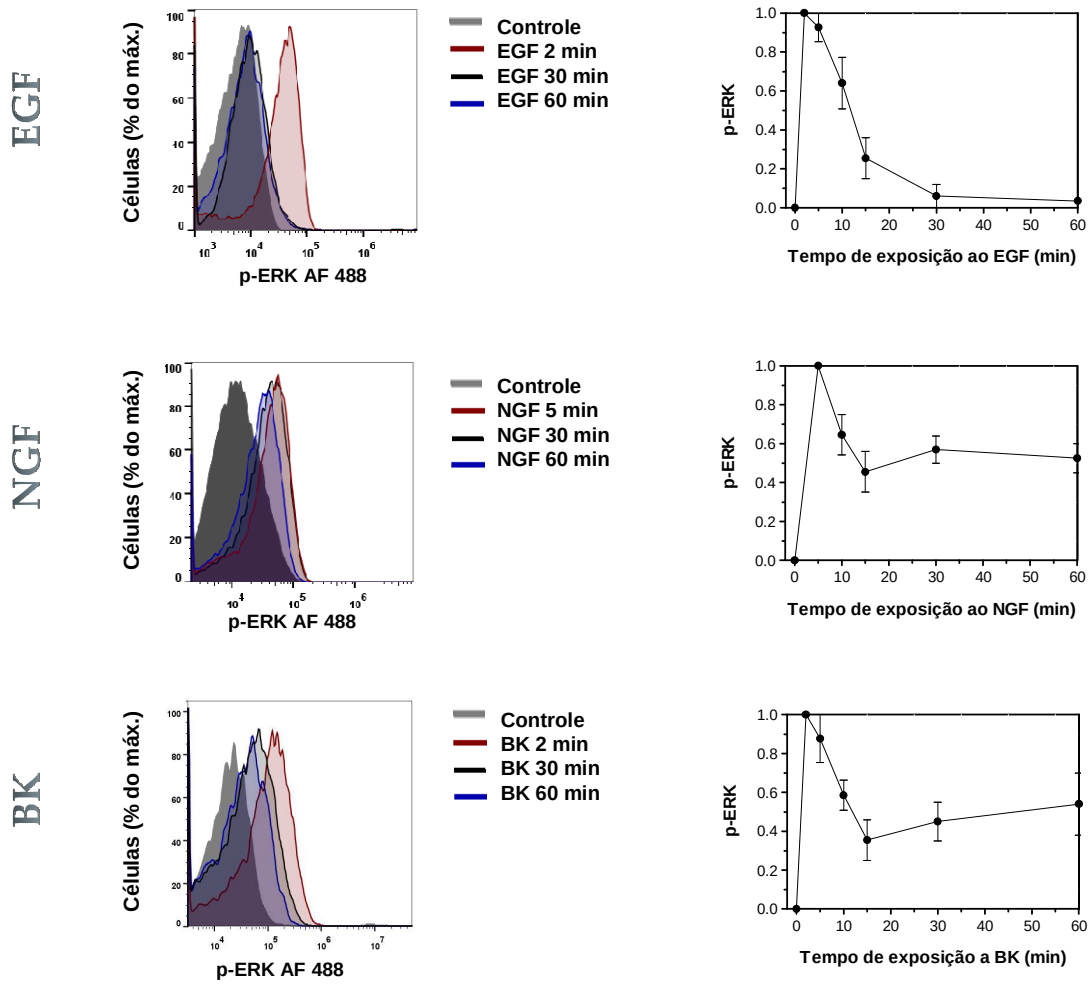


Figura 4.34: Cinética de ativação de ERK por EGF, NGF e BK. As células PC12 foram tratadas com EGF (100ng/mL), NGF (50ng/mL) e BK (1µM) por 2 à 60 minutos e então foram imediatamente fixadas com etanol. Em seguida as amostras foram marcadas com o controle de isotipo (IgG de coelho) ou com p-ERK e posteriormente com Alexa Fluor 488 seguindo o protocolo de citometria de fluxo. A expressão media de p-ERK por amostra foi estimado pela análise do MFI. Os resultados foram analisados no software Flowjo V10.

Como já mencionado, a translocação nuclear de ERK é comprovadamente necessária e em conjunto com a ativação sustentada dessa quinase são suficientes para induzir a neurogênese em células PC12. Assim como já avaliado em CPNs no tópico 4.4, avaliamos a sublocalização intracelular de ERK após estímulo com BK também em células PC12. Para tal, um dia antes dos experimentos as células foram mantidas em meio suplementado com 0,5% de SC. No dia seguinte, a BK (1µM) foi adicionada ao meio de cultura por 15 minutos. As células foram então imediatamente submetidas a baixas

temperaturas (gelo) e deu-se continuidade com os protocolos de fracionamento dos extratos protéicos nuclear e citoplasmático e WB conforme descritos nas subseções 3.11 e 3.12, respectivamente. Observamos um aumento da proporção de p-ERK no extrato nuclear após estímulo com BK em comparação às amostras não tratadas (Figura 4.35A e C). Não observamos alterações significativas na pERK no extrato citoplasmático (Figura 4.35A e C). Na Figura 4.35B, estão os controles da eficiência do fracionamente de proteínas nucleares e citoplasmáticas descrita. Esses dados de cinética e sublocalização da ERK pelo estímulo com BK, observados tanto em CPNs quanto em células PC12, podem compor o mecanismo efetor dessa cinina no favorecimento da neurogênese.

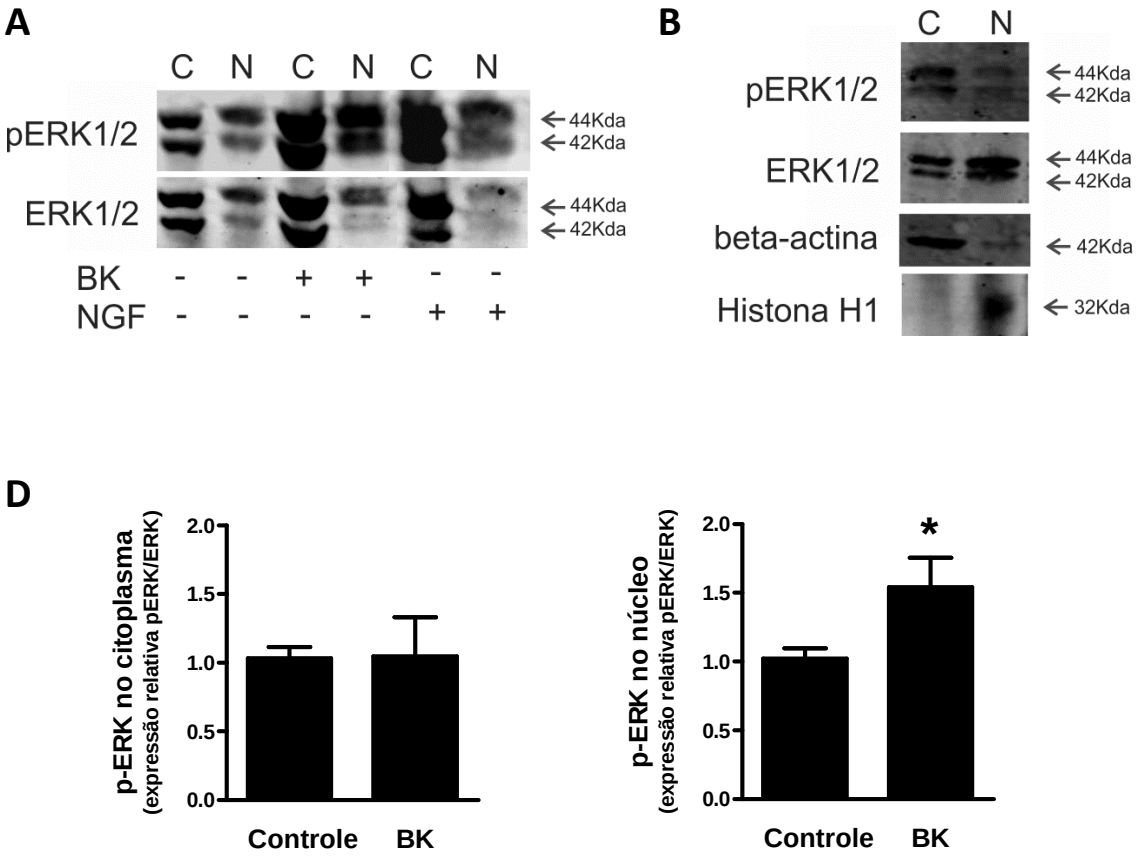


Figura 4.35: p-ERK citosólica e nuclear em células PC12 não tratadas e tratadas com BK. As células não foram tratadas ou foram tratadas com 1μM de BK por 15 minutos. A expressão de p-ERK citosólica e nuclear foi então determinada pelo fracionamento dos extratos proteicos citosólicos e nucleares seguido pelo *wetern blotting* conforme descrito na metodologia. A expressão relativa de p-ERK foi determinada pela comparação com ERK total através da densitometria óptica das bandas com auxilio do *software* ImageJ. C:

extrato citoplasmático. N: extrato nuclear. Os dados são representativos de cinco experimentos independentes (*$P < 0,05$).

Dessa forma, investigou-se também a diferenciação das células PC12 plaqueadas em matriz aderente de poli-*L*-lisina e laminina e mantidas sem tratamento (Controle; CTL) ou tratadas com NGF (50ng/mL) ou BK (1µM) por 48 horas. Como esperado, o NGF induziu a neurogênese uma vez que o tratamento com esse fator resultou em aumentou expressivo na proporção de células com mais de 3 neuritos e uma grande redução da presença de células sem neuritos em comparação as condições controle. O tratamento com BK também potencializou a diferenciação, visto que, foi observado um aumento significativo no número de células com mais de 3 neuritos e, em contrapartida, observou-se uma redução na porcentagem de células sem neuritos.

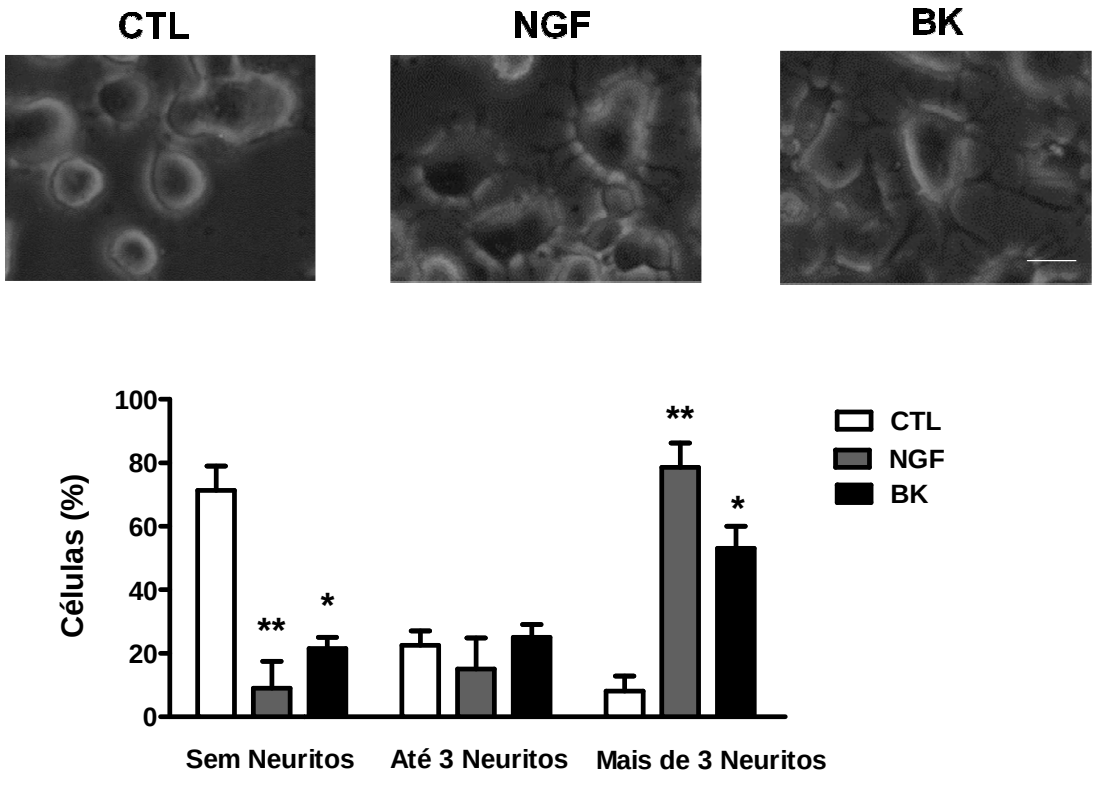


Figura 4.36: Diferenciação de células PC12 em poli-*L*-lisina e laminina sob influência de NGF e BK. As células foram diferenciadas por 2 dias na ausência (Controle; CTL) ou na presença de NGF (50ng/mL) e BK (1µM). Quinze imagens foram obtidas e mil células foram analisadas para cada condição experimental de 3 experimentos independentes. (*$P < 0,05$ e **$P < 0,01$). Escala de 10µm.

Tem se demonstrado que a ativação sustentada de ERK deve-se a ativação de duas ou mais vias que se integram ativando esta MAPK. Observou-se que a fase inicial (pico máximo de ativação da ERK) e a fase subsequente sustentada são mediadas por MAPKKK ativadoras de MEK diferentes (York et al, 1998). Essa última fase também pode ser influenciada por mecanismos de retroalimentação da via (Bhalla & Iyengar, 1999; von Kriegsheim et al, 2009).

Alguns estudos demonstram que a fase inicial da ativação de ERK por NGF, bem como a sua ativação por EGF, é mediada pelo ativador de MEK c-Raf1. Por outro lado, a fase de ativação sustentada de ERK tem sido atribuída a várias outras vias ativadoras de MEK atuando em conjunto, entre elas a da PKA, PKC, c-Raf1 e Rap1 (Bouschet et al, 2003; Santos et al, 2007; Sasagawa et al, 2005). Discute-se que essas vias são individualmente necessárias, mas isoladamente insuficientes para a ativação duradoura de ERK. Entre tais ativadores de MEK, a PKC tem sido bastante investigada e recentemente foi demonstrado que sua atividade é necessária para ativação sustentada de ERK por NGF e PACAP (molécula bem conhecida por induzir a diferenciação neuronal em PC12) (Bouschet et al, 2003; Santos et al, 2007).

Neste sentido, tentamos bloquear apenas a ativação sustentada de ERK pela BK, sem influenciar na fase inicial de sua ativação, e posteriormente, avaliamos os efeitos na diferenciação de tal modulação na cinética dessa MAPK. Assim, inibimos as PKCs clássicas (cPKCs; α, β e γ; ativadas por Ca^{2+}, DAG e fosfatidilserina) utilizando o inibidor Go6973 (Go) na concentração de 10μM por 1 hora e avaliamos a cinética de ativação da ERK pela BK bem como os efeitos na diferenciação neural nessas condições. Como pode ser visto na Figura 4.37, mesmo com as cPKCs bloqueadas, a BK permanece ativando a ERK em

apenas 2 minutos, no entanto, em seguida, observamos uma rápida desativação da via com retorno aos níveis basais em menos de 30 minutos. Em outras palavras, a BK passou a ativar a ERK somente de forma transiente. O tratamento prévio com Go, não impediu a fase inicial de ativação da ERK pela BK mas completamente bloqueou a sua ativação sustentada, indicando a importância da ativação de PKC pela BK na promoção dessa última fase.

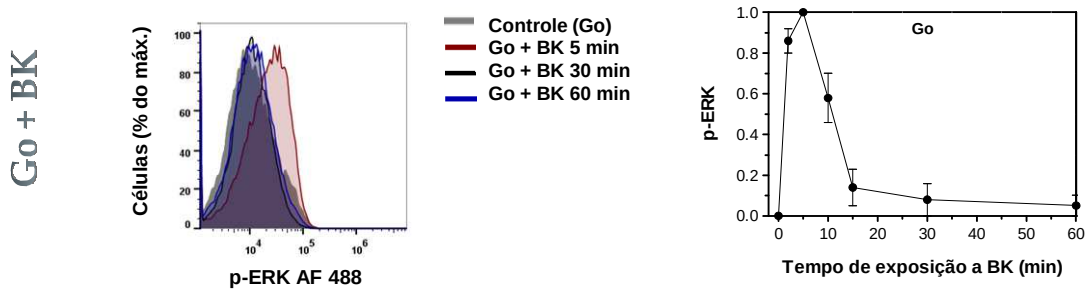


Figura 4.37: Cinética de ativação de ERK por bradicinina (BK) após inibição de PKC (Go). As células PC12 foram pré-tratadas com o inibidor de PKCs clássicas, Go6973 (Go; 10µM), por uma hora e, posteriormente, foram estimuladas com BK (1µM) por 2 à 60 minutos. Em seguida as amostras foram fixadas, marcadas com o controle de isotipo (IgG de coelho) ou com p-ERK e, posteriormente, com Alexa Fluor 488 seguindo o protocolo de citometria de fluxo. A expressão media de p-ERK por amostra foi estimado pela análise do MFI. Os resultados foram analisados no software Flowjo V10.

Tentamos também identificar a via de sinalização que se comunica com a ERK resultando na sua translocação nuclear. Como já mencionado (subseção 4.4), as vias PI3K/Akt e AMPc/PKA podem influenciar nesse processo. Assim, avaliamos se o bloqueio da PI3K pelo inibidor Ly impede a translocação de ERK induzida por BK. Observamos que o bloqueio dessa quinase não influencia na translocação de ERK (dados não mostrados). Dessa forma, no futuro pretendemos avaliar a influencia da PKA na translocação nuclear de ERK estimulada por BK.

Uma vez que o tratamento com Go modulou a cinética de ativação da ERK pela BK, avaliamos a influência de tal modulação na diferenciação neuronal.

Surpreendentemente, o tratamento com Go (10µM) + BK (1µM) resultou em uma redução no número de células com mais de 3 neuritos e um aumento na presença de células sem neuritos em comparação ao tratamento somente com BK (Figura 4.38). Em outras palavras, observou-se uma inibição substancial da diferenciação estimulada por BK quando as células foram previamente tratadas com Go, sugerem que a ativação sustentada de ERK possa ser um mecanismo indutor da neurogênese pela BK, assim como já comprovado pelos fatores NGF e PACAP.

Em conjunto, os resultados com células PC12 apontam a ativação sustentada e compartimentalização de ERK no núcleo, como mecanismos indutores da neurogênese pela BK.

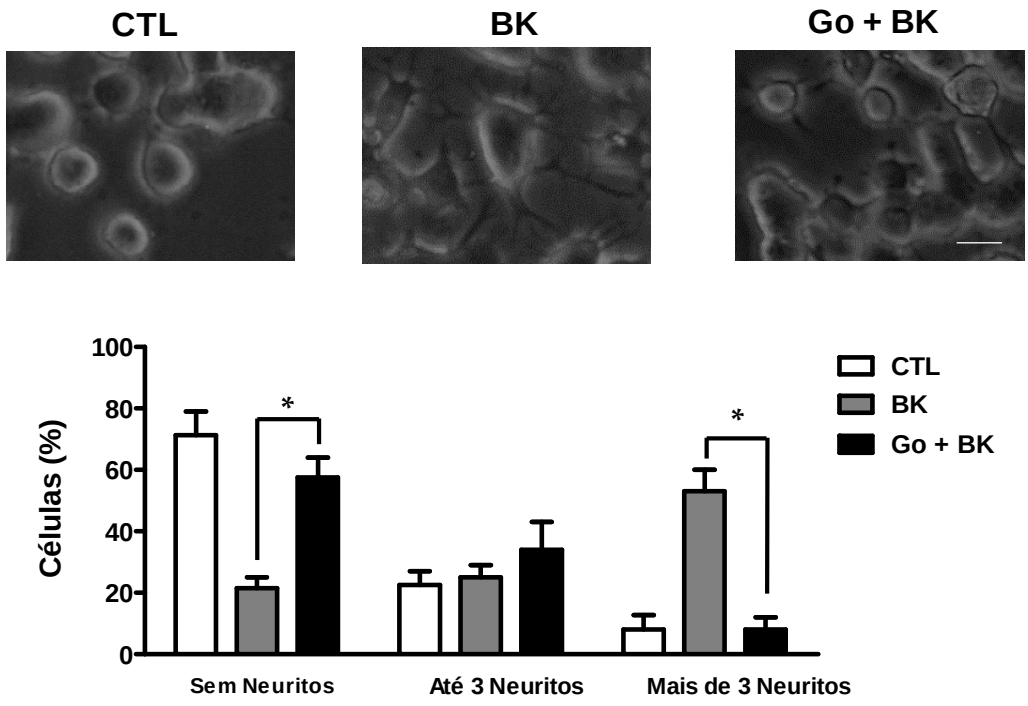


Figura 4.38: Diferenciação de células PC12 em laminina na presença de inibidor de PKC e bradicinina (BK). As células foram diferenciadas por 2 dias sob condições controle, na presença de BK (1µM) ou inibidor da PKC Go6973 (Go; 10µM) + BK. Quinze imagens foram obtidas e mil células foram analisadas para cada condição experimental de um *n* em um total de três experimentos independentes (**P* < 0,05 e ***P* < 0,01). Escala de 10µm.

5 Discussão

Na formação do sistema nervoso, todas as células têm a tarefa de proliferar, migrar, diferenciar, morrer ou amadurecer de forma precisa para formar estruturas complexas e com funções e atividades tão complexas quanto. Tal precisão é alcançada em decorrência da interação perfeita entre as células que comunicam-se constantemente pelo contato célula-célula ou por meio de mensageiros químicos no meio extracelular. Nesse contexto, nosso grupo tem investigado o envolvimento da bradicinina (BK) em processos do desenvolvimento neural (Martins et al, 2008; Martins et al, 2005; Trujillo et al, 2012). Observamos que a BK apresenta um papel importante na determinação do destino neural, favorecendo a migração celular e neurogênese em vários modelos de diferenciação. O oposto foi evidenciado em CPNs tratadas com HOE-140 ou *knockouts* para o seu receptor B2. Paralelamente, animais *knockouts* apresentaram uma diminuição no fenótipo neuronal em vários estágios do desenvolvimento, sugerindo o envolvimento desta cinina na determinação neural *in vivo* (Trujillo et al, 2012). Essas descobertas recentes do nosso grupo de pesquisa, em conjunto com descobertas sobre os efeitos das cininas na migração e neurogênese em animais pós-isquêmicos, motivaram a investigação, nessa tese, dos mecanismos subjacentes à BK que determinam tais efeitos.

5.1 Bradicinina na migração e destino neural de CPNs murinas

Nessa tese, demonstrou-se, a exemplo de dados anteriormente publicados, utilizando o modelo de neuroesferas de rato (Trujillo et al, 2012), que a BK também influencia efetivamente na diferenciação neural no modelo de CPNs murinas. Conforme apresentado na Seção 4.1, a BK favoreceu a migração radial que emerge da borda das neuroesferas, em comparação às amostras controle. Esse efeito pôde ser observado em

estágios iniciais (dia 1) e tardios (dia 7) da diferenciação. O tratamento crônico com BK e HOE-140 durante a diferenciação também refletiram na neuro-gliogênese. Os resultados obtidos pela técnica de PCR em tempo real, apresentados na Figura 4.2, demostram que o marcador de neurônio MAP2 apresentou uma expressão aumentada pelo tratamento com BK enquanto a expressão do marcador glial GFAP foi diminuída. Como esperado, o oposto foi observado após o tratamento com HOE-140. Apesar da β 3-Tubulina também ser um marcador para neurônios, a BK não influenciou na expressão gênica desse marcador, possivelmente porque no sétimo dia de diferenciação já se observa a presença de neurônios maduros ($MAP2^{+}$) e que o RNAm do marcador β 3-Tubulina é encontrado, principalmente, em neurônios jovens

Em complementação, os mesmos efeitos de BK (favorecendo a neurogênese em detrimento da gliogênese em CPNs murinas) puderam ser evidenciados pelos experimentos de imunocitoquímica e citometria de fluxo, apresentados nas Figuras 4.3 e 4.4, respectivamente. Esses resultados estão em conformidade com o observado no modelo de neuroesferas de rato, células P19 murinas e iPS humanas (Trujillo et al, 2012). Adicionalmente, nossos dados também estão em conformidade com os efeitos das cininas na neurogênese e migração em ratos adultos recém-isquêmicos, cujos estudos já foram detalhados na Seção 1.4.1 (Xia et al, 2004; Xia et al, 2006). Assim, os resultados aqui discutidos corroboram para a consolidação do papel da BK na diferenciação neural independentemente do modelo celular ou espécie avaliada estudada.

5.2 Bradicinina e a proliferação durante a diferenciação

No início da execução desse trabalho, não estava claro se a baixa taxa de proliferação observada no sétimo dia de diferenciação de neuroesferas de rato (Trujillo et

al, 2012) e camundongo (Figura 4.5) era causa direta e primária da ação de BK ou um efeito secundário ao enriquecimento de neurônios em cultura causado por esta cinina. Assim, dado que neurônios não proliferam e, portanto, encontram-se na fase G_0 do ciclo celular, a investigação da proporção de células nessa fase do ciclo poderia ser válida para indicar a relação causal entre os fenômenos (enriquecimento neural *versus* baixa proliferação). De fato, observou-se um enriquecimento na população na fase G_0 do ciclo celular, em amostras expostas à BK durante a diferenciação, sugerindo que os baixos níveis de proliferação observados poderiam ser um efeito secundário decorrente do enriquecimento de neurônios (Figura 4.6). A confirmação de tal relação causal foi obtida apenas quando se avaliou a proliferação em cada tipo celular, isoladamente. Assim, verificou-se que a BK não suprime a proliferação de nenhuma célula neural (glia, neurônio e CPN) nas condições propícias para a diferenciação, conforme evidenciado pelos experimentos representados na Figura 4.7. Isto demonstra que a supressão da proliferação observada no sétimo dia de diferenciação não é parte do mecanismo molecular disparado por BK para o enriquecimento neuronal. Sendo assim, visto que a BK não altera a viabilidade celular, parece razoável concluir que esse enriquecimento neuronal é proveniente apenas do favorecimento da neurogênese.

5.3 Papel e mecanismos da bradicinina na proliferação e ciclo celular de CNPs indiferenciadas

Conforme já discutido na seção de introdução, um mesmo sinal pode originar diferentes vias de sinalização de acordo com o contexto celular e, portanto, pode gerar respostas completamente diferentes. Este é o caso de BK, que pode desencadear respostas opostas quanto à proliferação. Esta cinina estimula tal processo em células

quiescentes, mas, por outro lado, o suprimi em células sob contexto mitogênico. Nesse sentido, Dixon e colaboradores (2002) verificaram que a BK suprime a proliferação estimulada por PDGF (*Platelet-Derived Growth Factor*) em células musculares e que este efeito se deu, principalmente, em decorrência da modulação dos níveis de pERK e do tempo prolongado de sua ativaçãona presença dos dois estímulos. Este cenário resultou na expressão de um inibidor do ciclo celular, o p27kip. Similarmente, Duchene e colaboradores (2002) demonstraram que a BK induz a proliferação de células mesangiais quiescentes, mas, por outro lado, produz um efeito antiproliferativo em células sob condições mitogênicas (na presença de sorro). Este último efeito foi dependente da interação da fosfatase SHP2 com o receptor B2 de cininas. Assim, os achados apresentados nessa tese, demonstrando que a BK suprime a proliferação estimulada pelos fatores de crescimento FGF2 + EGF (Figura 4.9), estão em concordância com a literatura técnica da área e demonstram, pela primeira vez, a participação da BK no efeito antiproliferativo de uma célula neural, mais especificamente, de células precursoras neurais.

Quanto ao ciclo celular, cabe lembrar que o equilíbrio entre divisão proliferativa e para diferenciação é fundamental para a formação do SNC (Caviness et al, 1995; Frisen et al, 1998). Os estímulos com GFs mantêm as CPNs multipotentes, fazendo divisões proliferativas, mas as deixam sujeitas as influencias de outros fatores ao longo da fase G_1. Nesse sentido, demonstrou-se que a BK é capaz de suprimir a proliferação estimulada pelos GFs (Figura 4.9), levando ao acúmulo de células na fase G_1 do ciclo celular (Figura 4.11). Este acúmulo não provém de um bloqueio do ciclo celular nessa fase, uma vez que foi observado uma proporção grande de células na fase S, subsequente à G_1 (Figura 4.10).

Embora testes mais específicos sejam necessários, parece plausível a hipótese de que o efeito de BK envolva um prolongamento da fase G_1 de CPNs e não o bloqueio da progressão do ciclo celular.

Esse aumento da proporção de células na fase G_1 do ciclo celular de CPNs, causado por BK, não é novidade entre os fatores neurogênicos. Similarmente, outro trabalho demonstrou que células precursoras corticais, obtidas de embriões de camundongos, apresentam um prolongamento da fase G1 e realizam divisão de diferenciação quando tratadas com neurotrofina 3 (Lukaszewicz et al, 2002), um agente indutor da diferenciação. Por outro lado, como esperado, quando essas células são estimuladas com FGF2, um fator que promove a proliferação, as mesmas apresentam uma fase G1 curta e realizam divisão proliferativa (Lukaszewicz et al, 2002). Conforme abordado na Introdução (Subseção 1.1), o alongamento da fase G1 resulta no comprometimento com a diferenciação neuronal por meio da substituição da divisão proliferativa pela divisão para diferenciação (Calegari & Huttner, 2003; Lako et al, 2009; Neganova et al, 2009). Nesse contexto, a BK poderia estar favorecendo a neurogênese por atuar prologando a fase G1 do ciclo celular e, dessa forma, removendo as CPNs da divisão proliferativa induzida por GFs.

5.4 Vias de sinalização da bradicinina em CPNs e células PC12

Outro aspecto dos mecanismos da BK na diferenciação neural se refere às vias de sinalização disparadas por esta cinina. Nesse contexto, para os processos de proliferação e diferenciação não existem respostas celulares intermediárias. As mesmas devem ser do tipo tudo ou nada. Dessa forma, sendo as vias de sinalização uma espécie de “cérebro” da célula, elas são responsáveis por transformar um sinal linear (um aumento gradual na

concentração de um mensageiro) em uma resposta digital (Bhalla & Iyengar, 1999). O mesmo não ocorre com a migração, já que uma célula pode apresentar respostas intermediárias para esse processo. Assim, tendo em vista a grande responsabilidade das vias de sinalização na resposta celular a um mensageiro, inclusive em respostas cruciais para a formação perfeita do sistema nervoso, essa tese investigou as vias ativadas por BK em CPNs e células PC12.

Dessa forma, observou-se que a BK promove a produção intracelular de AMPc. A concentração desse clássico segundo mensageiro elevou-se rapidamente (um minuto) após exposição das CPNs a esta cinina (Figura 4.12). A cinética rápida revela, antecipadamente, que, ao contrário do observado em neurônios do corno dorsal da medula espinhal (Kohno et al, 2008), a via que leva à ativação de AC por BK em CPNs não é dependente da síntese e estímulo autócrino da prostaglandina E2, uma vez que esse processo é bastante demorado. Sequencialmente, os resultados apresentados na Figura 4.13 evidenciam que a elevação da $[AMPc]_i$ não depende das proteínas Gs, Gq e PLC bem como, provavelmente, também não depende de seus clássicos alvos a jusante, PKC e Ca^{2+}/calmodulina. Entretanto, ensaios com inibidores específicos destas últimas enzimas são necessários para, de fato, descartar tal dependência. Por fim, observou-se que a produção de AMPc estimulada por BK foi dependente de proteínas G sensíveis à toxina pertussis (TP; Figura 4.13C). Muito provavelmente trata-se da proteína G_i, visto que suas subunidades βγ são capazes de ativar as ACs II, IV e VII, e, por outro lado, a proteína G_o (também sensível à TP) não interfere na atividade das ACs. Adicionalmente, estes dados sugerem que o receptor B2 de cininas acopla-se à proteína Gi em CPNs, indo de encontro aos resultados de Hanke e colaboradores (2006), obtidos em células COS-7. Estes últimos

comprovaram que o receptor B2 de cininas se acopla, simultaneamente, às proteínas Gq e Gi (resultados detalhados na Subseção 1.4).

Muitos grupos, incluindo o nosso, têm reportado que a BK induz o aumento transiente na $[Ca^{2+}]_i$ (Leeb-Lundberg et al, 2005; Trujillo et al, 2012; Zubakova et al, 2008). Nesse contexto, a resposta típica de um receptor acoplado à proteína G_q consiste no aumento desse íon mobilizado a partir dos depósitos intracelulares. Os dados apresentados nessa tese sugerem que o receptor B2 de cininas aciona tal resposta típica, visto que a BK resultou no aumento da $[Ca^{2+}]_i$ de maneira parcialmente dependente de proteína G_q sensível à YM-254890 (Figura 4.15) e esse íon foi mobilizado dos retículos sarcoendoplasmáticos, conforme evidenciado pelos resultados apresentados na Figura 4.16, após tratamento com tapsigargina. Mais detalhadamente, esses resultados sugerem que o receptor B2 de cininas se acopla à proteína G_q em CPNs e que, após exposição à BK, a subunidade α da proteína G_q ativa a PLCβ. Esta última cliva fasfatidil inositois, originando DAG e IP3. O DAG, por sua vez, ativa isoformas de PKCs. Já o IP3 se liga a seus receptores específicos na membrana dos retículos sarcoendoplasmáticos, liberando Ca^{2+} desses compartimentos.

Adicionalmente, observamos que o aumento na $[Ca^{2+}]_i$, induzido por BK em CPNs, também foi dependente de proteína G sensível à toxina pertussis (TP) (Figura 4.15). Esta resposta não é classicamente associada à proteína G_i, porém, estudos recentes demonstram uma relação íntima entre $[AMPc]_i$ e $[Ca^{2+}]_i$, considerando que, por exemplo, a PKA é capaz de fosforilar diretamente o receptor de IP3 no retículo sarcoplasmático (Borodinsky & Spitzer, 2006; Gorbunova & Spitzer, 2002; Tang et al, 2003). Outro mecanismo plausível para o aumento na $[Ca^{2+}]_i$ através de proteína G_i consiste na

ativação de PLCβ pelas subunidades βγ dessa proteína, conforme mecanismo descrito por Park e colaboradores (1993). A proteína G_o também é sensível à TP, no entanto, ela ativa canais de cálcio na membrana plasmática (Liu et al, 1994). Observou-se, em CPNs, que o aumento da $[Ca^{2+}]_i$ induzido por BK provém dos estoques intracelulares e não do meio extracelular (Figura 4.17), portanto, é improvável que a proteína G_o esteja envolvida com a mobilização desse íon pela BK. Em conjunto, esses dados sugerem que tanto a proteína G_q quanto a proteína G_i são ativadas pelo estímulo com BK para a obtenção da resposta máxima em termos de $[Ca^{2+}]_i$ em CPNs.

Continuando o estudo da rede de sinalização acionada por BK em CPNs e células PC12 indiferenciadas, observamos que esta cinina leva à ativação de Akt e MAPK p38, mas não leva à ativação de STAT3 e MAPK JNK (Figura 4.17; 4.33). A ativação de Akt por BK foi dependente de PI3K (PI3K→Akt) (Figura 4.22). Estes dados estão de acordo com resultados previamente publicados, que reportam a ativação dessa via pelo receptor B2 de cininas (Xu et al, 2012). Mesmo não tendo sido investigado nesse trabalho, cabe ressaltar que as subunidades βγ da proteína G e Ras-GTP são possíveis elementos a montante de PI3K. A ativação de Ras em resposta ao estímulo com BK é frequentemente observada e reportada na literatura (Blaukat et al, 2000; Chen et al, 2004). Entre as vias a jusante de Ras, encontra-se a cascata da MAPK p38. Similarmente aos nossos resultados, outros grupos também verificaram a ativação de p38 em resposta ao estímulo com BK (Wilk-Blaszczak et al, 1998).

Adicionalmente, como ocorre em inúmeros tipos celulares que o receptor B2 é expresso (Dixon et al, 2002; Moughal et al, 1995; Yang et al, 2004), o estímulo com BK acionou a cascata da MAPK ERK em CPNs e células PC12 (Figura 4.18; 4.33; 4.34).

Evidenciou-se também que essa MAPK é ativada em resposta à BK, especificamente via receptor B2 de cininas. Isto pôde ser concluído porque CPNs *knockout* para esse receptor perderam completamente a capacidade de ativar ERK quando exposta a BK (Figura 4.19). A Figura 5.1 sumariza alguns achados da presente tese a respeito da complexa rede de vias de sinalização acionada por BK em CPNs indiferenciadas.

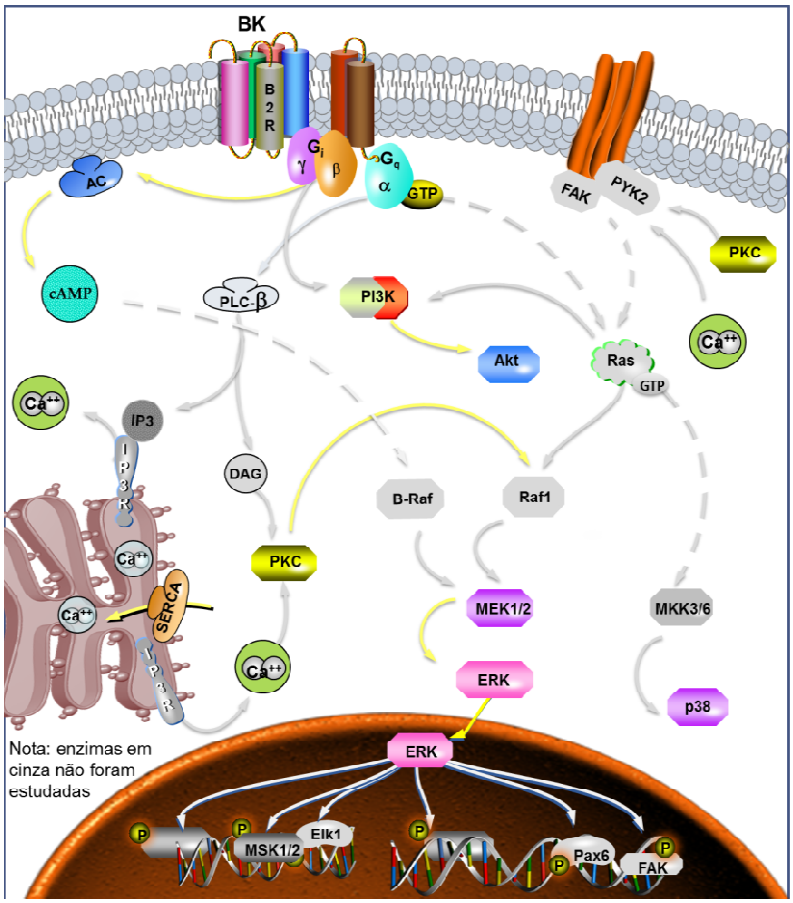


Figura 5.1: Representação esquemática, com base nos resultados dessa tese, da possível rede de vias de sinalização acionada em resposta à bradicinina em células precursoras neurais. Significado das abreviaturas e mais detalhes estão presentes no texto.

Como já abordado nas Subseções 1.3.2 e 1.4, a rota de ERK certamente é uma das mais importantes cascatas responsáveis por integrar vias de sinalização, inclusive, vias acionadas pelo receptor B2 de cininas. Nesse sentido, observou-se, em células PC12, que a ativação de ERK, sobretudo sua ativação sustentada, foi parcialmente dependente da

atividade de uma PKC da família de PKCs clássicas (cPKCs) (Figura 4.37). Esta família é especialmente importante no contexto de receptores que mobilizam o aumento na $[Ca^{2+}]_i$, como o receptor B2 de cininas, pois ela agrega todas as isoenzimas de PKCs (α, β e γ) que são ativadas por Ca^{2+} (também são ativadas por fosfatidilserina e DAG)(Newton, 1995). Portanto, considerando os resultados apresentados nessa tese, parece razoável concluir que, a jusante ao Ca^{2+} e/ou DAG, uma cPKC é ativada e esta, por sua vez, resulta na ativação da cascata de ERK.

O tratamento com o inibidor de cPKCs Go6973 aboliu totalmente a segunda fase de ativação de ERK (ativação prolongada), após estímulo com BK. Entretanto, conforme evidenciado na Figura 4.37, esse tratamento não bloqueou a ativação inicial da MAPK. Isso demonstra que existe outra via disparada por BK ativando a cascata de ERK, e essa via é independente das cPKCs. Além disso, esses dados apontam para a importância da MAPK na integração de vias disparadas por BK, em células PC12. Observou-se que a ativação de ERK por BK é independente de PI3K (Figura 4.22a). Assim, existe a possibilidade que a ativação da rota de ERK, independente de cPKC, proceda da via do AMPc (Gi→AMPc→PAK/Epac→Rap1→B-Raf), de uma via de Ras (Gi→→Ras→Raf1; Ca2+→Pyk2→→Ras→Raf1), ou ainda, de ambas. Contudo, estas interpretações devem ser consideradas inconclusivas, pois testes funcionais ainda não foram realizados.

Nesse sentido, os resultados desse trabalho são similares aos resultados apresentados por Blaukat e colaboradores (2000). Eles verificaram que o estímulo com BK em células HEK293T e HF-15 resulta na ativação de ERK, através das vias G_q → PKC e G_i → Ras, mas ocorre de maneira independente de PI3K, subunidades βγ e tirosinas quinases. Além disso, Dikic e colaboradores (1996) identificaram uma larga dependência, porém

parcial, da via da PyK2 (ativada por PKC e Ca^{2+}) →→Ras na ativação de ERK, em células PC12 acionadas por BK.

Além das vias que convergem para a cascata de ERK, é importante considerar a multiplicidade de regulações dessa rota. Nesse sentido, informações cinéticas e de sublocalização intracelular abrangem um amplo contexto, podendo ser úteis para rejeitar ou admitir um certo conjunto de mecanismos. O presente estudo é o primeiro a investigar a cinética e sublocalização de ERK após estímulo com BK em CPNs e células PC12. Interessantemente, verificou-se que o estímulo das células com BK faz com que a ERK seja ativada por um tempo prolongado, de até 60 minutos (Figuras 4.20 e 4.35), e que ela transloque para o núcleo (Figura 4.21 e 4.36). Essa cinética e sublocalização de ERK são comparáveis às induzidas por fatores neurogênicos, como o NGF e PACAP, em células PC12, e o ácido retinóico (AR) em células P19 (Bouschet et al, 2003; Ebisuya et al, 2005; Reffas & Schlegel, 2000).

Em um perfil, aparentemente simples, de ativação de ERK, como o originado por BK, NGF, PACAP e AR, há um conjunto complexo de mecanismos que são regulados de maneira coordenada para a existência de tal perfil. Entre estes mecanismos, pode-se citar: a desativação de vias de retroalimentação negativa; desativação de fosfatases das MAPKs, principalmente as nucleares, como a MPK1 (Reffas & Schlegel, 2000); ativação de vias de retroalimentação positiva (Santos et al, 2007)); ativação de vias de comunicação cruzada (*crosstalk*) que acionam a translocação de ERK para o núcleo, tais como a Akt e PKA (Impey et al, 1999; von Kriegsheim et al, 2009), etc. Assim, conforme abordado na Seção 1.3.2, muitos estudos suportam a hipótese de que a cinética de ativação prolongada, em conjunto com a translocação nuclear de ERK, fornecem especificidade ao

sinal dessa quinase e compõem a forma de sua ativação, que é necessária e suficiente para resultar na resposta neurogênica em células PC12 e, possivelmente, em células P19 (Bouschet et al, 2003; Ebisuya et al, 2005; Santos et al, 2007; Sasagawa et al, 2005). Dessa forma, considerando os efeitos da BK na neurogênese de CPNs e células PC12, sugere-se que essa forma de ativação da ERK tratar-se de um mecanismo da ação desta cinina para tal efeito.

5.5 Influências das vias de sinalização da bradicinina na diferenciação neural de CPNs e células PC12

Identificou-se, em CPNs, que a BK atua através de sua ligação ao receptor B2 de cininas, que parece se acoplar tanto à proteína Gq quanto à proteína Gi, ativando a produção/mobilização dos clássicos segundos mensageiros AMPc e Ca^{2+}. Já em nível de efetores, identificou-se que a BK induz a ativação de PI3K/Akt, p38 e ERK. Considerando que quinases estão, supostamente, mais a jusante que segundos mensageiros, optou-se por avaliar apenas a influência das primeiras nos efeitos de BK durante a diferenciação neural.

Os resultados apresentados nessa tese mostraram que a BK, com ou sem o inibidor de PI3K, Ly294002, favoreceu a migração e neurogênese e suprimiu a proliferação de CPNs indiferenciadas (Figuras 4.24; 4.29; 4.32). Este inibidor bloqueou a ativação de Akt por BK, demonstrando sua funcionalidade (Figura 4.22b), e não alterou a proporção de células viáveis (Figura 4.23). Portanto, PI3K/Akt não estão implicadas nos mecanismos efetores desses três eventos da diferenciação neural impostos por BK. Entretanto, nossos resultados também mostraram fosforilação de Akt por BK. Logo, essa indução de Akt por Bk deve seguir outros efeitos celulares, por exemplo, a

neuroproteção. Este evento é reconhecidamente induzido pela ativação do receptor B2 de cininas e é amplamente relacionado com a atividade de Akt (Su et al, 2012; Xia et al, 2004).

Por outro lado, os resultados apresentados mostraram que a BK, com o inibidor de p38 SB203580, perdeu a capacidade de suprimir a proliferação de CPNs indiferenciadas e de favorecer a migração durante a diferenciação. Estes dados demonstram que a p38 está implicada nos mecanismos impostos por BK que resultam nestas respostas celulares. Além disso, tais dados estão de acordo com o papel da p38 na regulação negativa da proliferação de CPNs e no favorecimento da migração de muitos tipos celulares (Roussa et al, 2006; Sato et al, 2008).

Por fim, investigou-se o envolvimento de ERK na diferenciação de CPNs e células PC12. A inibição farmacológica dessa MAPK bloqueou completamente o efeito neurogênico e antiproliferativo de BK (Figuras 4.25; 4.26; 4.38). Observou-se que tanto a inibição direta de MEK/ERK com U0126 e PD98059, quanto a inibição de uma de suas vias a montante (Go6973; inibidor das cPKCs), impediu o favorecimento da neurogênese desencadeado por esta cinina. A inibição das cPKCs também aponta para a importância da ativação sustentada de ERK, disparada por BK na neurogênese em células PC12. Isso pode ser sugerido, uma vez que o bloqueio da primeira quinase aboliu a ativação prologada de ERK e, como resultado celular, observou-se a perda da resposta à BK na neurogênese (Figuras 4.37 e 4.38). É sabido que esta abordagem experimental possui falhas, visto que ela descarta as possíveis influências de cPKCs na neurogênese de maneira independente de ERK. Entretanto, este experimento, também realizado por outros trabalhos (com outros fatores) (Santos et al, 2007), contribuiu para propor o papel

da ativação sustentada de ERK no favorecimento da neurogênese, em resposta à BK. Em suma, estes resultados demonstram que a ERK compõe os mecanismos efetores da neurogênese, impostos por BK.

Entender mecanisticamente os efeitos da BK na diferenciação neural é potencialmente muito importante, pois pode ter promissoras implicações na terapia de doenças e lesões do SNC, como por exemplo, na doença de Parkinson, Alzheimer e Esclerose Múltipla e nas lesões isquêmicas. Nesse sentido, a demonstração da presença de células-tronco em áreas específicas do encéfalo adulto, como a zona subventricular e o hipocampo trouxeram novas perspectivas para a terapia celular e a neuroregeneração (Svendsen & Smith, 1999). Em outras palavras, a abordagem terapêutica sobre o reestabelecimento da circuitaria neural pela neurogênese de CPNs transplantadas ou endógenas, tem sido amplamente estudada. Nesse contexto, como detalhado na Seção 1.4.1, estudos pioneiros sugere que a BK induz a neuroregeneração/neurogênese de CPNs endógenas em modelo animal da doença de Parkinson (lesão da via nigro-estriatal por 6-hidroxidopamina; estudo do nosso laboratório ainda em preparação) e em ratos recém-isquêmicos (Xia et al, 2004; Xia et al, 2006). Assim, prevendo possíveis aplicações da BK em terapias de reparo neuronal, as elucidações apresentadas nessa tese sobre os seus mecanismos na diferenciação neural podem fornecer caminhos promissores para novas abordagens terapêuticas utilizando a própria BK e/ou alvos subjacentes desta.

Conclusões 6

Essa tese elucidou alguns mecanismos de ação da bradicinina em processos da diferenciação neural *in vitro.* Em suma, entre os resultados mais relevantes, pode-se destacar a influência de BK sobre a proliferação (auto-renovação) de CPNs indiferenciadas, conforme apresentado na Figura 6.1. Essa cinina exibiu um efeito antiproliferativo, dependente da atividade de ERK e p38, em decorrência do acúmulo de células na fase G_1 do ciclo celular. Considerando que o prolongamento dessa fase induz a divisão para diferenciação em CPNs, esses resultados podem compor um mecanismo efetor da neurogênese disparado por BK.

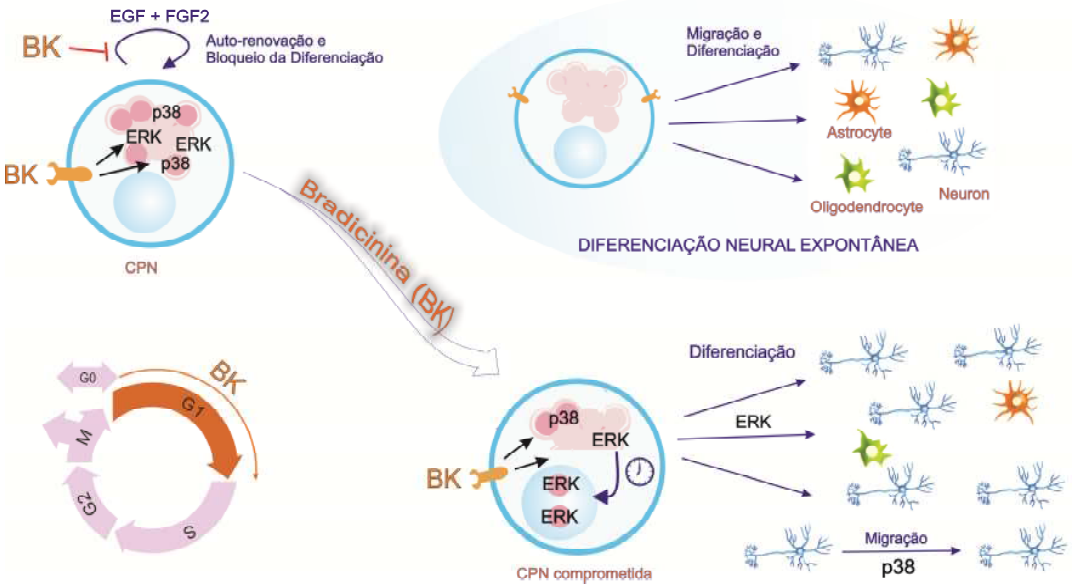


Figura 6.1: Representação esquemática dos possíveis mecanismos de ação da BK na diferenciação neural *in vitro*. Mais detalhes no texto.

Adicionalmente, destaca-se a dependência da atividade de ERK para os efeitos de BK sobre à neuro-gliogênese. Essa cinina disparou a ativação de ERK por tempo prolongado com concomitante translocação da mesma para o núcleo, em CPNs e células PC12. Essa forma/topologia de ativação de ERK é conhecida por resultar na neurogênese

em células CP12. Os resultados dessa tese também sugerem que tal forma de ativação de ERK compõe um mecanismo efetor da neurogênese disparado por BK. Além disso, observou-se que a p38 está implicada na ação desta cinina sobre o favorecimento da migração durante a diferenciação.

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FORMAÇÃO ACADÊMICA/TITULAÇÃO

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Universidade de São Paulo (USP), Instituto de Química, São Paulo, SP, Brasil. Laboratório: Desenvolvimento Neural
Título: Mecanismos de Ação da Bradicinina na Diferenciação Neural *in vitro.*
Orientador: Prof. Dr. Alexander Henning Ulrich
Bolsista do: Conselho Nacional de Desenvolvimento Científico e Tecnológico

2007 - 2009 Mestrado em Biologia Celular e Molecular (Capes 5).

Pontifícia Universidade Católica do Rio Grande do Sul, PUC-rs, Porto Alegre, RS, Brasil.
Laboratório: Imunologia Celular e Molecular.
Título: Aspectos da proliferação celular na infecção por HTLV-I e sua relação com o quadro clínico e a sensibilidade aos glicocorticoides.
Orientador: Moisés Evandro Bauer

Bolsista do: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2007 - 2008 Especialização em Farmácia Hospitalar.

Escola Superior de Gestão e Ciências da Saúde, Porto Alegre, RS, Brasil.
Título: Liderança: Uma avaliação do nível de entendimento de profissionais farmacêuticos.
Orientador: Josue Schostack

2003 – 2006 Graduação em Farmácia

Universidade Regional do Noroeste do Estado do Rio Grande do Sul- UNIJUÍ, Rio Grande do Sul, Brasil.
Título: Determinação do Teor de 17ß-Estradiol e Acetato de Noretisterona Dissolvido em Medicamentos Manipulados e Industrializados Usados na Terapia Hormonal na Menopausa.
Orientadora: Angélica Cristiane Moreira

IDIOMAS

Inglês Compreende Bem, Fala Razoavelmente, Escreve Bem, Lê Bem.

Espanhol Compreende Razoavelmente, Fala Razoavelmente, Escreve Razoavelmente, Lê Bem

PRÊMIOS E TÍTULOS

2013 Prêmio Viagem Internacional (2000 dólares) para doutorando destaque do Programa de Pós-graduação em Bioquímica - Universidade de São Paulo.

2012 Honorable Mention (poster), III Meeting of the Brazilian Purine Club.

2010 John N. Whitaker Award the Best Paper in Clinincal Neuroimmunology - Paper "Change in T cell phenotype and activated MAPKs are correlated to impaired... ", International Society of Neuroimmunology / **Prêmio de pesquisadora Junior para Pillat, MM** pelo melhor artigo do ano na área de Neuroimunologia Clinica. 2000 dólares.

PUBLICAÇÕES

Artigos Publicados em Revistas Científicas

1. Pillat, Micheli M., Correa, B.L., Lampert, S.S., Lopes, R.P., Müller, G.C., Rocha, C.F.K., Menna-Barreto, M., Teixeira, A. L., Bauer, M.E. Changes in T cell phenotype and activated MAPKs are correlated to impaired cellular responses to antigens and glucocorticoids during HTLV- I infection. **Journal of Neuroimmunology**, v.216, p.76 - 84, 2009.

2. Pillat, M. M., Moreira, A. Determinação do Teor de 17ß-Estradiol e Acetato de Noretisterona Dissolvido em Medicamentos Manipulados e Industrializados Usados na Terapia Hormonal no Climatério. **Revista Contexto & Saúde**, v.6, p.17 - 26, 2007.

3. LAMEU, C., TRUJILLO, C. A., SCHWINDT, T. T., NEGRAES, P. D., PILLAT, M. M., MORAIS, K. L. P., LEBRUN, I., ULRICH,H. Interactions between the NO-Citrulline Cycle and Brain-derived Neurotrophic Factor in Differentiation of Neural Stem Cells. **Journal of Biological Chemistry**, v.287, p.29690 - 29701, 2012.

4. PILLAT, MICHELI MAINARDI, Bauer, Moisés E., OLIVEIRA, A. C. P., ULRICH, HENNING, CASSEB, J. HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP): Still an Obscure Disease. **Central Nervous System Agents in Medicinal Chemistry** v.11, p.239 - 245, 2011.

5. TRUJILLO, C. A., NEGRAES, P. D., SCHWINDT, T. T., LAMEU, C., CARROMEU, C, MOUTRI, A.L., PESQUEIRO, J.B., CERQUEIRA, D.M., PILLAT, M. M., DE SOUZA. H.D.N., TURAÇA, L.T., ABREU, J.G., ULRICH, H. Kinin-B2 Receptor Activity Determines the Differentiation Fate of Neural Stem Cells**. The Journal of Biological Chemistry**, v.287, p.44046 - 44061, 2012.

6. GLASER, TALITA, CAPPELLARI, ANGÉLICA REGINA, PILLAT, MICHELI MAINARDI, ISER, ISABELE CRISTIANA, WINK, MÁRCIA ROSÂNGELA, BATTASTINI, ANA MARIA OLIVEIRA, ULRICH, HENNING. Perspectives of purinergic signaling in stem cell differentiation and tissue regeneration. **Purinergic Signalling**. v.8, p.523 - 537, 2012.

7. OLIVEIRA, SOPHIA L. B., Pillat, Micheli M., CHEFFER, ARQUIMEDES, LAMEU, CLAUDIANA, SCHWINDT, TELMA T., ULRICH, HENNING. Functions of neurotrophins and growth factors in neurogenesis and brain repair. **Cytometry. Part A**. v.83A, p.76 - 89, 2012.

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BOFF, E. T. O., HAMES, C., FRISON, M. D., AUTH, M. A., FERREIRA, F. W., PILLAT, M. M., NONENMACHER, S. E., ZANON, L. B. Fundamentos e propostas para educação em química no ensino fundamental. Em: **Alimentos: Produção e Consumo.** Editora Unijuí. 2007 p.88p.

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1. Muller, G.C., Correa, B. L., PILLAT, M. M., Pires, P. J., Matias, R. F., Pitrez, P. M. C. Glucocorticoid Resistance in children with asthma In: **VII Congress of the International Society for Neuro Imuno Modulation**, 2008, Rio de Janeiro, Brazil.

2.Pillat, M. M., MOREIRA, A. Verificação in Vitro da Eficácia de Medicamento Contendo Hormônio Obtido Magistralmente Comparando-se com Medicamento Obtido por Processo Industrial para a TRH In: **XIV Seminário de Iniciação Científica UNIJUÍ**, 2006, Ijuí. Livro de Resumos do XIV Seminário de Iniciação Científica da UNIJUÍ. Ijuí, 2006.

3 PILLAT, M. M., LAMEU, C., SCHWINDT, T. T., ULRICH, H. Bradykinin Signaling Suppresses Proliferation and Induces Neurogenesis of Mouse Neural Progenitor Cells In: International Summer School: **Advanced Molecular Biology Methods in Biotechnology**, 2011, Piran, Eslovênia.

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7. Pillat, M. M. , Trujillo, C.A., Schwindt, T.T., Ulrich, H. Promotion of Neurogenese by Bradykinin- Induced ERK Signaling. In: **Congresso Brasileiro de Células-Tronco e Terapia Celular,** 2010, Rio Grande do Sul, Brasil. p.111

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10. CHEFFER, A, PILLAT, MM, ULRICH, H. Importance of tyrosine and threonin residues in the regulation of human P2X4 receptor activity In: **III Meeting of the Brazilian Purine Club** 'Purinergic Signaling: Structure, Functions and Therapeutic Applications', 2012, Ouro Preto, MG, Brazil.

11. Hutton G. on Behalf of the CAMMS223 Study Group (Pillat, MM) .The CARE-MS-SM II Trial (comparison of alemtuzumab and rebif® Efficacy in multiple sclerosis): updated design of a phase 3, open-label, rater-blinded study of alemtuzumab in patients with multiple sclerosis who have relapsed on therapy. In: **19th Meeting of the European Neurological Society**. 2009. Milan, Italy.

12 NASCIMENTO, I. C., PILLAT, M. M., ANDRADE, A., ULRICH, H. Modulation of neural differentiation of mouse embryonic stem cells by bradykinin. In: IBRO's 2011 **World Congress of Neuroscience**, 2011, Florence, Italy.

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14.Pillat, M. M., Correa, B. L., Rocha, C. F. K.,Muller, G.C., Lopes, R. P., Lampert, S.S., Menna-Barreto, M., Bauer, M. E. Influence of ERK and p38 on glucocorticoid response in HTLV-I-infected patients. In: **III Iberoamerican Congress of NeuroImmunoModulation,** 2009, Buenos Aires. Argentina p.109.

15.Lampert, SS., Correa, B. L., Pillat, MM., Bauer, ME. Correlation of Psychoneuroimmunological markers of chronic stress in older caregivers. In**: XXXIV Congress of the Brazilian Society for Immunology** and X International Symposium on Allergy and Clinical Immunology, 2009, Bahia. Brazil, 2009. p.44

16.Lampert, S.S., Pillat, M. M., Correa, B. L., Bauer, M. E. Psychoneuroimmunological correlates of chronic stress in older caregivers. In: **III Iberoamerican Congress of NeuroImmunoModulation**, 2009, Buenos Aires.

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Demais tipos de produção técnica

1. Pillat, MM; Ulrich,H, Lardner, NCSP, Augusto, O. Bioquímica Experimental Avançada. Universidade de São Paulo USP, São Paulo, SP, Brasil. (Desenvolvimento de **material didático** ou instrucional - Caderno) 2010.

2. Boff, Eva Teresinha de Oliveira; Pillat, Micheli M.; Brandão, L; Stolz, Eveline. Bioquímica I. Universidade Regional do Noroeste do Estado do Rio Grande do Sul, Unijuí, Ijuí, RS, Brasil. (Desenvolvimento de **material didático** ou instrucional - Caderno) 2005.

APRESENTAÇÃO DE TRABALHO E PALESTRA

1. Mecanismos de Indução da Neurogênese pela Bradicinina
Cidade: São Paulo; 45 minutos, 2013.
Evento: III Reunião anual do Núcleo de Apoio à Pesquisa em Neurociência Aplicada da Universidade de São Paulo (NAPNA/USP); Inst.promotora: NAPNA/USP

2. **BRADYKININ SIGNALING SUPPRESSES PROLIFERATION AND INDUCES NEUROGENESIS IN MOUSE NEURAL PROGENITOR CELLS**.
Cidade: Piran, Eslovênia; 40 min; 2011.
Evento: International Summer School: Advanced Molecular Biology Methods in Biotechnology; Inst.promotora: National Institute of Biology

3. **Aspectos da Proliferação Celular Espontânea na Infecção por HTLV-I e sua Relação com o Quadro Clínico e a Resistência aos Glicocorticóides**,
Local: Pontifícia Universidade Católica do Rio Grande do Sul; Porto Alegre; 30min; 2008.
Evento: III Mostra de Pesquisa da Pós-Graduação da PUCrs; Inst.promotora: PUCrs.

4. **Verificação in Vitro da Eficiência de Medicamento Contendo Hormônio Obtido Magistralmente Comparando-se com Medicamento Obtido por Processo Industrial para a THM**.
Áreas do conhecimento: Análise e Controle de Medicamentos; 20 min; 2006.
Local: Universidade Federal do Rio Grande do Sul (UFRGS); Cidade: Porto Alegre; Inst.promotora: UFRGS.

4. **Verificação in Vitro da Eficiência de Medicamento Contendo Hormônio Obtido Magistralmente Comparando-se com Medicamento Obtido por Processo Industrial para a THM**.
Áreas do conhecimento: Análise e Controle de Medicamentos; 20 min; 2006.
Local: Universidade Regional do Noroeste do Estado do Rio Grande do Sul (Unijuí); Cidade: Ijuí; Inst.promotora: Unijuí.

PARTICIPAÇÃO EM CONGRESSOS E EVENTOS

Participação e apresentação de Poster na **Gordon Research Conference:Cell Grow th & Proliferation**, 2013. Título: Bradykinin-induced ERK and Akt signaling modulate proliferation and neurogenesis of neural stem cells. Vermont, Estados Unidos.

Participação e apresentação oral na **International Summer School: Advanced Molecular Biology Methods in Biotechnology**, 2011. Apresentação oral: Bradykinin Signaling Suppresses Proliferation and Induces Neurogenesis of Mouse Neural Progenitor Cells. Piran, Eslovênia.

Apresentação de Poster no **V Congresso Brasileiro de Células-Tronco e Terapia Celular**, 2010. Título: Promotion of Neurogenese by Bradykinin- Induced ERK Signaling. Gramado, RS, Brasil.

Homenageada com prêmio e participação no **10th INTERNATIONAL CONGRESS OF NEUROIMMUNOLOGY**, 2010. Prêmio: 2010 John N. Whitaker Aw ard the Best Paper in Clinincal Neuroimmunology. Sitges, Espanha.

Participação no Meeting on **Stem Cell Research: Perspectives of Stem Cells,** 2010. São Paulo, SP, Brasil.

Participação e apresentação de poster no **XXXIV Congress of the Brazilian Society for Immunology and X International Symposium on Allergy and Clinical Immunology**, Título: Possible Involvement of the CD8+CD28- Regulatory T Cells on Lymphocyte Response to Antigens and Glucocorticoids During HTLV-I infection. 2009. Salvado, Brasil.

Participação e apresentação de Poster no **XII Encontro Gaúcho de Imunologia**, Título: Determinação da Taxa Molar HspBP1/ HSP70 em Amostras de Melanoma Murino (B16F10). 2008. Porto Alegre, Brasil.

Participação e apresentação de Poster no **VII Congress of the International Society for Neuro ImunoModulation**, Título: Resistência a Glicocorticóides em crianças com asma. 2008. Rio de Janeiro, RJ, Brasil.

Participação no **XI Encontro Gaúcho de Imunologia**, 2007. Porto Alegre, RS, Brasil.

Participação no **Simpósio Gaúcho sobre Terapia Gênica e Celular**, 2007. Porto Alegre, RS, Brasil.

Participação no **FARMAPOLIS** 13 edição, 2005. Florianópolis, SC, Brasil

Participação no **V Encontro de Estudos Farmacêuticos**, 2005. Ijuí, RS, Brasil

Participação no **IV Encontro de Estudos Farmacêuticos**, 2004. Ijuí, RS, Brasil

Participação em cursos e seminários

Participação e apresentação de Poster na **Gordon Research Seminar: Cell Growth & Proliferation**, 2013. Título: Bradykinin-induced ERK and Akt signaling modulate proliferation and neurogenesis of neural stem cells. 2013. Vermont, Estados Unidos.

Curso de **Protemics Methods and Appraches for Protein Identification and Quantification**. Ministrado pelo prof. Dr. Pierre Thibault, na Faculdade de Ciências Farmacêuticas - FCF-USP.2010, São Paulo, SP, Brasil.

Curso de **Electrophysiology for immunologists**. XXXIV Congress of the Brazilian Society for Immunology and X International, SBI, 2009, Salvador, Brasil

Simpósio sobre Terapias Inovadoras. Universidade Federal do Rio Grande do Sul, UFRGS, 2009, Porto Alegre, Brasil

Curso de **Radicais Livres: verdades, angústias e expectativ**. Universidade Federal do Rio Grande do Sul, UFRGS, 2009 Porto Alegre, Brasil

Curso Básico de Citometria de Fluxo. Instituto de Cardiologia do Rio Grande do Sul, IC/FUC, 2008, Porto Alegre, Brasil

II Curso de Extensão de Inverno de Ciências Farma. Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, 2008 Porto Alegre, Brasil

Curso de **Administração de Medicamentos Injetáveis**. Escola Francisco de Assis, EFA, 2005, Ijuí, Brasil

Curso de **Síntese de Novos Fármacos.** Universidade Regional do Noroeste do Estado do Rio Grande do Sul, 2005, Ijui, Brasil

Curso de **Biologia Molecular Aplicada a Biotecnologia**. FARMAPOLIS - 13 edição, FARMAPOLIS, 2005, Florianópolis, SC Brasil

Curso de **Triagem Neonatal: Hemoglobinopatias.**
Universidade Regional do Noroeste do Estado do Rio Grande do Sul, 2005, Ijui, Brasil

Curso de **Comprimidos Matriciais**. Universidade Regional do Noroeste do Estado do Rio Grande do Sul, 2004, Ijui, Brasil

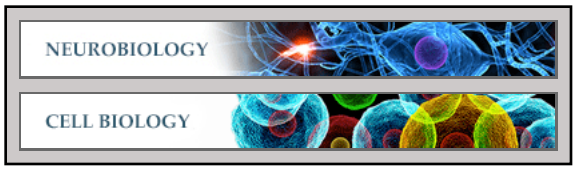
Curso de **Diagnóstico Laboratorial das Leucemias**. Universidade Regional do Noroeste do Estado do Rio Grande do Sul, 2004, Ijui, Brasil

I Conferência Regional da Política de Medicamentos e Assistência Farmacêutica, 2003. Ijuí, RS, Brasil

IV Semana Acadêmica da Saúde. Universidade Regional do Noroeste do Estado do Rio Grande do Sul, 2003, Ijui, Brasil

Neurobiology:

Kinin-B2 Receptor Activity Determines the Differentiation Fate of Neural Stem Cells



Cleber A. Trujillo, Priscilla D. Negraes, Telma T. Schwindt, Claudiana Lameu, Cassiano Carromeu, Alysson R. Muotri, João B. Pesquero, Débora M. Cerqueira, Micheli M. Pillat, Héllio D. N. de Souza, Lauro T. Turaça, José G. Abreu and Henning Ulrich

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Kinin-B2 Receptor Activity Determines the Differentiation Fate of Neural Stem Cells*

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Background: Recent studies point at functions of bradykinin in the CNS including neuromodulation and neuroprotection.
Results: Bradykinin augments neurogenesis of neural stem cells from embryonic telencephalon, whereas bradykinin receptor inhibition promotes gliogenesis.
Conclusion: Bradykinin acts as switch for phenotype determination using an *in vitro* system of migrating cells, closely reflecting conditions of cortex development.
Significance: Novel functions are described for bradykinin with therapeutic relevance.

Bradykinin is not only important for inflammation and blood pressure regulation, but also involved in neuromodulation and neuroprotection. Here we describe novel functions for bradykinin and the kinin-B2 receptor (B2BkR) in differentiation of neural stem cells. In the presence of the B2BkR antagonist HOE-140 during rat neurosphere differentiation, neuron-specific *β3-tubulin* and *enolase* expression was reduced together with an increase in glial protein expression, indicating that bradykinin-induced receptor activity contributes to neurogenesis. In agreement, HOE-140 affected in the same way expression levels of neural markers during neural differentiation of murine P19 and human iPS cells. Kinin-B1 receptor agonists and antagonists did not affect expression levels of neural markers, suggesting that bradykinin-mediated effects are exclusively mediated via B2BkR. Neurogenesis was augmented by bradykinin in the middle and late stages of the differentiation process. Chronic treatment with HOE-140 diminished *eNOS* and *nNOS* as well as *M1–M4 muscarinic receptor* expression and also affected purinergic receptor expression and activity. Neurogenesis, gliogenesis, and neural migration were altered during differentiation of neurospheres isolated from *B2BkR* knock-out mice. Whole mount *in situ* hybridization revealed the presence of *B2BkR* mRNA throughout the nervous system in mouse embryos, and less *β3-tubulin* and more glial proteins were expressed in developing and adult *B2BkR* knock-out mice brains. As a underlying transcriptional mechanism for neural fate determination, HOE-140 induced up-regulation of *Notch1* and *Stat3* gene expression. Because pharmacological treatments did not affect cell viability and proliferation, we conclude that bradykinin-induced signaling provides a switch for neural fate determination and specification of neurotransmitter receptor expression.

The central nervous system is originated from a monolayer of neuroepithelial cells from which single neural progenitors arise, proliferate, and differentiate into a complex neural network (1–3). One of the most important steps during brain development is the generation of cellular diversity, *i.e.* the decision to form neurons or glial cells. This dynamic process is tightly regulated by spatial and temporal patterns (4, 5). The mechanisms underlying progenitor proliferation and differentiation during development are related to both extrinsic and intrinsic factors (6). Extrinsic factors, including neurotransmitters, cytokines, hormones and growth factors, have been shown to influence the acquisition of neuronal or glial phenotypes (7, 8). These diffusible factors activate membrane-bound receptors, which act as morphogens and regulate the progress of neural differentiation (9).

One factor that may play a role in neural differentiation that has not been previously studied in this context is bradykinin (Bk).[7] Kinins are biologically active peptides released into the

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[5] Supported by a fellowship from Conselho Nacional de Desenvolvimento Cientifico e Tecnológico.
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[7] The abbreviations used are: Bk, bradykinin; NPC, neural progenitor cell; iPS, induced pluripotent stem; ACE, angiotensin-converting enzyme; ASS, argininosuccinate synthetase; eNOS, endothelial nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; GFAP, glial fibrillary acidic protein; NO, nitric oxide; CNS, central nervous system.

plasma or interstitial fluid after proteolytic cleavage of kininogens by kallikreins. The kallikrein-kinin system is best known for its involvement in cardiovascular homeostasis, coagulation, inflammation, pain, and development (10–12). Moreover, there are also effects on neuronal physiology of Bk and related kinins (13, 14). B1 (B1BkR) and B2 (B2BkR) G protein-coupled receptors are present in the CNS and participate in many signaling cascades and physiological consequences including NO formation and glutamate release (15–18).

Previously, we have shown that Bk secretion and *B2BkR* expression are regulated during *in vitro* neuronal differentiation of P19 embryonal carcinoma cells. Receptor expression and activity as well as generation of Bk rose with ongoing neuronal differentiation. Carbachol-induced intracellular calcium transients and gene expression of muscarinic receptors were suppressed following chronic treatment of differentiating cells with HOE-140, a specific B2BkR-antagonist (19). Thus, B2BkR activity was essential for differentiation of P19 cells into neurons with a cholinergic phenotype.

Here we report novel functions for Bk in phenotype determination whether a neural progenitor cell (NPC) differentiates into a neuron or a glial cell. Three *in vitro* differentiation models, P19 mouse embryonal carcinoma cells, rat NPCs, and human induced pluripotent stem cells were used to demonstrate the importance of B2BkR in neural fate and neurotransmitter receptor expression determination. As an underlying mechanism, we found that migration of NPCs was largely restricted when B2BkR activity was inhibited. These results were confirmed in migration assays with neurospheres obtained from *B2BkR* knock-out mice, which also revealed reduced migration. We also observed a strong expression of *B2BkR* in the developing mouse brain, and reduced β3-*tubulin* expression in *B2BkR* knock-out embryos. Together, these results indicate a novel function of Bk in the determination of cell fate in the process of neural differentiation.

EXPERIMENTAL PROCEDURES

Animals—This work was approved by the Ethics on Animal Care and Use Committee of the Instituto de Química of the Universidade de São Paulo. Wistar Hannover rats, wild type and *B2BkR*$^{-/-}$ C57BL/6 mice (provided by Instituto de Química and Center for Development of Experimental Models for Medicine and Biology, UNIFESP, respectively), were used for neural progenitor isolation and neurosphere formation. Animals were housed under optimal light, temperature, and humidity conditions, with food and water provided *ad libitum*. Timed-pregnant animals were obtained by overnight mating. The efficiency of mating was confirmed by the presence of sperm after vaginal smear or appearance of the vaginal plug. Comparison of the *B2BkR*$^{-/-}$ mice was made with their wild-type littermates. Following 14 (rats) and 12.5 days (mice) of gestation, females were sacrificed in a chamber with a saturated CO_2 atmosphere. Genotyping of the *B2BkR*$^{-/-}$ mice was performed using polymerase chain reaction (PCR) of genomic DNA extracted from tails. Detailed genotyping procedure and primers for PCR have been previously described (20).

Cortical Primary Culture—Newborn rats were decapitated and their brains removed aseptically in ice-cold phosphate-buffered saline (PBS). Briefly, after removal of meninges, the cerebral cortex was dissected and dissociated by incubation with 0.05% trypsin solution at 37 °C for 5 min followed by light trituration. After cell counting, cells were plated in DMEM/F-12 (Life Technologies) with 10% fetal bovine serum (FBS) at a density of 3×10^5 cells/ml in poly-L-lysine (1 mg/ml) pretreated dishes. The medium was replaced every other day for 7 days, and the cells remained in the incubator at 37 °C with controlled humidity and 5% CO_2.

Neurosphere Culture and Differentiation—NPCs were isolated from telencephalon of E14 rats or E12.5 mice embryos, using techniques previously described (21). After brain dissection, telencephalon was subjected to mechanical and enzymatic dissociation. Cells were grown in suspension at a density of 2×10^5 cells/ml in DMEM/F-12 in the presence of 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine, 5 μg/ml of heparin, 20 ng/ml of FGF-2, 20 ng/ml of EGF, and 2% B-27 (Life Technologies) at 37 °C in 95% humidity and 5% CO_2. Cultures were grown for 10 days with one passage prior to neural differentiation. For differentiation studies, primary whole neurospheres were allowed to attach to poly-L-lysine and laminin-coated coverslips or culture flasks with DMEM/F-12, 2% B-27 in the absence of FGF-2 and EGF. Progenitor cells were differentiated for 7 days and treated with 1 μM HOE-140 (Tocris Bioscience) or 1 μM Bk (Tocris Bioscience). The migration assay was evaluated on the seventh day of differentiation as the distance of the foremost cells to the neurosphere boundary. Neurospheres of similar diameter were used in this assay.

P19 Embryonal Carcinoma Cell Culture and Neural Differentiation—P19 mouse embryonic carcinoma cells were grown and differentiated as described previously (19, 21). In brief, for the induction of neural differentiation, 1 μM all-*trans*-retinoic acid was added to 5×10^5 cells/ml, kept in suspension to form embryoid bodies (DMEM supplemented with 2 mM glutamine, 2 mM sodium pyruvate, 2.4 μg/ml of sodium bicarbonate, 5 μg/ml of insulin, 30 μg/ml of human apo-transferrin, 100 mM ethanolamine, 30 nM sodium selenite, 100 IU/ml of penicillin, 100 mg/ml of streptomycin, and 10 mM HEPES, pH 7.4). After 2 days of treatment, embryoid bodies were transferred to culture flasks, and the medium was replaced with DMEM supplemented with 10% FBS to allow cell adhesion. After another 2 days, the medium was replaced by defined medium and maintained until the end of differentiation (day 8).

Human iPS Cell Formation and Neural Differentiation—The human-induced pluripotent stem (iPS) cell lineage was obtained and characterized as described previously (22). Human fibroblasts were generated from dermal biopsies of healthy individuals following informed consent under protocols approved by the University of California, San Diego. Briefly, fibroblasts were infected with retrovirus containing *OCT4*, c-*MYC*, *KLF4*, and *SOX2* human cDNAs (23). After 2 days, fibroblasts were plated on mitotically inactivated mouse embryonic fibroblasts (Millipore) with human embryonic stem cell medium. Following formation of iPS cell colonies, they were directly transferred into Matrigel-coated dishes (BD) containing mTeSR1 (StemCell Technologies). After embryoid body formation in low-adherence dishes in the absence of FGF-2, cell aggregates were allowed to attach to polyornithine-

and laminin-coated dishes in DMEM/F-12 (Life Technologies) supplemented with 1% N2 (Life Technologies). Following rosette visualization, they were dissociated with accutase (Millipore) and plated into coated dishes with NPC medium (DMEM/F-12 supplemented with 0.5% N2; 1% B-27 and FGF-2) to achieve a homogeneous population of NPC. Neural differentiation was induced with 1 μM retinoic acid in NPC medium in the absence of FGF-2 for 3 weeks. Mature embryoid bodies were dissociated and plated in polyornithine- and laminin-coated dishes in NPC media without FGF-2.

Immunocytochemistry—Immunofluorescence procedures have been described in detail elsewhere (24, 25). Plated neurospheres were fixed in 4% paraformaldehyde (PFA) for 20 min and then blocked/permeabilized in 3% FBS, 0.1% Triton X-100 in PBS for 30 min. After 2 h of incubation with primary antibodies against β3-tubulin (Sigma), MAP-2 (Cell Signaling), S100β (Calbiochem), nestin (Millipore), GFAP (DAKO) at 1:500 dilutions, and against B2BkR (1:1000, BD) in PBS with 3% FBS, 0.1% Triton X-100, NPCs were washed, and anti-mouse Alexa 555-conjugated and anti-rabbit Alexa 488-conjugated secondary antibodies (Life Technologies) at 1:500 dilutions were added. After washing with PBS, DAPI solution (Sigma; 0.3 μg/ml) was used as a nuclear stain. Coverslips were mounted, and slides were analyzed under a fluorescence microscope (Axiovert 200, Zeiss).

BrdU Incorporation Assay—Cell proliferation was measured following incubation with 0.2 μM 5-bromo-2-deoxyuridine (BrdU; Sigma) for 14 h. Antigen retrieval was performed following fixation of cells with 4% PFA. Cells were incubated for 30 min in 1.5 M HCl, washed in PBS, and incubated for 2 h with rat anti-BrdU (1:500, Abcam). Alexa 488-conjugated secondary antibodies were used at 1:500 dilutions. After washing with PBS, DAPI solution (0.3 μg/ml) was used as a nuclear stain. Slides were mounted and analyzed by fluorescence microscopy. In this assay, only migrated cells were considered for analysis. The percentages of BrdU-positive cells were calculated as the ratio of immunolabeled cells over the total number of DAPI-stained cells.

Western Blot Analysis—In vitro neural-differentiated cells obtained from different sources or cells from cortical primary cultures were washed once with PBS then incubated in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.1% Nonidet P-40 supplemented with protease inhibitors mixture (Amresco)). Cells were harvested and homogenized on ice. The lysates were then centrifuged for 10 min at 14,000 $\times$ *g*. The concentration of soluble protein in the supernatant was determined by using the Bradford reagent. For Western blot analysis, 10 μg of soluble protein extracts were separated in a 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted using antibodies against β3-tubulin (1:1000, Sigma), GFAP (1:1000, DAKO), tyrosine hydroxylase (1:1000, Millipore), 5-hydroxytryptamine (1:1000, Abcam), GAD65 (glutamic acid decarboxylase, 1:1000, Millipore), and β-actin (1:2000, Sigma). Horseradish peroxidase-conjugated secondary antibodies were added (1:2000, Jackson ImmunoResearch), and antibody binding was detected by using the enhanced chemiluminescence Luminol reagent (Santa Cruz). Autoradiography films were exposed to the membranes and developed using a Kodak film processor. Band intensities were determined by densitometry and reported as ratios of neuronal and glial markers over β-actin contents. Densitometry analysis was performed using ImageJ software (NIH). Background values were subtracted from all densitometric determinations.

Flow Cytometry Analysis—Flow cytometry procedures were in agreement with previously published protocols (24, 26). Neurospheres and cortical primary cultures were centrifuged for 5 min at 200 $\times$ *g* and dissociated to a single cell suspension. Cells were fixed for 20 min in ice-cold 1% PFA in PBS, washed with PBS supplemented with 2% FBS, and incubated for 2 h with primary antibodies specific for neural markers (β3-tubulin, GFAP, nestin, and neuronal specific enolase (NSE, BioMeda, Foster City, CA)) at 1:500 dilutions. Following a washing step with PBS, cells were incubated with 1:500 Alexa 488- or 555-conjugated secondary antibodies (Life Technologies) and then analyzed on a flow cytometer (Fc500, Beckman Coulter, Fullerton, CA). An argon laser line was used for fluorescence excitation (FL1 525 nm and FL2 575 nm, band pass filter). Fifty-thousand events were acquired per sample with fluorescence measured in logarithmic scales. Background fluorescence was measured using unlabeled cells and cells labeled with secondary antibody alone and used to set gating parameters between positive and negative cell populations. Forward and side light-scatter gates were used to exclude cell aggregates and small debris.

Data were analyzed using the Cyflogic software and plotted in a histogram format. All histograms were smoothed by the software. Fluorescence gates were set below 2% of blank histogram and events corresponding to a fluorescence signal exceeding this percentage were considered as positive events. The results are reported as mean $\pm$ S.D. of positively stained cells.

TUNEL Assay—The effect of HOE-140 treatment on NPC viability was determined using the In Situ Cell Death Detection Kit (Roche Applied Science), according to the protocol provided by the manufacturer. For the negative control, instead of being incubated with the TUNEL reaction mixture, cells were kept in the absence of terminal transferase. For the positive control, cells were incubated with DNase I (3 units/ml, Ambion) for 10 min at room temperature. Thirty-thousand events were acquired in a flow cytometer (Beckman Coulter, Fc500) and analyzed with the Cyflogic software.

Reverse Transcription and Quantitative Polymerase Chain Reaction—Total neurosphere RNA was extracted using the TRIzol reagent (Life Technologies). Following DNase I treatment, 3 μg of RNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Life Technologies). Quantitative SYBR Green real-time PCR was performed with the Step One Plus Instrument (Life Technologies). Each 25 μl of SYBR Green reaction consisted of 25 ng of cDNA, 12.5 μl of 2$\times$ SYBR Green Universal PCR Master Mix (Life Technologies), and 200 nM of each forward and reverse primers. Unless otherwise stated, primer sequences were designed using Primer Express Software and can be found in Table 1. Real-time PCR were performed using the temperature protocol 50 °C for 2 min, 95 °C for 10 min, and 50 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a dissociation curve protocol for evalua-

TABLE 1
Primer sequences and amplicon sizes (base pairs, bp) of cDNAs coding for neurotransmitter receptors, nitric oxide-related enzymes, neural markers, transcription factors, and GAPDH used for real-time PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	bp	Ref.
P2X1	GAGAGTCGGGCCAGGACTTC	GCGAATCCCAAACACCTTGA	233	66
P2X2	TCCCTCCCCCACCTAGTCAC	CACCACCTGCTCAGTCAGAGC	149	66
P2X3	CTGCCTAACCTCACCGACAAG	AATACCCAGAACGCCACCC	150	66
P2X4	CCCTTTGCCTGCCCAGATAT	CCGTACGCCTTGGTGAGTGT	145	66
P2X5	GGATGCCAATGTTGAGGTTGA	TCCTGACGAACCCTCTCCAGT	81	66
P2X6	CCCAGAGCATCCTTCTGTTCC	GGCACCAGCTCCAGATCTCA	152	66
P2X7	GCACGAATTATGGCACCGTC	CCCCACCCTCTGTGACATTCT	171	66
P2Y1	AACCGTGATGTGACCACTGA	TTCAACTTGTCCGTTCCACA	216	67
P2Y2	TGCTGGGTCTGCTTTTTGCT	ATCGGAAGGAGTAATAGA	209	67
P2Y4	TCGATTTGCAAGCCTTCTCT	CCATAGGAGACCAGGGTGAT	215	67
P2Y6	TGCTGCTACCCCCAGTTTAC	TGGCATAGAAGAGGAAGCGT	246	67
P2Y12	CTGTTTTTTGCTGGGCTCATC	GCGGATCTGGAAGAAAATCCT	60	
P2Y13	GGATGCAGGGCTTCAACAA	GCAGCTGTGTCATCCGAGTGT	60	
P2Y14	GGTGGGTTTCGCCTCATGT	CCTCAGGTGACCGGCATCT	56	
M1 mAChR	CTGTCACGGTCATGTGTACACTGT	CCGGGCTCGGTTTTCTGT	62	
M2 mAChR	CAAAAATGGCAGGCATGATG	GGCCCAGAGGATGAAGGAAA	59	
M3 mAChR	CGACGTGGTGTGATGATTGG	ATGGCAGGAGCCCATAGGA	62	
M4 mAChR	CACCAAACCCCTCACCTATCC	CGATCATGAGACCTGCCATCT	59	
M5 mAChR	CCATAATCCTGTCCGAATGGA	ATCCCGTGGCATTAGTTAGCA	64	
eNOS	GACTTTTAAGGAAGTAGCCAATGCA	CCATACAGGATAAGTCGCCTTCAC	93	
nNOS	CCAATGTTCACAAAAAACGAGTCT	TCGGCTGGACTTAGGGCTTT	77	
Argininosuccinate synthetase	TGCACTCTATGAGGACCGCTATC	AGGCCTGGCGAGAGAGGTGCCTAG	49	
B1BkR	CCAGGGTTCGTCATCACTATCTG	GCAAAAGGAAGAAGGACAAGACTAA	73	68
B2BkR	CCCTTCCTCTGGGTCCTCTT	CAGAACACGCTGAGGACAAAGA	65	68
β3-Tubulin	GAGACCTACTGCATCGACAATGAAG	GCTCATGGTAGCAGACACAAGG	111	25
GFAP	AAGAGTGGTATCGGTCCAAGTTTG	CAGTTGGCGGCGATAGTCAT	107	25
S100β	TGGTTGCCCTCATTGATGTCT	CCCATCCCCATCTTCGTCC	179	
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC	117	66
Ngn1	CAGTAGTCCCTCGGCTTCAG	AAGCAGGGTGTCGTATGGAG	102	69
Notch1	CACCACGCCTCTCCCACCTGCCTGTAGC	TGCCTGTGTGCTTAGTGTGCCCGGAGTC	209	70
Stat3	TATCTTGGCCCTTTGGAATG	GTGGGGATACCAGGATGTTG	284	71
NeuroD1	CTTCCCGGTGCATCCCTACTCCTACC	AGGAAGGGCTGGTGCAATCAGTTAGG	167	70

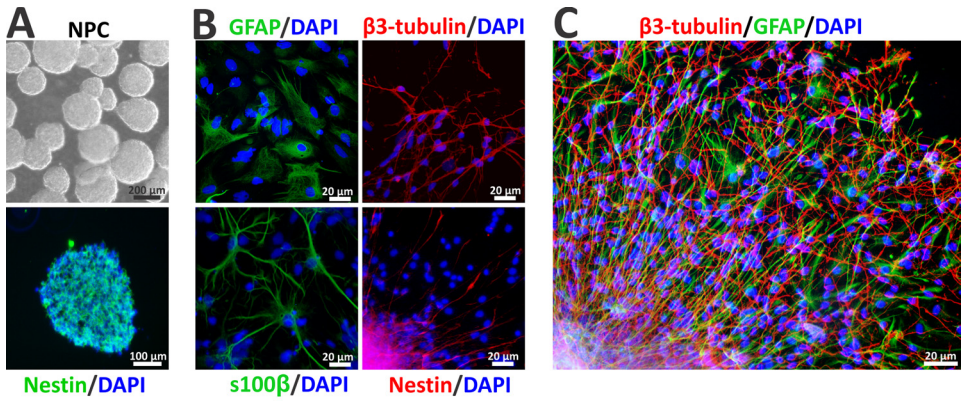
tion of the specificity of the amplicon produced in each reaction. A distinct peak indicated that a single DNA sequence was amplified during PCR.

Standard curves were measured for each primer set and cDNA sample to verify the efficiency of the reaction. As the efficiency of all reactions was >95%, the $2^{-\Delta\Delta Ct}$ parameter was used for relative quantification of gene expression. The data shown were obtained from three independent samples and RT-PCR real-time reactions were prepared in triplicates for each analyzed gene. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene expression was determined as endogenous control.

Calcium Imaging by Confocal Microscopy—Intracellular calcium transients were measured by fluorescence imaging of differentiated cells using the calcium indicator dye fluo-3 AM as described elsewhere (19). Differentiated neurospheres were loaded with 5 μM fluo-3 AM in 0.5% DMSO and 0.1% of F-127 pluronic acid for 1 h at 37 °C. After three washes with culture medium, cells were placed in a warm chamber and fluorescence emissions were captured by a LSM 510-Meta confocal microscope (Zeiss). Following chronic treatment with HOE-140, the inhibitor was removed from the cell culture 1 h prior to calcium measurements by medium change and washing the cell layers five times. Fluo-3 AM was excited using the 488 nm line of argon ion laser, and the emitted light was detected at 515–530 nm using a band-pass filter. Time kinetics of free intracellular calcium ($[Ca^{2+}]_i$) variations were constructed from over 300 images collected in 1-s intervals. The fluorescence intensities (F) were calibrated in a solution containing 5 mM ionophore (F_{max}) and 10 mM EGTA (F_{min}) to provide an estimation of the absolute change in the intracellular calcium concentration using the following equation: $[Ca^{2+}]_i = K_d[(F - F_{min})/(F_{max} - F)]$; assuming a 450 nM K_d for fluo-3 AM. $[Ca^{2+}]_i$ levels of cell populations prior and following stimulation were calculated using the average value of at least five fields of observation in independent experiments.

Whole Mount in Situ Hybridization—Whole mount *in situ* hybridizations were adapted from a protocol described elsewhere (27). In summary, mouse embryos were fixed in 4% PFA, treated with proteinase K, re-fixed with 4% PFA, 0.1% glutaraldehyde and hybridized overnight with 1 μg of digoxigenin-labeled RNA sense and antisense probes. After the wash, embryos were treated with a solution containing 10% goat serum, 1% Boehringer Block, and 0.1% Tween 20 in PBS at 4 °C for 2 h and then incubated overnight with anti-digoxigenin alkaline phosphatase antibodies at 4 °C. Finally, the embryos were washed in 0.1% BSA and stained overnight with alkaline phosphatase substrate at 4 °C. *B2BkR* sense (5′-GGACTCCCTACAACACAGAAC-3′) and antisense (5′-GGACAAAGAGGTTCTCCAGTG-3′) probes were generated by linearization and *in vitro* transcription of pBluescript II KS-*B2BkR* (NM_009747.2) with XbaI/T3 and XhoI/T7, respectively.

Statistical Analyses—The results were expressed as mean ± S.D. from three or more independent experiments, unless otherwise stated. Statistical comparisons between different treatments were done by either a Student's t test or one-way analysis of variance by using GraphPad Prism 5.1 software (Graph-Pad Software Inc.). For quantification of immunolabeled $BrdU^+$ cells, a minimum of 300 and up to 800 cells per sample was analyzed using ImageJ software. For



NPC undifferentiated NPC differentiated

Marker	Mean ± SD (%)	Median	Mean ± SD (%)	Median
β3-tubulin	35.1±6.3	62	76.4±7.0	180
GFAP	19.2±4.7	56	36.4±8.4	97
Nestin	42.2±9.2	19	23.2±7.1	25

FIGURE 1. ***In vitro* neural progenitor differentiation.** *A,* neural progenitor was obtained from rat embryo telencephalon (E14) induced for 7 days to proliferation for formation of neurospheres. *Upper panel*, phase-contrast image of primary undifferentiated neurosphere (NPC). *Lower panel*, nestin is highly expressed in undifferentiated neurospheres. *B,* typical immunofluorescence images of neurospheres on day 7 of differentiation. Differentiated neurospheres express specific protein markers for progenitor cells (nestin), astrocytes (GFAP and s100*β*), and neurons (*β3-tubulin*). *C*, radial cell migration pattern and neural maturation. The radial migration observed near the neurospheres consists mainly of precursor cells and astrocytes, whereas neuronal migration occurs to form a distal network. *D*, detection of co-expression of B2BkR and MAP-2, indicating that B2BkR are expressed in mature neurons. *E*, flow cytometry analysis of neural markers expression of undifferentiated (*red lines*) and differentiated (*blue lines*) neurospheres. Events with higher fluorescence as those in the control histograms (within the area delimited by *bars*) were considered positive and quantified in the table below. The data shown are representative of at least three independent experiments.

flow cytometry, a minimum of 30,000 cells was analyzed per sample. The criteria for statistical significance were set at $p < 0.05$.

RESULTS

B2BkR and Neural-specific Protein Expression Profile during Neurosphere Differentiation—Rat telencephalon cells were cultured in growth medium to allow neural stem cells and NPCs to proliferate and form neurospheres (Fig. 1*A*). Consistent with Martins *et al.* (24), undifferentiated neurospheres expressed high levels of GFAP and nestin, in some cases co-expressed in the same cell. Following induction of differentiation, the number of nestin-positive cells in the outer layers of migrating cells decreased, whereas cells within the neurosphere remained undifferentiated (24). Neuron-specific protein β3-tubulin, and astrocyte-specific s100*β* were expressed at high levels in differentiated cells (Fig. 1*B*). Cells elongated in a radial pattern with intense staining for GFAP and nestin. Network-forming differentiated cells were located most distally (Fig. 1*C*). Double-immunostaining against MAP-2 and B2BkR on day 7 of differentiation revealed that the B2BkR was expressed by mature neurons, as shown in Fig. 1*D*.

Analysis by flow cytometry clearly confirmed the difference in the expression of proteins specific for un- or differentiated neurospheres (Fig. 1*E*). Expression of the neuronal marker *β*3-tubulin was detected in 35 and 76% of undifferentiated and differentiated cells, respectively. GFAP and nestin were present in 19 and 42% of undifferentiated cells, respectively. In differentiated cells, the expression of GFAP increased to 36%, and nestin expression was reduced to 23% of the cell population.

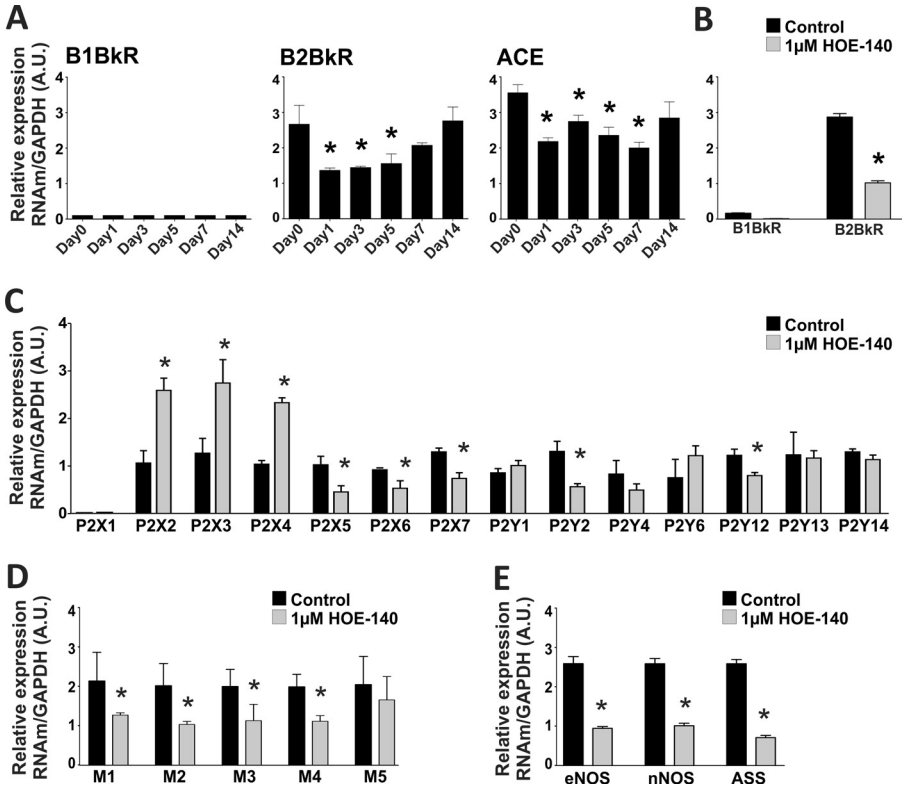


FIGURE 2. **Gene expression of components of the kallikrein-kinin system and neurotransmitter receptors after chronic treatment with 1 μM HOE-140 along rat neural differentiation.** *A*, *B1BkR* gene expression could not be detected during 14 days of neurosphere differentiation, whereas *B2BkR* and angiotensin-converting enzyme (*ACE*) expression showed an initial reduction after differentiation induction. *B2BkR* expression increased again during the final differentiation. *B*, *B2BkR* gene expression after NPC treatment with HOE-140 during 7 days. *C*, specific B2BkR inhibition for 7 days caused an alteration in ionotropic and metabotropic purinergic receptor gene expression. *D* and *E*, chronic HOE-140 treatment led to reduced muscarinic receptor gene expression as well as key proteins involved in nitric oxide formation (neuronal nitric-oxide synthase, nNOS; endothelial nitric-oxide synthase, eNOS; argininosuccinate synthetase, ASS). The data are representative for three independent experiments conducted in triplicate and shown as mean ± S.D. (*, $p < 0.05$).

Throughout differentiation, percentages of neuronal and glial phenotypes increased in the cell population, whereas percentages of NPCs decreased.

B2BkR Inhibition during Differentiation Alters Expression and Activity of Neurotransmitter Receptors—A large number of membrane receptors are expressed to initiate complex sets of sequential transcriptional events important for cell fate determination. The expression of kinin, purinergic, and muscarinic receptors during rat differentiation was quantitatively evaluated by real-time PCR. The expression of the *B1BkR* was lower than the detection limits of the methodology employed, whereas *B2BkR* expression decreased initially and increased during later differentiation. The transcriptional levels of angiotensin-converting enzyme (*ACE*) mRNA controlling lifetime of biologically active kinins remained stable (Fig. 2*A*). Chronic treatment of differentiating rat neurospheres with HOE-140, a specific antagonist of the B2BkR, significantly decreased the gene expression of *B2BkR* (Fig. 2*B*). The expression of other components of the kallikrein-kinin system in neurospheres and Bk release were already reported in a previous publication of our group (24). Quantitative real-time PCR analysis revealed a significant increased expression of rat *purinergic P2X2*, *P2X3*, and *P2X4* receptor subunits and decreased expression of *P2X5*, *P2X6*, *P2X7*, *P2Y2*, and *P2Y12* subtypes (Fig. 2*C*). The expression of *M1–M4 muscarinic receptors* decreased following chronic treatment with HOE-140, corroborating previous data obtained from P19 cells (19) and supporting the existence of an interrelationship between cholinergic and kallikrein-kinin systems (Fig. 2*D*). Thus, Bk influences the expression of purinergic and cholinergic receptors during neural differentiation. We also investigated the presence of transcripts of *endothelial* and *neuronal nitric-oxide synthase* (*eNOS* and *nNOS*) and *argininosuccinate synthetase* (*ASS*) along neural differentiation. Considering the role of NO in neural differentiation and proliferation (28–30), the inhibitory effects of HOE-140 on gene expression of *eNOS, nNOS*, and *ASS* further indicate functions for the B2BkR during neural differentiation (Fig. 2*E*).

HOE-140-induced effects on iono- and metabotropic receptors in differentiated rat neurospheres were also studied by using calcium imaging. HOE-140 was completely removed from the cells following several washes 1 h before the beginning of the experiment. ATP- and UTP-induced receptor responses diminished in the presence of HOE-140, reflected by changes in $[Ca^{2+}]_i$ peak values from Δ1695 ± 190 to Δ1044 ± 279 nM ($p = 0.0371$) and from Δ1320 ± 126 to Δ703 ± 189 nM ($p = 0.0055$), respectively (Fig. 3). Effects of chronic B2BkR blockade on muscarinic receptor activity were even more evident. Muscarine-induced $[Ca^{2+}]_i$ transients with peak values of Δ2023 ± 304 nM were reduced to Δ243 ± 205 nM ($p = 0.0016$) when HOE-140 was present during the course of differentiation. B2BkR activity was also reduced by 37% following chronic blockade of

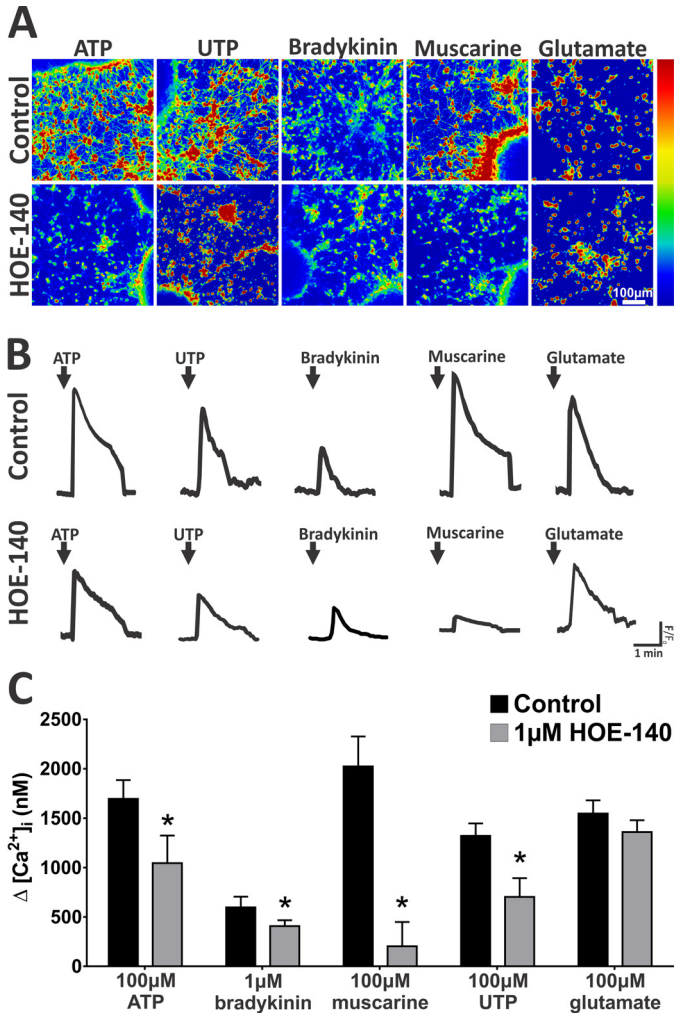


FIGURE 3. **Effects of chronic B2BkR blockade on neurotransmitter-induced $[Ca^{2+}]_i$ transients in differentiated rat neurospheres.** *A*, representative images following stimulation by 100 μM ATP, 100 μM UTP, 1 μM Bk, 100 μM muscarine, or 100 μM glutamate in cells differentiated for 7 days in the absence or presence of 1 μM HOE-140. HOE-140 was removed from cell cultures by medium change and washing the cell layers five times 1 h before staining cells with fluo-3 AM. $[Ca^{2+}]_i$ levels were monitored using calcium imaging by confocal microscopy, calculated using the average value of at least five fields of observation and represented in a color gradient. *B*, kinetics of $[Ca^{2+}]_i$ transients. *Arrows* indicate the time point of agonist application (F_o values represent basal $[Ca^{2+}]_i$ levels of nonstimulated cells). *C*, mean values of $[Ca^{2+}]_i$ peak amplitudes in differentiated neurospheres pre-treated or not with 1 μM HOE-140 were calculated as described under "Experimental Procedures" and shown as mean ± S.D. ($n = 3$) (*, $p < 0.05$ by Student's *t* test. ATP, $p = 0.0371$; Bk, $p = 0.0058$; muscarine, $p = 0.0016$; UTP, $p = 0.0055$; glutamate, $p = 0.1688$).

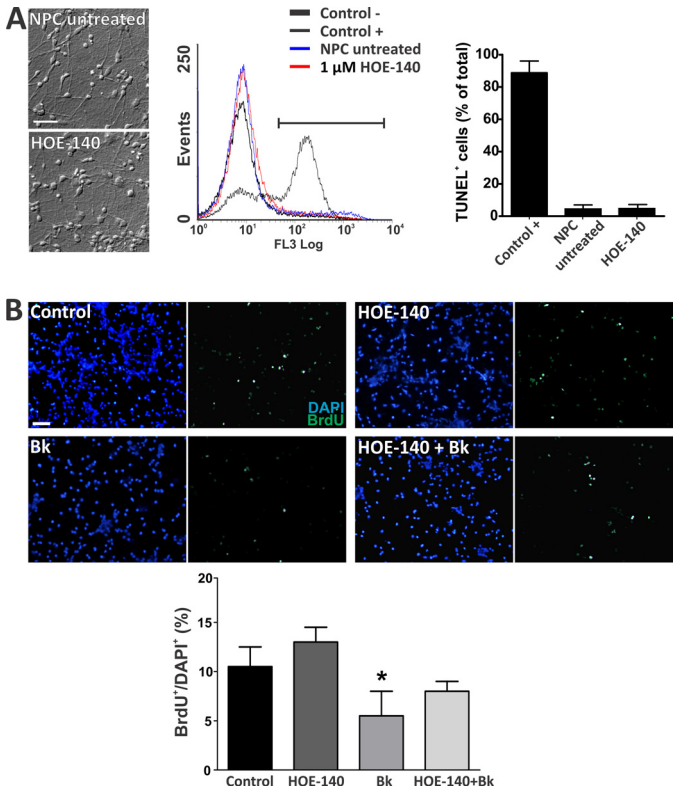
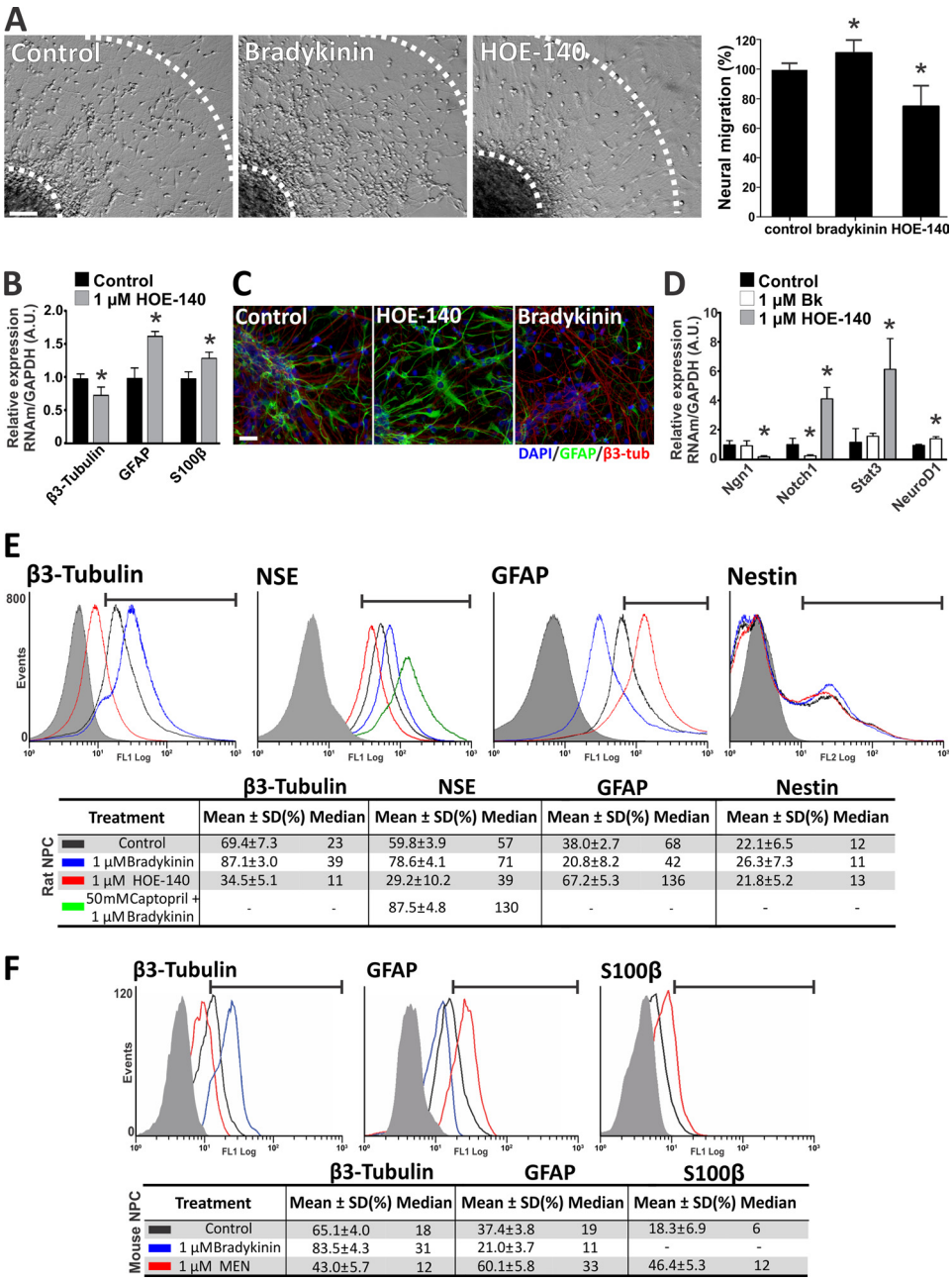


FIGURE 4. **Effects of B2BkR inhibition on rat neural progenitor cell death and proliferation.** *A*, the images show the cellular morphology of rat NPCs differentiated for 7 days in the presence or absence of 1 μM HOE-140. Percentages of cells on day 7 of differentiation undergoing cell death were determined by flow cytometry using the TUNEL assay. For negative control, we used only the marker reagent. For the positive control, NPCs were treated with DNase I to induce DNA strand breaks and verify their positive staining. Thirty-thousand events were acquired in a flow cytometer (Beckman Coulter, Fc500) and analyzed with the Cyflogic software. Cell death measured by the TUNEL assay was not significantly altered in the presence of 1 μM HOE-140 ($n = 2$). *Scale bars* = 20 μm. *B*, immunodetection of BrdU (0.2 μM) after a 14-h pulse in differentiated neurospheres in the presence of 1 μM Bk in the absence or presence of 1 μM HOE-140. BrdU incorporating nuclei are shown in *green*. The graph shows the quantification of proliferation in different treatments as the ratio of $BrdU^+$ over $DAPI^+$ cells. The percentage of proliferating $BrdU^+$ cells is significantly lower in NPCs treated with bradykinin. Six fields were evaluated for each treatment (*, $p < 0.05$). *Scale* = 50 μm.

kinin-B2 receptors followed by wash-out of the antagonist prior to calcium measurements (control = Δ596 ± 109 nM; treated with HOE-140 = Δ407 ± 59 nM) ($p = 0.0058$). The observed changes did not affect all signaling systems as no significant changes in glutamate-induced $[Ca^{2+}]_i$ peak values were observed in cells treated with HOE-140 ($p = 0.1688$).

Effects of Bradykinin and HOE-140 on Cell Death and Proliferation—We evaluated the effects of B2BkR activation and inhibition on cellular proliferation and whether HOE-140 treatment would induce cell death or have any visible effects on cellular morphology and cell viability. To this end, rat neurospheres were differentiated for 7 days in the absence or presence of 1 μM HOE-140 (Fig. 4) and analyzed by TUNEL staining. Flow cytometry analysis revealed that the chronic treatment with 1 μM HOE-140 did not affect the number of $TUNEL^+$ cells when compared with control experiments (5.1 ± 0.3% $TUNEL^+$ cells) (Fig. 4*A*). The effect of B2BkR blockade on cell proliferation was analyzed by the BrdU incorporation assay (Fig. 4*B*). Approximately 11% of the cells on day 7 of differentiation were proliferative. Similar values were obtained in cells co-treated with 1 μM HOE-140 and 1 μM Bk (9.8 ± 1.3%) or treated with HOE-140 alone (12.5 ± 1.7%). However, in the presence of Bk, cell proliferation was inhibited by ~40% (5.4 ± 2.7% of the cell population; $p = 0.0187$).

Bradykinin Favors Neurogenesis in Distinct Cell Models—The progress of neural differentiation is closely related to cell migration and neuron-glia interactions (31–33). In this process, different factors act on neural progenitor cells for defining their fate. Thus, we studied whether the effects of B2BkR activation or blockade would influence migration prior to neuronal and glial maturation. Seven days after rat neurospheres were plated onto adherent surfaces in medium deprived of growth factors;

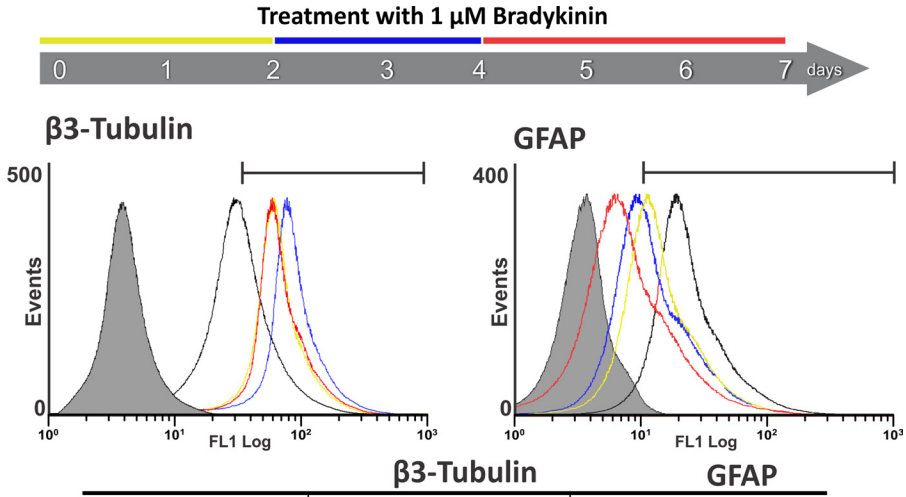


	Treatment	β3-Tubulin Mean ± SD(%)	β3-Tubulin Median	NSE Mean ± SD(%)	NSE Median	GFAP Mean ± SD(%)	GFAP Median	Nestin Mean ± SD(%)	Nestin Median
Rat NPC	Control	69.4±7.3	23	59.8±3.9	57	38.0±2.7	68	22.1±6.5	12
	1 μM Bradykinin	87.1±3.0	39	78.6±4.1	71	20.8±8.2	42	26.3±7.3	11
	1 μM HOE-140	34.5±5.1	11	29.2±10.2	39	67.2±5.3	136	21.8±5.2	13
	50 mM Captopril + 1 μM Bradykinin	-	-	87.5±4.8	130	-	-	-	-

	Treatment	β3-Tubulin Mean ± SD(%)	β3-Tubulin Median	GFAP Mean ± SD(%)	GFAP Median	S100β Mean ± SD(%)	S100β Median
Mouse NPC	Control	65.1±4.0	18	37.4±3.8	19	18.3±6.9	6
	1 μM Bradykinin	83.5±4.3	31	21.0±3.7	11	-	-
	1 μM MEN	43.0±5.7	12	60.1±5.8	33	46.4±5.3	12

FIGURE 5. **Bradykinin enhances neurogenesis, whereas HOE-140 promotes gliogenesis in neurosphere differentiation.** *A*, phase-contrast images representing radial migration pattern after 7 days of neural differentiation in the presence of 1 μM bradykinin (Bk) or 1 μM HOE-140. The region enclosed *between the dotted lines* comprises ~95% of migrated cells. Scale = 100 μm. *B*, neural markers gene expression was changed upon B2BkR inhibition. Note that the GFAP and S100β expression levels were increased, whereas β3-tubulin expression was decreased. The data are representative of three independent experiments conducted in triplicate and show as mean ± S.D. ($n = 4$) (*, $p < 0.05$). *C*, immunostaining of rat neurospheres differentiated in the presence of 1 μM Bk or 1 μM HOE-140. *Scale*, 20 μm. *D*, transcription factor and neural marker gene expression was changed upon B2BkR inhibition or activation. The data are representative of three independent experiments conducted in triplicate and shown as mean ± S.D. (*, $p < 0.05$ by Student's *t* test compared with control data. *Ngn1* (*neurogenin 1*), Bk, $p = 0.8304$; HOE-140, $p = 0.0098$; *notch 1*, Bk, $p = 0.0139$; HOE-140 $p = 0.0038$; *Stat3*, Bk, $p = 0.0178$; HOE-140, $p = 0.0008$; *NeuroD1*, Bk, $p = 0.0302$). *E*, flow cytometry analysis of GFAP, β3-tubulin, neuronal specific enolase (*NSE*), and nestin expression in rat neurospheres differentiated for 7 days in the presence of Bk, HOE-140 or captopril, and Bk. Representative histograms compare expression levels of neural markers in differentiated rat neurospheres, treated with Bk, HOE-140, and captopril + Bk. *F*, analysis of β3-tubulin, GFAP, and S100β expression in mouse neurospheres differentiated for 7 days in the presence of 1 μM bradykinin or 1 μM MEN-11270 (MEN), a B2BkR antagonist. The data shown are representative of at least two independent experiments. The *blank histograms in gray* reveal fluorescence emission data in the absence of primary antibodies. The data shown are representative of at least five independent experiments.

NPCs presented a radial migration pattern closely linked to a gradient of maturation (Fig. 5). Fig. 5*A* shows representative images of differentiated neurospheres, where the region enclosed *between the dotted lines* comprises ~95% of migrating cells. Cells migrated 15% farther from the edge of neurospheres in the presence of Bk compared with control cultures. Conversely, blockade of B2BkR by HOE-140 treatment resulted in a 25% smaller migration distance despite displaying the same radial pattern. These results suggest that alteration of migration may also influence neurogenesis and gliogenesis.



Treatment	β3-Tubulin Mean ± SD (%)	β3-Tubulin Median	GFAP Mean ± SD (%)	GFAP Median
Untreated NPC	65.1 ± 5.3	32	43.6 ± 8.5	25
1µM Bradykinin 0-2	74.6 ± 3.8	58	20.1 ± 3.7	14
1µM Bradykinin 2-4	84.8 ± 5.2	75	17.8 ± 2.8	11
1µM Bradykinin 4-7	75.3 ± 5.7	57	11.3 ± 4.1	6

FIGURE 6. **Role of B2BkR on different days of rat neurospheres differentiation.** Bradykinin was applied between days 0–2, 2–4, and 4–7, and was removed at the end of each period. Representative flow cytometry histograms comparing the expression of neural markers in control differentiated neurospheres (*black*) or at different times of treatment with 1 μM bradykinin (*Bk*), between days 0–2 (*yellow*), 2–4 (*blue*), and 4–7 (*red*). The most significant effects on neural differentiation were observed in intermediate and final phases. The data shown are representative of at least four independent experiments.

Additionally, chronic treatment of NPCs with HOE-140 decreased the expression of *β3-tubulin* by 25 ± 13% (p = 0.0477), whereas *GFAP* and *S100β* expression levels were significantly increased by 65 ± 9 (p = 0.0024) and 31 ± 8% (p = 0.0032), respectively (Fig. 5*B*). These results indicate, for the first time, an important role of the B2BkR in neural fate determination, where inhibition of B2BkR activity favors gliogenesis over neurogenesis. B2BkR-mediated effects were confirmed by microscopic analysis of immunostained cells (Fig. 5*C*). Although gliogenesis was reduced, neurogenesis visualized by β3-tubulin expression was much more evident in neurospheres treated with 1 μM Bk throughout the course of differentiation when compared with neurospheres differentiated in the absence of the Bk or in the presence of HOE-140.

We further investigated the expression of neurogenic and transcription factors genes, such as *ngn1* (*neurogenin 1*), *notch1*, *Stat3* (signal transducer and activator of transcription 3), and *NeuroD1*, in differentiated NPCs in the absence or presence of HOE-140 or Bk. Real-time PCR revealed that the treatment with HOE-140 significantly increased the expression of genes related to gliogenesis (*notch 1* and *Stat3*), whereas in the presence of Bk a significant difference of *NeuroD1* expression was obtained compared with the control group (Fig. 5*D*). Conversely, *Ngn1* expression levels were decreased with HOE-140 treatment and *notch 1* levels diminished after Bk treatment.

Flow cytometry analysis revealed that the expression of the neuronal markers β3-tubulin and NSE following Bk treatment were increased from 69.4 ± 7.3 to 87.1 ± 3.0% and 59.8 ± 3.9 to 78.6 ± 4.1%, respectively (Fig. 5*E*). Co-treatment with captopril and Bk greatly increased the percentage of NSE$^+$ cells, reaching 87.5 ± 4.8% after ACE inhibition, given by the increased availability of Bk. In contrast, prolonged activation of B2BkR decreased the glial population from 38.0 ± 2.7 to 20.8 ± 8.2%, whereas the population of nestin$^+$ cells did not show any significant variation, remaining at ~22%. Chronic treatment with HOE-140 also altered the phenotypic population features; however, this treatment showed a bias of gliogenesis. The percentage of GFAP$^+$ cells almost doubled from 38.0 ± 2.7 to 67.2 ± 5.3%. Percentages of nestin$^+$ cells did not change significantly under Bk treatment. Similar results were obtained by flow cytometry analysis of mouse NPCs differentiated in the presence of Bk or MEN-11270 (another B2BkR specific inhibitor) (Fig. 5*F*).

Effects of Bk on neural fate determination may depend on the time of application, *i.e.* its action could be more evident at the beginning or end of differentiation, considering other external and internal factors participating in this process. Thus, Bk was added and removed at specific times during differentiation: 0–2, 2–4, or 4–7 days. Quantification of glia and neuronal populations by flow cytometry revealed that most significant favoring of neurogenesis by Bk occurred in intermediate and late days of differentiation (Fig. 6). Although discrete, the expression of β3-tubulin during this period peaked (84.8 ± 5.2%) and was comparable with those obtained by chronic treatment with Bk along the whole course of differentiation. This may be related mainly to the migration of cells from neurospheres, which is enhanced at intermediate and late stages of differentiation. On the other hand, the decreased GFAP expression was most evident when cells were treated with Bk between days 4 and 7 (11.3 ± 4.1%), in agreement with reversal of the proliferation blockade at the end of differentiation in the presence of HOE-140.

Additionally to immunocytochemistry and flow cytometry, we used Western blot analysis to evaluate relative protein con-

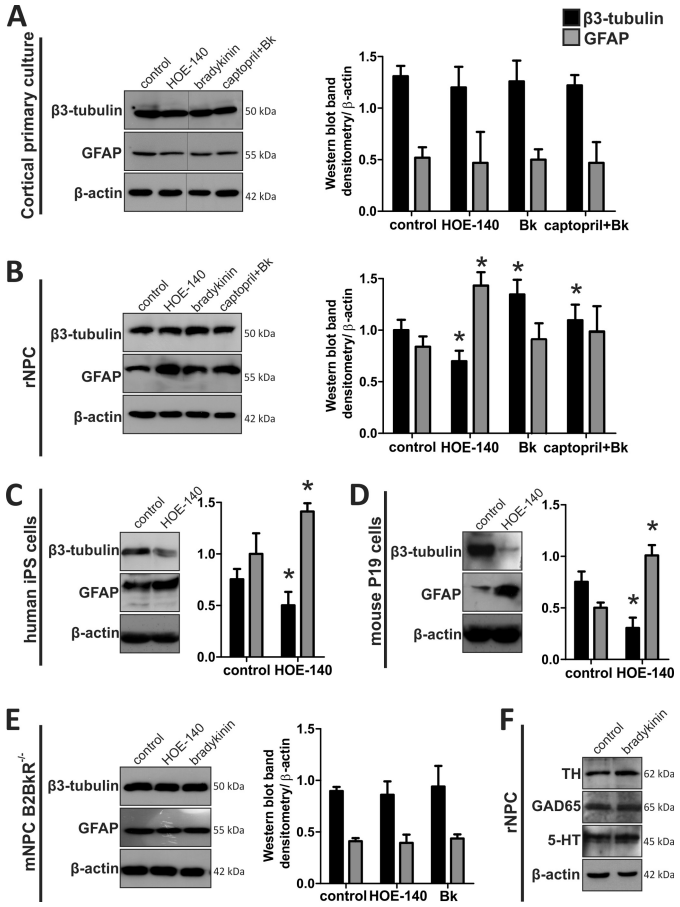


FIGURE 7. **Western blot analysis of neural marker expression.** *A*, immunoblots of protein extracts from newborn rat cortical primary culture after treatment with 1 μM Bk, 1 μM HOE-140, or 50 mM captopril + 1 μM bradykinin. *B*, densitometry of protein extracts from differentiated rat neurospheres ($n = 4$). *C*, densitometry of protein extracts from human-induced pluripotent stem cells ($n = 2$). *D*, densitometry of protein extracts from differentiated mouse P19 carcinoma cells ($n = 3$). *E*, densitometry of protein extracts of differentiated mouse neurospheres obtained from *B2BkR* knock-out mice ($n = 2$). *F*, immunoblots of protein extracts from differentiated rat neurospheres after treatment with 1 μM bradykinin along differentiation (TH, tyrosine hydroxylase; 5-HT, 5-hydroxytryptamine; GAD65, glutamic acid decarboxylase).

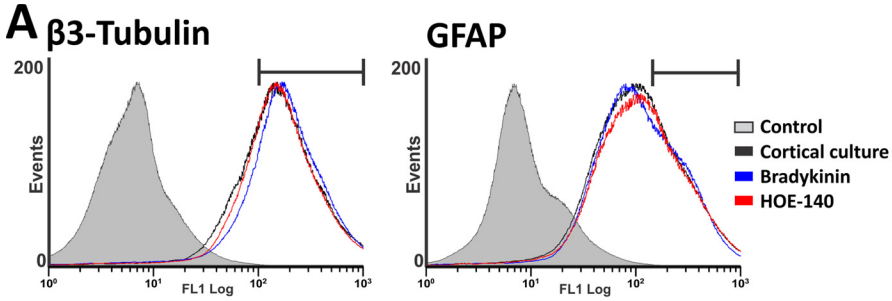
tents of β3-tubulin and GFAP in different cell models (Fig. 7). In accordance with the results so far shown, Western blot analysis showed no change in the number of neuronal and glial proteins in primary cortical cultures previously treated with HOE-140 or Bk. We noticed a clear increase in β3-tubulin protein after treatment with Bk or captopril + Bk in rat neurosphere, murine P19, and human iPS cell differentiation. The opposite effect, increased GFAP content, could be observed after differentiation of the same models of rat, mouse, and human cells in the presence of the specific blocker of B2BkR, HOE-140 (Fig. 7, *A–D*). To verify that promotion of neurogenesis resulted from B2BkR activation, neurospheres from the B2BkR knock-out mice were differentiated in the presence of 1 μM Bk or 1 μM HOE-140. After these treatments we found no significant change in neural marker protein content, confirming that neural differentiation was specifically modulated by B2BkR and not by possible side effects of HOE-140 treatment (Fig. 7*E*). The presence of Bk along differentiation did not induce any changes of subpopulation-specific neurotransmitter expression. Immunoblotting against tyrosine hydroxylase (dopaminergic marker), 5-hydroxytryptamine (serotoninergic marker), and GAD65 (GABAergic marker) in neurospheres did not reveal any differences in expression of these markers (Fig. 7*F*).

Neurogenesis Is Favored via B2BkR Only in Differentiating Cells—For confirming specific neurogenic roles of Bk, we investigated whether these effects occurs only in the differentiation process or could also be observed in differentiated neural cells. For this purpose, we used cortical primary cultured cells of newborn rats treated for 7 days without or with 1 μM Bk or 1 μM HOE-140. Flow cytometry analysis of cortical primary cultures pre-treated with Bk or HOE-140 did not reveal any change in percentages of neuronal and glial cells (Fig. 8*A*). Thus, the occurrence of neurons and glia remained constant regardless of treatment, suggesting that the effect of neuronal cell enrichment by Bk via B2BkR occurs only during the process of differentiation.

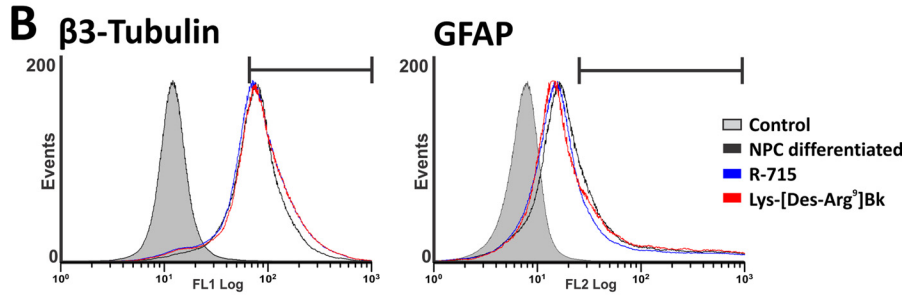
Considering the influence of the B2BkR in modulating neural differentiation by promoting neurogenesis, we assessed whether this effect would be caused by enzymatic cleavage of Bk with consequent formation of metabolites and activation of the B1BkR. In this context, rat neurospheres were plated and differentiated in the presence of 1 μM Lys-[des-Arg^9]-Bk, an agonist of the B1BkR, or 1 μM R-715, a specific B1BkR antagonist. After this period, we performed flow cytometry analysis to quantify neural marker expression (Fig. 8*B*). There was no change in the number of cells expressing neural markers β3-tubulin and GFAP. Thus, the phenotypic fate determination during neural differentiation does not appear to be influenced by the B1BkR. It is noteworthy that mRNA transcription coding for B1BkR during neural differentiation could not be detected in real-time PCR analysis.

Differentiated Neurospheres Derived from B2BkR$^{-/-}$ Mice Show a Reduction in Neural Migration—Further confirmation of modulation of neurogenesis by Bk and its receptor was obtained in *B2BkR* knock-out mice. The use of *B2BkR*$^{-/-}$ mice to obtain neurospheres allowed further study of the process of cell differentiation and neural migration. To verify the homozygosis in knock-out mice, genomic DNA was extracted from small biopsies of the animals and amplified by PCR with specific primers for *B2BkR* and rate genes. *B2BkR*$^{-/-}$ mice-derived neurospheres revealed the same growth rates of wild-type neurospheres, without visible morphological changes. After plating and induction of neural differentiation of *B2BkR*$^{-/-}$ neurospheres, we observed the same radial pattern, although with decreased migration when compared with control neurospheres from wild-type animals (Fig. 9). The quantification of cell migration between the dotted lines is shown in Fig. 9*A*. In addition, immunocytochemical analysis revealed the same pattern characterized mainly by radial GFAP$^+$ cells and a low migration of β3-tubulin$^+$ cells in *B2BkR*$^{-/-}$ mice neurospheres (Fig. 9*B*). The immunostaining also reveals less β3-tubulin$^+$ cells (~72%) and a high content of GFAP$^+$ cells (47%) in B2BkR$^{-/-}$.

Developmental Expression of B2BkR and Its Effect on Neural Marker Expression during Brain Development—The *B2BkR* is ubiquitously and constitutively expressed in adult healthy tissues. To assess whether it is also expressed in developing mice,



	β3-Tubulin		GFAP	
Treatment	Mean ± SD(%)	Median	Mean ± SD(%)	Median
Control	76.4±9.8	172	38.4±7.5	197
1μM bradykinin	81.2±5.4	198	37.3±2.4	193
1μM HOE-140	78.5±7.3	185	36.8±4.1	202



	β3-Tubulin		GFAP	
Treatment	Mean ± SD(%)	Median	Mean ± SD(%)	Median
Control	72.0±4.3	71	31.4±9.5	29
1μM Lys-[Des-Arg^9]-Bk	75.8±8.0	68	36.9±3.2	22
1μM R-715	76.2±6.3	72	34.8±6.1	29

FIGURE 8. **Flow cytometry analysis of β3-tubulin and GFAP expression in cortical primary culture and neurosphere differentiated in the presence of 1 μM Lys-[des-Arg^9]-bradykinin and R-715.** *A*, cortical cells of newborn rats were cultured in the absence or presence of 1 μM bradykinin or 1 μM HOE-140 for 7 days. Representative flow cytometry histograms comparing the expression of neural markers in differentiated neurospheres (*black*), treated with bradykinin (*blue*) or HOE-140 (*red*) ($n = 4$). *B*, representative flow cytometry histograms comparing the expression of neural markers in differentiated neurospheres (*black*), treated with a B1BkR inhibitor, R-715 (*blue*), or a B1BkR agonist, Lys-[des-Arg^9]-bradykinin (*red*) ($n = 3$). The *blank histogram* represents data obtained in the absence of primary antibodies.

expression of *B2BkR* was determined in embryos removed from pregnant dams at various neurogenic developmental time points (E9.5–E12.5) by whole mount *in situ* hybridization with antisense RNA probes (Fig. 10). Mouse *B2BkR* transcripts were detected in neural cells at day E9.5, starting in the optic vesicle (Fig. 10*A*), then increasing their expression pattern to the whole nervous system at days E11.5 (Fig. 10*B*) and E12.5 (Fig. 10*C*). Negative controls with *B2BkR* sense probes did not reveal any specific labeling (Fig. 10*D*). Here, we show for the first time that *B2BkR* is strongly expressed in the developing mouse brain, including telencephalon, diencephalon, and ventral region of midbrain and hindbrain as well as in the spinal cord. For further analysis of the role of B2BkR in developing brains, we verified the expression of *β3-Tubulin* in the telencephalon and cortex at several time points during WT and *B2BkR*$^{-/-}$ mice development (E9.5-adult) (Fig. 10*E*). The developing knock-out mice brains showed significantly less expression of *β3-Tubulin* from E11.5 until adulthood (*, $p < 0.05$; adult, $p = 0.0334$; E9.5, $p = 0.0861$; E11.5, $p = 0.4349$; E14.5, $p = 0.0004$; E17.5, $p = 0.0008$; P0, $p = 0.0001$). Adult *B2BkR*$^{-/-}$ brain express more glial markers, such as *GFAP* (*, $p < 0.05$; adult, $p = 0.0083$) and *S100β* (*, $p < 0.05$; adult, $p = 0.0001$) (Fig. 10*F*). These data indicate that the *B2BkR*$^{-/-}$ brain expresses less neuronal marker and higher levels of glial markers, indicating that Bk-induced actions occur not only during *in vitro* neural differentiation, but are also important for *in vivo* neurogenesis.

DISCUSSION

Bk actions in neurogenesis are suggested based on its participation in determining the cholinergic phenotype of differentiating cells (19), induction of calcium waves (34, 35), neurite formation (36–38), and cell migration. Moreover, Bertram *et al.* (39) demonstrated increased migration of human monocytes induced by Bk. In glioma cells, Lu *et al.* (40) reported augmented migration in the presence of Bk, but this effect was reproduced by B1BkR agonists. In another study, increased migration of chondrosarcoma cells was related to the Bk-activated signaling cascade (41). In summary, Bk or

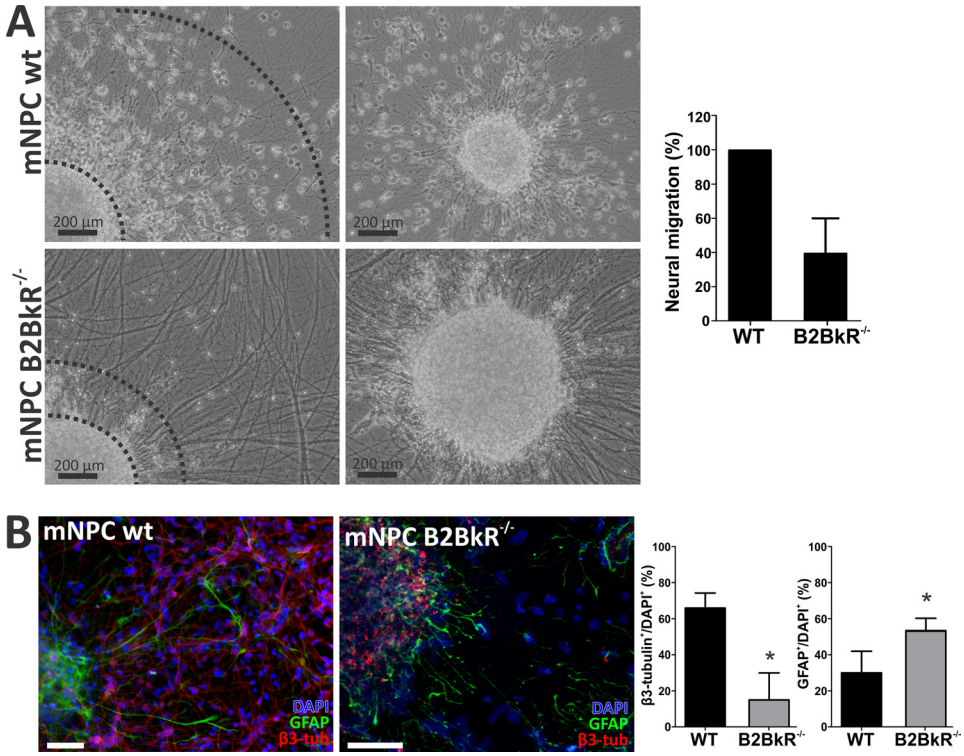


FIGURE 9. **Neural migration and differentiation of neurospheres obtained from *B2BkR*$^{-/-}$ mice.** Differentiation of neurospheres obtained from embryonic telencephalon (*E12.5*) of wild type (*mNPC wt*) and *B2BkR* knock-out mice (*B2BkR*$^{-/-}$ *mNPC*). *A*, phase-contrast images of radial migration pattern after 7 days of neural differentiation. The region enclosed *between the dotted lines* comprises ~95% of cells that migrated. *Scale*, 200 μm ($n = 2$). *B*, immunofluorescence staining of dissociated *B2BkR*$^{-/-}$ mNPC against β3-tubulin and GFAP protein revealed an increase in the number of glial cells compared with wild type mNPC. *Scale*, 20 μm ($n = 2$) (*, $p < 0.05$).

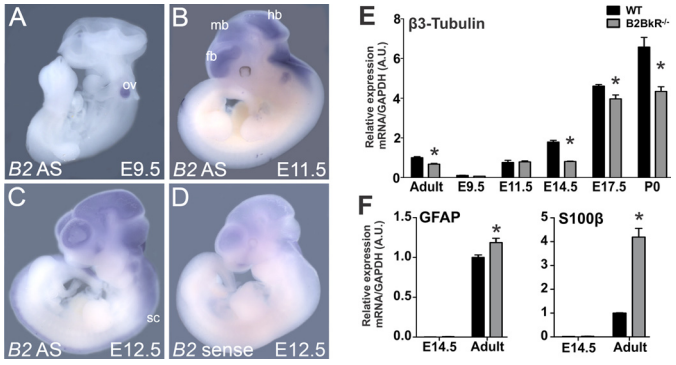


FIGURE 10. **Expression pattern of *B2BkR* during mouse embryo development and neuronal marker expression in telecephalons from *B2BkR* knock-out and wild-type mice.** Whole mount *in situ* hybridization of mouse embryos with *B2BkR* antisense probe (*A–C*) and *B2BkR* sense probe (used as a control, *D*). At stage E9.5, *B2BkR* expression is restricted to the optic vesicle (*A*), strong *B2BkR* expression was observed in the developing nervous system (*B* and *C*), *fb*, forebrain; *hb*, hindbrain; *mb*, midbrain; *ov*, optic vesicle; *sc*, spinal cord. *E*, *β3-tubulin* neuronal marker gene expression during WT and *B2BkR*$^{-/-}$ mouse brain development. The *B2BkR*$^{-/-}$ embryos express less of the marker during several time points of brain development ($n = 6$) (*, $p < 0.05$ by two-way analysis of variance with Bonferroni post-test compared with WT. *Adult*, $p = 0.0334$; *E9.5*, $p = 0.0861$; *E11.5*, $p = 0.4349$; *E14.5*, $p = 0.0004$; *E17.5*, $p = 0.0008$; *P0*, $p = 0.0001$). *F*, *GFAP* and *S100β* glial marker gene expression of WT and *B2BkR*$^{-/-}$ mice brain. Adult *B2BkR*$^{-/-}$ brain reveal more gene expression of glial proteins, such as *GFAP* (*, $p < 0.05$ by two-way analysis of variance, *Adult*, $p = 0.0083$) and *S100β* (*, $p < 0.05$ by two-way analysis of variance, *Adult*, $p = 0.0001$).

its degradation products participate via B1BkR or B2BkR activation in processes similar to those occurring during neurogenesis, such as neurite outgrowth, cell migration, and maturation.

In this context, several other factors can participate in early cell fate determination induced by Bk, including hormones (42) and amyloid-β precursor protein (43). Gallego and co-workers (42) demonstrated that inhibiting hormone signaling prevented the differentiation of embryonic stem cells aggregates into neuroectodermal cells. Porayette and co-workers (43) showed that the inhibition of amyloid-β precursor protein formation significantly suppressed human embryonic stem cell proliferation and promoted NPC formation. Interestingly, there is evidence that sex steroids alter B2BkR expression, and that Bk affects amyloid-β precursor protein processing (44, 45) and increases its secretion. Moreover, due to possible regulation of production and secretion of hormones, growth factors and other substances by Bk, both, direct and indirect effects evoked by this peptide in neural fate determination are possible. In this regard, further investigation of the changes in muscarinic and cholinergic receptor expression and activity in conditions of chronic B2BkR inhibition will provide clues on these mechanisms.

Here we have defined novel functions for Bk and its receptor using rat embryonic telencephalon neurospheres as an *in vitro* model for early cortex neurogenesis and gliogenesis (Fig. 11). Besides intracellular calcium signaling, Bk promotes NO production, essential for the progress of neurogenesis. In agreement with a recently published study of our group, any interference with the production of arginine, the substrate for NO production, or with NOS activity interferes with the differentiation process (30). Subsequently, deficient B2BkR signaling in the presence of HOE-140, resulting in impaired neurogenesis,

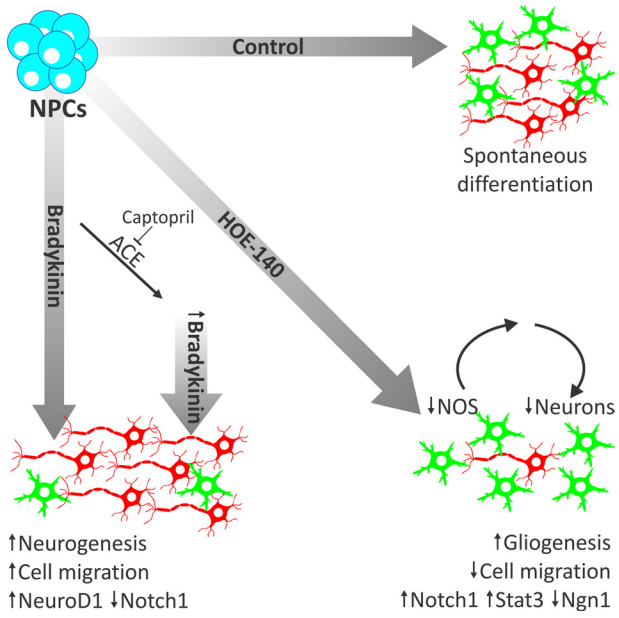


FIGURE 11. **Bradykinin promotes neurogenesis via B2BkR activation.** Following plating, neural stem cells spontaneously differentiate into neurons (*red*) and glial cells (*green*). However, when B2BkR activity is blocked by HOE-140, the progress of neurogenesis is inhibited. The addition of Bk to NPC cultures decreases proliferation and promotes migration and neural differentiation following activation of *NeuroD1* and down-regulation of *Notch1* expression, whereas specific inhibition of the B2BkR reduces neurogenesis and augments gliogenesis following up-regulation of *Notch1* and *Stat3* and down-regulation of *Ngn* (*neurogenin 1*) expression. The increase in neurogenesis of NPCs by Bk is yet enhanced in the presence of captopril, an inhibitor ACE, augmenting the half-time of this peptide in the culture medium. Neurogenic actions exerted by Bk also involved NO production, because expression of key enzymes of the NO-citrulline cycle was down-regulated as result of B2BkR inhibition (30).

is also reflected by down-regulated expression of NOS and the step-limiting enzyme ASS.

B2BkR expression was evident throughout differentiation of neurospheres into neurons and glial cells accompanied by reduction of expression of the neural progenitor marker nestin and an increase in expression of neuronal β3-tubulin and NSE as well as of GFAP and S100β, identifying glial cells. Bk was released into the culture medium during phenotypic transition of undifferentiated cells into specialized neural cells (24). Further evidence for a functional kallikrein-kinin system is given by the expression of ACE, limiting Bk half-life in the extracellular fluid; however, the B1BkR could not be detected, both on expression and activity levels.

These data agree with previous work of our laboratory suggesting the presence of an autocrine loop system of Bk secretion and receptor activation during neuronal differentiation of P19 embryonal carcinoma cells, in which the blockade of receptor activation suppressed Bk liberation into the medium and led to inhibition of *M1–M3 muscarinic receptor* expression in neuronal-differentiated P19 cells (19). Based on these observations during differentiation of an embryonic cell model, we questioned now whether these B2BkR functions are also present in an *in vitro* model closely reflecting conditions occurring during embryonic cortex development in a network of migrating cells.

As found during *in vitro* neurogenesis of P19 cells, gene expression of *M1–M4 receptors* and muscarine-induced $[Ca^{2+}]_i$ transients were reduced following inhibition of B2BkR activity during neurosphere differentiation. Phenotypic changes observed in neurospheres differentiated in the presence of HOE-140 included alterations in purinergic receptor expression and activities. Suppression of *P2X5* and *P2X6* receptor subunit expression, known to be regulated during neuronal development (46), is consistent with an inhibitory effect of B2BkR blockade on the progress of neurogenesis. Scemes *et al.* (47) reported a reduction in neural outgrowth by blocking P2Y1 receptor activity during neurosphere differentiation, whereas the relative population of neurons and glial cells remained unchanged (48). These results are in agreement with the down-regulation of P2Y1 receptor expression due to HOE-140 treatment and subsequent decreased neural migration, agreeing with important roles of this receptor in neural proliferation and migration (49).

There are growing evidence that points at regulatory functions of NO in the development of the CNS, including cell proliferation and fate determination (50–52). The mechanism of regulating proliferation/differentiation depends on the NOS isoform involved in NO production (29). In this context, expression of enzymes of the citrulline-NO cycle including eNOS, nNOS, and argininosuccinate synthetase was also down-regulated in the presence of HOE-140. As a possible mechanism, B2BkR activity controls key events including expression of the machinery necessary for NO formation, which is essential for cell fate determination and guidance of maturation into neurons expressing specific neurotransmitter receptors (50).

Effects of B2BkR inhibition on final neural phenotype determination did not result in increased cell death rate or in the permanence of differentiating cells in the progenitor stage. Moreover, neurogenesis, measured by an increase in the number of β3-tubulin$^+$ cells, augmented with the distance of migration from undifferentiated neurosphere cell aggregates, whereas cells that migrated less showed higher labeling for nestin and GFAP. Therefore, migration is linked directly to neuronal differentiation, and gliogenesis yet occurs due to proliferation of GFAP$^+$ cells. A direct participation of B2BkR in cell migration was confirmed with neurospheres isolated from *B2BkR* knock-out mice, where just as in the presence of HOE-140 migrated distances were reduced. On the other hand, changes in the percentages of β3-tubulin$^+$ and GFAP$^+$ cells induced by chronic treatment with HOE-140 were not observed in primary cultures of postnatal cortex neurons indicating that effects only occur during neural development and not when final neural fate determination and differentiation have already happened.

A possible molecular mechanism for Bk-induced neural fate determination can be delineated by the expression of neural markers and transcription factors related to neurogenesis/gliogenesis switches *in vivo*. Wnt activation in proliferating neural progenitors followed by up-regulation of *Ngn1* expression promotes the expression of genes related to neurogenesis such as *NeuroD1* (53). At the same time, gliogenesis controlled by Ngn1 is induced by activation of Stat3 and expression of GFAP (54). Actually, the cooperation between Smad, Stat, and p300 protein is particularly effective for promoting gliogenesis in NPCs (55, 56). Associated to this molecular machinery, notch 1 regu-

lates interactions between physically adjacent cells and its activation leads to a potent inhibition of neurogenesis, whereas committing the cells to an astrocyte phenotype (57, 58). In this context, activation and inhibition of B2BkR can interfere with the expression and activity of some of these transcription factors, thereby changing cell fate. However, the cause-consequence relationship between B2BkR downstream signaling and the expression of neurogenic genes is not well understood.

Neurogenesis was even more enhanced when Bk was added to the culture medium together with captopril, increasing Bk half-life. In fact, this observation reveals new strategies for strengthening neurogenesis, even in the adult organism following insults like stroke and in neurodegenerative diseases. In view of that, stable B2BkR agonists and ACE inhibitors may gain therapeutic applications for cellular therapy. It is expected that these compounds will also induce endogenous neurogenesis and provide adequate niches for transplanted stem cells to survive.

Less β3-tubulin expression during development of *B2BkR* knock-out animals points to crucial participation of B2BkR during *in vivo* neurogenesis, being in line with previous results showing that neurogenic activities of exogenously added kallikrein or kallikrein gene transfer in an animal model depends on B2BkR activity (59–61). Xia *et al.* (61) suggested that the insertion of tissue kallikrein genes by viral infection in newborn mice promotes ischemic neuroprotection by stimulating glial migration, neurogenesis, and inhibition of apoptosis in the injured area, mainly related to increased levels of phospho-Akt, Bcl-2, and NO, in addition to decreased activation of caspase-3. The observed effects can be explained by the increased availability of Bk and subsequent activation of B2BkR, because they were reversed by pretreatment with HOE-140. Such neuroprotective features were recently described for an *in vitro* model of hippocampal neurons where Bk reversed apoptosis induced by NMDA-mediated excitotoxicity (62).

Bk-induced changes in neural fate determination do not involve alterations in populations of excitatory glutamatergic and inhibitory GABAergic neurons nor of dopaminergic neurons as judged by comparison of global expression levels of neurotransmitters. These results agree with those of calcium imaging assays showing no interference with glutamate receptor activity following chronic treatment with HOE-140 along differentiation. On the other hand, purinergic and muscarinic acetylcholine-receptor expression and activity were affected by the presence of HOE-140. These results are again in line with the suggestion for neurogenic actions of Bk, having in mind that both receptor systems contribute to the progress of neuronal differentiation (63, 64).

França *et al.* (65) showed that expression of B2BkR increases during early rat organogenesis (E8) and stabilizes during fetal growth (E15). Most importantly, besides being strongly expressed in the whole nervous system during the neurogenic stage of embryo development, B2BkR-induced neurogenesis and inhibition of gliogenesis were conserved throughout different models of neurogenesis, even in iPS cells reprogrammed to pluripotency from adult somatic cells. Our work provides new tools for directing differentiating cells into homogeneous populations of neurons *in vitro* for posterior transplantation. In this regard the results obtained with human iPS cells are extremely valuable. In summary, neurogenic properties of Bk described herein may open novel avenues for therapy of neurodevelopmental and neurodegenerative diseases.

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HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP): Still an Obscure Disease

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Abstract: Human T-cell leukemia virus type 1 (HTLV-1) is the ethiologic agent of the neurological disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Although the majority of HTLV-1–infected individuals remain asymptomatic during their lifetime, approximately one percent of this population develops a myelopathy consisting of a chronic inflammation of the white and gray matter of the spinal cord. Glucocorticoids are widely used for treatment because of their anti-inflammatory properties, improving symptoms mainly in those patients with only a few years from onset of the disease, when inflammation is more prominent. Interferon-alpha and vitamin C are other therapies presenting some benefits in clinical practice, probably due to their anti-viral and immunomodulatory activities observed *ex vivo*. Furthermore, inhibitors of histone deacetylase, which increase virus expression but result in a substantial decline in the proviral load, have also been proposed. This review is intended to bridge the gap between clinical and basic science by presenting recent findings on HAM/TSP disease, mechanisms of drug action, and benefits of these therapies in HAM/TSP patients.

Keywords: HTLV-1, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), glucocorticoids; neuroinflammation, pathogenesis, HAM/TSP therapy.

INTRODUCTION

Retroviruses are important causes of human morbidity and mortality and have evoked pandemics in the last three decades. Among these retroviruses, the human T-lymphotropic type 1 (HTLV-1) and type 2 (HTLV-2) have long-term silent viral persistence in the host for several decades. It is estimated that 10 to 20 million people are infected worldwide [1], and Brazil has about two million people infected with HTLV-1, corresponding to up to 2% of the infected blood bank samples in some regions of the country [2]. Although this virus has worldwide distribution, Japan, Africa, the Caribbean Basin and South America are considered to be endemic areas for HTLV-1 infection [3].

Although most HTLV-1-infected carriers remain healthy, 2.5% will eventually develop HTLV-1 associated myelopathy/ Tropical spastic paraparesis (HAM/TSP), adult T-cell leukemia/lymphoma (ATL) and inflammatory disorders correlated with HTLV-1 infection, such as myositis, arthritis, dermatitis, uveitis and alveolitis [4, 5]. There is no accurate number of HAM/TSP or ATL cases since these diseases are not considered reportable by the World Health Organization (WHO), although Japan reports approximately 800 cases of ATL yearly [4]. The major histopathological characteristic of HAM/TSP is a chronic inflammation of the white and gray matter of the spinal cord followed by a degenerative process that preferentially affects the white matter in the lower spinal cord [3, 6]. HAM/TSP is characterized by a chronic slowly progressive spastic paraparesis with bladder disturbances, absent or mild sensory loss and low back pain, with seropositivity for HTLV-1 antibodies, in the absence of spinal cord compression [4, 7]. Despite the more usual presentation characterized by a slow progression, 21.5% of the patients may experience a rapid progression, with severe disability two years after the onset of symptoms [5]. This phenomenon is related to older age of onset, parenteral HTLV-1 transmission route, high viral loads, and high antibody titers [8, 9].

In contrast to ATL, the incidence of HAM/TSP decreases with age. Thus, HAM/TSP progress is similar to multiple sclerosis (MS), where few cases have been reported among people over 60 years of age [10]. This could be explained by age-related lack of CD8+ cell hyperactivity and associated lower risk for clinical development of HAM/TSP [11].

HAM/TSP PATHOGENESIS

Although previous studies have investigated the potential underlying factors for the HAM/TSP pathogenesis, no consensus has been reached so far. There have been many tentative explanations, but HTLV-1 tax viral load, genetic background or immune disturbances have been implicated as

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likely major causes. Fig. (**1**) summarizes the changes in blood and spinal cord of HAM/TSP patients. There is a mounting evidence for high levels of serum/ cerebrospinal fluid (CSF) inflammatory cytokines in HAM/TSP patients. In fact, MIP-1α levels are higher in HAM/TSP patients than in asymptomatic carriers [12]. Thus, in addition to the pro-inflammatory cytokines, including IFN-γ, IL-2, and IL-15 [13], and the high activity of cytotoxic T lymphocytes (CTL), MIP-1α released by peripheral blood mononuclear cells (PBMC) may have an important role in HAM/TSP pathogenesis. This process can also influence the inflammatory activity in the spinal cord and thus contribute to the HAM/TSP progress. Alternatively, type 3 CXC chemokine receptors (CXCR3), which are expressed at high levels on activated and memory T lymphocytes, selectively respond to some of these chemokines. Those memory cells are inducible by IFN-γ, which is found at high levels in HTLV-1-infected subjects [14]; these cells may in turn migrate to the spinal cord, leading to a potential damage to the myelin membrane [15]. Thus, two pathways could be implicated in HAM/TSP development, including: (i) memory cells directly involved with cytolytic damage driven by IFN-γ, and (ii) RANTES and MIP-1α acting as chemotactic factors, all produced mainly by $CD8^+$ T cells. We have observed that pro-inflammatory soluble factors, such as MIP-1α, may have an important role in mediating tissue-specific leukocyte recruitment and T-cell stimulation. This may result in damage to the myelin membrane, a major characteristic of myelopathy in HTLV-1-infected patients. A chemokine-induced activation of T cells via CCR5 leads to activation of focal adhesion kinases, which also have important roles in cell motility including cell spread and migration [16]. These CCR5 ligands are critical for T cell proliferation and the transcriptional activation of cytokine genes [17]. This mechanism may be directly involved as a cytolytic damage in the adult T cell leukemia (ATL), since high levels of RANTES were described in these patients [18], and one *in vitro* study showed a role of *tax* induction of MIP-1α [19]. The predilection of neuroinflammation by HTLV-1 in the thoracic cord may be due to slower local blood flow, allowing a better opportunity for cells expressing adhesion molecules to transmigrate [20], while other, more watershed areas of the central nervous system usually remain clinically silent.

TREATMENTS/STRATEGIES

There is no standard therapy for HAM/TSP. Several studies have proposed the use of anti-inflammatory drugs, immune modulator drugs and others. In our experience in the last 15 years in a clinical setting in São Paulo (Brazil), corticosteroids have yielded better results in patients with less than 5 years from HAM/TSP onset.

Glucocorticoids

Despite no standard therapy for HAM/TSP is available, glucocorticoids (GCs) are the most widely prescribed class of drugs for its treatment. Their clinical efficacy is attributed to their ability to reduce the expression of proinflammatory genes, resulting in several benefits including a reduction of inflammatory cells migration, regulation of Th1/Th2 cytokine balance and potential reduction in HTLV-1 proviral load.

Most GC-related effects are mediated by the intracellular receptors (GRs) which are ligand-activated transcription factors. The GRs can act either by enhancing or repressing transcription of target genes [21, 22]. Genes that are negatively regulated by GR often involve the negative interference of GR with the activity of other transcription factors, such as NF-κB, CREB, STAT, GATA-3, interferon regulatory factor 3 (IRF3), activating protein (AP)-1, nuclear factor of activated T cells (NFAT), and T-box expressed in T cells (T-Bet). Typical target genes that are negatively regulated include several inflammatory proteins such as IL-1β, IL-2, IL-6, IL-8, IL-12, IL-5, IL-18, COX-2, E-selectin, interferon-γ (IFN-γ), TNFα, and intercellular adhesion molecule (ICAM), monocyte chemoattractant protein 1 (MCP-1) chemokine (C-C motif), vascular cell adhesion molecule (VCAM) [reviewed by [21, 23]. On the other hand, there are several recently characterized anti-inflammatory genes

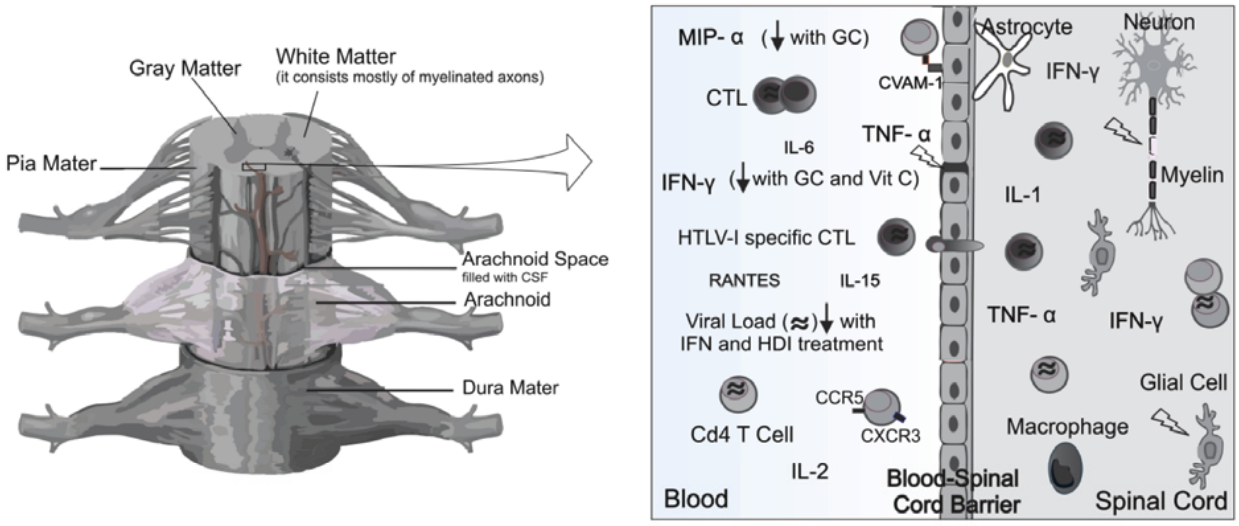

Fig. (1). Schematic representation of the spinal cord, alterations in blood and spinal cord from HAM/TSP patients and effects of glucocorticoids (GC), interferon-alpha (IFN), vitamin C (Vit C) and histone deacetylase inhibitor (HDI) therapies. ↓ = reduction.

whose expression is up-regulated by GR, such as IκB, IL-10, the genes coding for MKP-1, lipocortin-1, secretory leukoprotease inhibitor (SLPI), annexin A1, and GC-induced leucine zipper (GILZ) [23, 24].

As discussed above, HAM/TSP is a chronic inflammation as long as patients show high levels of proinflammatory proteins and some of them may be inhibited by GC. These proteins include IFN-γ, TNFα, IL-2, MIP1α, IL-15, IL-6, and IL-16 and may have direct effects in white matter lesions [13, 25-27]. TNF-α, for example, induces cytotoxic damage to endothelial cells. MIP-1α and 1β can enhance transendothelial migration of lymphocytes into the nervous system, IL-16 is a chemoattractant for $CD4^+$ cells, and $CD4^+$ cells produce IL-2 that is required by IL-2 non-producer $CD8^+$ cells for proliferation [28]. In this context, one study showed that IFN-γ and MIP1α levels decreased after treatment with prednisolone in PBMCs from HAM/TSP patients while IL-4 and IL-10 levels increased [25].

In our experience, GCs have been used with better results in patients with less than five years of HAM/TSP onset. This possibly occurs because of changes in inflammatory context during HAM/TSP disease. In patients with a clinical history of up to three years it has been observed a high expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelium, TNF-α, IL-1β and IFN-γ in perivascular infiltrating cells in spinal cord and IL-1β predominantly expressed in the infiltrating macrophage and parenchymal astrocytes [29, 30]. However, in those patients with longer time of disease, the myelin and axons are equally degenerated and lost and there is a small number of inflammatory cells (mostly $CD8^+$ cells) with downregulation of proinflammatory cytokine expression (with the exception of IFN-γ) [3, 31]. Therefore, therapies with GCs present better result in patients with less than 5 years of HAM/TSP onset, probably because of stronger inflammatory context in the early phase of disease.

The GCs also suppress the T lymphocyte proliferation mainly through its role in lowering the synthesis of IL-2 [21, 32]. These drugs may also reduce spontaneous proliferation of peripheral blood mononuclear cells (PBMCs) from patients with HAM/TSP [33]. This is important since the quantification of provirus reflects the number of HTLV-1-infected cells, which defines the proviral load. Therefore, suppressing the counts of HTLV-1-infected cells reduces the proviral load and may provide an improvement of clinical symptoms [34].

However, previous studies addressing the clinical efficacy of corticosteroids have yielded conflicting results. A stduy conducted in Japan, where several therapies have been tested in a group of 200 patients with HAM/TSP, revealed improved motor activity in 69% of patients with orally admininstered prednisolone, in 40% of those who underwent an intrathecal injection of hydrocortisone, and in 30% of those who underwent pulse therapy with methylprednisolone (10 patients) [35]. In Brazil, Araújo and colleagues conducted a clinical trial with 23 patients in Rio de Janeiro, with improvement for only one patient who reported shorter time of illness [36]. In Japan, a study conducted with patients with radiological changes (leukoencephalopathy on MRI) following intravenous injection of methylprednisolone (1 g / day for 3 days) revealed therapeutic effects in the short term, but without any radiological and clinical benefits in the long-term [37]. Recently, we reported a study with 39 patients with HAM/TSP treated with methyl-prednisolone (1g/day bolus for 3 days). They received an average of 3.4 pulses during follow-up. Some patients also received concomitant physical therapy, and symptomatic drugs such as baclofen, tricyclic antidepressants, vitamin B and oxibutinine. During this period we noticed a 24.5% improvement from baseline on the ISS scale, after a mean of 2.2 years of follow-up [38]. To date, data describing the real clinical benefits of GC treatment for HAM/TSP in the literature are limited. This is partially explained by the fact that few studies have had a longer follow-up time. It is possible that corticosteroids produced only a limited improvement, as suggested by a better performance in the scores only up to visit two. However, HAM/TSP is a chronic and progressive illness. One study showed that the median times from onset of the disability until the assignment of scores 6, 6.5, and 8 on the Disability Status Scale (DSS) scale were 6, 13 and 21 years, respectively [39]. We believe that slowing down the disability progression in the long run may itself represent a clinical benefit. An explanation for the apparent efficacy of corticosteroids could be that HAM/TSP is considered an immune-mediated disease, mainly driven by IFN-γ overproduction. Thus, the anti-inflammatory properties of corticosteroids may have exerted a significant impact on the myelin membrane inflammation process, improving some symptoms, mainly in those patients with only a few years from onset, when inflammation is more prominent [36]. In addition, corticosteroids decrease the number of mononuclear cells and α-2 microglobulin in the CSF, and probably decrease the HTLV-1 DNA proviral load [40, 41].

It should be kept in mind that clinical efficacy of GC treatment is related to tissue sensitivity to these hormones. Several mechanisms of tissue sensitivity and resistance to GCs have been described and include altered expression or distribution of GRs on peripheral lymphocytes, activation of mitogen-activated protein (MAP) kinases, cytokine-mediated pathways, activation of the transcription factor activator protein 1 (AP1), histone deacetylase-2 (HDAC2) expression, and increased P-glycoprotein-mediated drug efflux [42]. HTLV-1 (tax or HBZ proteins) activates several signaling pathways that have the potential to alter the sensitivity to GCs, such as mitogen-activated protein (MAP) kinase p38, AP-1 and pathways activated by IL-2 [43, 44]. However, despite some evidence, no study has showed the direct action of these factors in altering tissue sensitivity to GCs in HAM/TSP. In addition, previous studies demonstrated an overexpression and higher activity of P-glycoprotein, encoded by the multidrug resistance gene (MDR1), in T lymphocytes from HTLV-I-infected subjects (asymptomatic individuals and HAM/TSP and ATL patients) [45, 46]. The P-glycoprotein is a drug efflux pump for xenobiotic compounds including GCs. It is responsible for decreasing intracellular drug accumulation and often mediates the development of resistance to drugs, including resistance to GCs. Moreover MDR1 gene promoter is transcriptionally activated by the HTLV-I tax protein [45]. These observations open up the possibility of new therapeutic approaches for HAM/TSP through the use of P-glycoprotein inhibitors along with GCs.

Histone Deacetylase Inhibitor

Histone deacetylation or acetylation is an important mechanism of gene expression regulation. Acetylation of the lysine residues at the N-terminus of histone proteins removes positive charges, reducing the affinity between histones and DNA (negatively charged) facilitating access of the promoter region by RNA polymerase and transcription factors. Therefore, histone acetylation enhances transcription and histone deacetylation represses transcription. Histone acetylation is catalyzed by histone acetyltransferases (HATs) enzyme and histone deacetylation is catalyzed by histone deacetylase (HDAC). Inhibition of HDAC activity therefore results in histone hyperacetylation and an increase in gene expression. Some different forms of HATs and HDACs have been identified, including CBP/p300 forms. Some studies showed that Tax recruitment of p300 (a HAT enzyme) to the HTLV-1 promoter enhances the level of viral transcription *in vitro* and is directly correlated with histone acetylation [47, 48]. Others studies showed Tax excludes HDAC enzyme from HTLV LTR DNA sequence preventing histone deacetylation and chromatin inactivation [49]. So, histone acetylation is an important mechanism of HTLV-1 gene expression regulation.

Interestingly HTLV-1 proviral expression is stable over periods of several years [8]. This equilibrium, appears genetically determined and is tightly controlled by host immune responses [50]. The selective pressure of immune response maintaining this equilibrium seems effective since the viral load consists basically of infected cells containing transcriptionally silent viruses [51, 52]. In support of this, very little viral expression has been detected in the peripheral blood [53], but HTLV is expressed during short-term culture *ex vivo* [54]. So, there is a hypothesis that infected cells escape from immune surveillance after silencing virus transcription. Thus, the utilization of a HDAC inhibitor, that increase virus expression [55], would result in a better immune response against HTLV.

Based on this, a study showed that treatment of 16 HAM/TSP patients with a standard clinical dose of valproate [HDAC-1 inhibitor) produces a substantial decline in the proviral load [52]. Although HTLV-1 proviral load is transiently increased as expected, a significantly decreased viral load is observed following that (mean of 24 fold) in all patients. Valproate treatment also induced the reduction of spasticity in all patients. Other study showed that inhibition of HDAC doubled Tax expression in naturally infected lymphocytes after overnight culture [55]. However, the rate of $CD8^+$ cell-mediated lysis of Tax-expressing cells *ex vivo* was halved. In addition, valproate treatment was mildly toxic to lymphocytes from HTLV-1–infected individuals. It is important to mention that the impact of this inhibitor on HTLV-1 proviral load *in vivo* cannot be accurately predicted and that caution is recommended when using HDAC inhibitors for nonmalignant cases of HTLV-1 infection [55].

Interferon-Alpha

It is well accepted that interferon-alpha (IFN-α) is a cytokine with powerful antiviral, antiproliferative and immunomodulatory activities. IFN-α appears to be the major cytokine responsible for the amplification of the $CD8^+$ T cell response and resistance to viral infections and additionally regulate the $CD4^+$ Th1 cells, which are both crucial to development of protective immune responses [56]. Its endogenous production has been shown to occur during exposure of cells to viruses, double-stranded RNA, and other cytokines [57]. IFN-α exerts its therapeutic effects both via direct cellular effects via action on cell surface receptors and by indirect mechanisms involving induction of host antiviral responses. Among the mechanisms of direct inhibition of viral replication, it is the induction of cellular enzymes such as 2'5'oligoadenylate synthetase resulting in viral RNA degradation [58]. IFN-α also inhibits viral replication indirectly, by altering cytokine synthesis, which can amplify the cytotoxic T cell and natural killer (NK) cell response against infected cells.

IFNs were able to prevent virus production and release from a cell line chronically infected with HTLV-1 [59]. IFN-α also revealed an *in vitro* modulation of the spontaneous proliferation, and this phenomenon coincided with a clinical improvement in patients with HAM/TSP treated with this cytokine [33]. Another study showed that lymphocytes from patients with HAM/TSP cultured with IFN-α decreased the levels of proviral DNA and viral mRNA, and that this cytokine was able to modulate a proliferative response of $CD8^+$ T cells [60]. Indeed, Saito *et al.* [61] showed that HTLV-I proviral load was significantly decreased concomitantly with the reduction of memory $CD8^+$ T cells.

Interestingly, the IFN-α therapy in nine patients with HAM/TSP reduces $CD4^+$ cell subsets (i.e. Th1 cells) in the cerebrospinal fluid [39, 62]. After therapy, the percentages of $CXCR3^+$ and $CCR5^+$ cells in $CD4^+$ cells significantly decreased in the cerebrospinal fluid as well as in the blood. [39]. In addition, the therapy also lowered the intracellular IFN-γ/IL-4 T cell ratio in the blood [62]. These results suggest that IFN-α suppresses Th1 responses in HAM/TSP and that patients with higher Th1 immunity and proviral load may be better responders to the therapy [62]. One multicenter, randomized, double-blind and controlled clinical trial used three different doses (0.3; 1.0 ; 3.0 MU) of IFN-α , daily for 4 weeks, with 48 HAM/TSP patients. Clinical evaluation included motor dysfunction, urinary disturbances, and changes of neurologic signs. The frequency of therapeutic response judged as excellent to good, 4 weeks after starting therapy and 4 weeks after completion of therapy, were 66.7% [10 of 15] and 61.5% (8 of 13) for the 3.0 MU group. The therapeutic response in the 3.0 MU group was significantly higher than in others groups [63]. These data are important, demonstrating that IFN-α is effective in the treatment of HAM/TSP.

The HTLV-I infection seems to result in low levels of endogenous IFN-α production. Smith *et al.* [64] have demonstrated low or undetectable levels of this cytokine from five HTLV-1 infected lines. Another study demonstrated that HTLV-I infection results in the inhibition of type I IFN gene expression (that includes IFN-α] [65]. In this same study, suppressor of cytokine signaling (SOCS1) was highly expressed in $CD4^+$ T cells from HAM/TSP and AC patients, but not in ATL. Subsequent analysis demonstrated that HTLV-1-induced SOCS1 expression played a positive role in viral replication through inhibition of the IFN response.

SOCS1 directly interacted with IRF3 (interferon regulatory factor 3) and promoted its proteasomal degradation, thus blocking IFN-α synthesis [65]. Therefore, in this context, IFN-α therapy seems to be still more important in HAM/TSP treatment. However, experimental evidence has been obtained that SOCS1 can suppress IFN signaling (including exogenous IFN -α) by direct binding to phosphorylated type I IFN receptor [66]. This would be a possible explanation for the observation that some patients do not present a significant improvement with IFN-α therapy.

Vitamin C

Vitamin C supplementation has been shown to be of therapeutic value in specific clinical conditions, and devoid of serious side effects. The first study about vitamin C and HAM/TSP evaluated seven patients. Grades of the disability score improved at 9 months after therapy from 7-1 (3.3) to 3-6 (2.0) and serum immunosuppressive acidic protein (IAP) decreased from 747 (316) to 398 (86) µg/ml [67]. The levels of this protein correlated closely with the impairment of the host's immunity. A second study showed a modest clinical benefit in HAM/TSP patients after therapy with ascorbic acid. Motor disability grades were improved by more than one grade in 20.0% (4/20) of patients and 50% (10/20) showed a fair response to treatment [35]. In another recent study *ex vivo,* ascorbic acid induced a dramatic 95% decrease in spontaneous proliferation, a decrease in tax and LTR transcription, and a decrease in IFN- γ production in HAM/TSP PBMC [68]. Obviously, the combination therapy with high-dose vitamin C in HAM/TSP should be further explored.

CONCLUSIONS

In our experience, GCs may improve the neurological symptoms and therefore, the quality of life of HAM/TSP patients. More important, they can prevent the HAM/TSP disease progression that can occur if no therapeutic measure is taken. Another major advantage of this therapy is its feasibility, especially for developing countries. The low cost, the relatively easy administration, and the reversible side effects indicate that this therapy is a promising strategy protecting a certain number of patients against more severe disabilities. Anyway, only a double-blinded placebo-controlled clinical trial can ultimately determine the potential role of GCs in HAM/TSP, a disease for which very few clinical trials have been published. Additionally, physical and psychological rehabilitation should be performed for all patients, regardless of their clinical status. Furthermore, new approaches such as immune therapies like those used for multiple sclerosis, rather than anti-retroviral therapy, may be used for treating this condition.

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REVIEW ARTICLE

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Functions of Neurotrophins and Growth Factors in Neurogenesis and Brain Repair

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Abstract

The identification and isolation of multipotent neural stem and progenitor cells in the brain, giving rise to neurons, astrocytes, and oligodendrocytes initiated many studies in order to understand basic mechanisms of endogenous neurogenesis and repair mechanisms of the nervous system and to develop novel therapeutic strategies for cellular regeneration therapies in brain disease. A previous review (Trujillo et al., Cytometry A 2009;75:38–53) focused on the importance of extrinsic factors, especially neurotransmitters, for directing migration and neurogenesis in the developing and adult brain. Here, we extend our review discussing the effects of the principal growth and neurotrophic factors as well as their intracellular signal transduction on neurogenesis, fate determination and neuroprotective mechanisms. Many of these mechanisms have been elucidated by in vitro studies for which neural stem cells were isolated, grown as neurospheres, induced to neural differentiation under desired experimental conditions, and analyzed for embryonic, progenitor, and neural marker expression by flow and imaging cytometry techniques. The better understanding of neural stem cells proliferation and differentiation is crucial for any therapeutic intervention aiming at neural stem cell transplantation and recruitment of endogenous repair mechanisms.

Key terms

neural stem cells; neurotrophins; growth factors; neurogenesis; brain repair

Introduction

Cells from neural tube and crest form the nervous system during embryo development. In this process neural stem cells (NSC) extensively proliferate and differentiate into oligodendrocytes, astrocytes, and neurons that can be identified by expression of specific marker proteins (1). For a long time scientists believed that no new neurons were born in the adult central nervous system (CNS). This view was changed during the first half of the 20th century, when many research groups identified cell division in the brain of birds and rodents, but only in the 1990's the idea of neurogenesis was accepted (2). In 1992, Reynolds and Weiss isolated NSC from the striatum of adult mice and cultivated them in the presence of epidermal growth factor (EGF) and observed some clusters of dividing neural stem and progenitor cells. After EGF removal, cells were able to differentiate and expressed neural or glial markers, an indicative of stem cells in the adult brain (3). Those findings gave rise to a very useful in vitro model to study cell fate determination, called neurosphere.

Previous published research and review articles of our group focused on functions and signaling mechanisms of the kinin-kalikrein, cholinergic and purinergic systems in triggering neural differentiation and phenotype determination (4–8). However, the mechanisms how neurotrophins and growth factors determine cell fate is still far from being completely understood. This review aims to highlight the new findings about roles of neurotrophins and growth factors on the modulation of NSC proliferation, survival, and differentiation (Fig. 1). Another goal is to provide an update of new markers

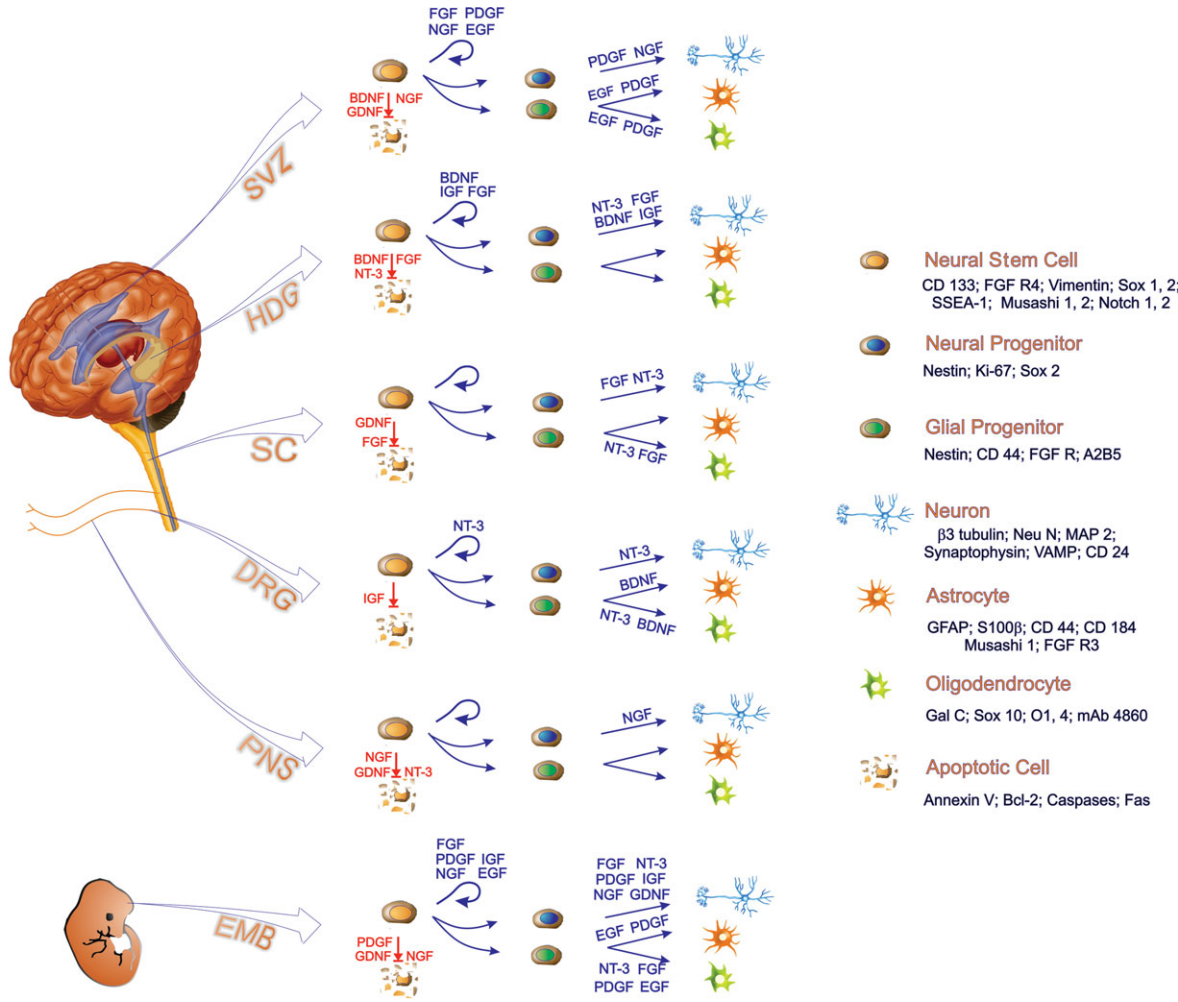

Figure 1. Effects of growth factors and neurotrophins on neural stem and progenitor cells from different regions of adult and embryonic (EMB) brain. In contrast to the developing nervous system, the adult CNS maintains NSC only in defined neurogenic areas in the brain, such as the subventricular zone (SVZ), the dentate gyrus (HDG) and the subgranular zone (SZ) of the hippocampus, the olfactory bulb (OB), and the spinal cord (SC). Moreover, NSC also persists in the adult peripheral nervous system (PNS), where they can originate dorsal root ganglion (DRG) cells and other peripheral neural phenotypes. The diagram shows the influence of growth factors and neurotrophins on proliferation, survival and differentiation of NSC into progenitor cells, following differentiation into neurons, astrocytes, or oligodendrocytes. Markers for cell differentiation and apoptosis are also indicated.

characterizing neural stem and progenitor cells (Fig. 1, Table 1). Cellular phenotypes are identified, to great extent, through imaging or flow cytometry analysis of neural cell marker expression. Several classical markers are used, such as Nestin for neural progenitor cells, GFAP for glial cells, β3-Tubulin for neuronal cells, microtubule associated protein 2 (MAP2), neuron-specific enolase (NSE), and NeuN for mature neurons, S100β for mature astrocytes, and Gal C for oligodendrocytes (Fig. 1). Recently, novel markers were identified, contributing to the understanding of roles and effects of grow factors and neurotrophins in neural stem cell fate determination.

Nerve Growth Factor (NGF)

In 1951, Levi-Montalcini and Hamburger first noticed that a mouse sarcoma stimulated the growth of sympathetic and sensory neurons and, together with Cohen, they isolated NGF (9). Pro-NGF, a precursor form, is processed to the mature form by furins, that then activates two types of receptors: the tyrosine kinase receptor TrkA, which binds specifically NGF, and the member of the tumor necrosis factor receptor family, p75NTR, which binds any neurotrophin. (10,11). The expression pattern of these receptors and the concentration of NGF determines effects exerted by this polypeptide (12,13). The proliferative effect of NGF on NSC proliferation was reported by Cattaneo and McKay. They found that cells exposed to fibroblast growth factor 2 (FGF2), followed by NGF treatment, displayed an increase in the Nestin$^+$ population (14). Later it was demonstrated that a previous exposition to growth factors is necessary for TrkA expression (15). NGF promotes proliferation through the phosphorylation of ERK1/2 in NSC (16). Zhang et al. demonstrated that lower concentrations of NGF (2–5 ηg/ml) are more effective to promote proliferation (13).

Table 1. Markers utilized for flow cytometry and immunohistochemistry to identify neural stem, progenitor, and differentiated cells

MARKERS PROFILE	CELL IDENTIFIED	CELL ORIGIN	REFERENCE
$CD44^+$	Astrocyte progenitor cells	Postnatal mouse cerebellum	(195)
$CD133^+/CD15^+$	Neural stem cells	Human fetal brain E50-55	(196)
E-PHA binding *N*-glycans	Neural stem cells	Mice fetal brain E12, E14, E16	(197)
GD3	Neural stem cells	Mouse striata and subventricular zone	(198)
$HRD1^+/nestin^+/GFAP^+$	Neural stem cells	Mice subventricular zone	(199)
$HRD1^+/nestin^+$	Neural stem cells	Mice dentate gyrus	(199)
$Id1^{high+}/GFAP^+$	Type B1 astrocytes	Mice subventricular zone	(200)
Ki-67	Neural progenitor cells	Pig subventricular zone	(201)
QKF	Neural stem cells	Mice subventricular zone	(202)
$CD184^+$ $CD271^-$ $CD44^-$ $CD24^+$	Neural stem cells	Human embryonic stem cells	(203)
$CD184^-$ $CD44^-$ $CD15^{Low}$ $CD24^+$	Neuron	Human embryonic stem cells	(203)
$CD184^+$ $CD44^+$	Glial cells	Human embryonic stem cells	(203)
$Vimentin^+$ $nestin^+$ $Sox2^+$	Radial glial cells	Rat germinal zone E16,5	(204)
$CD140a^+/CD9^+$	Oligodendrocyte progenitor cells	Fetal human forebrain	(205)
mAb 4860	Oligodendrocyte	Mouse telencephalus E13	(206)
$CD15^+$ $CD29^{High}$ $CD24^{Low}$	Neural stem cells	Human embryonic stem cells	(207)
$CD15^-$ $CD29^{High}$ $CD24^{Low}$	Neural crest-like cells	Human embryonic stem cells	(207)
$CD15^-$ $CD29^{Low}$ $CD24^{High}$	Neuroblast and neurons	Human embryonic stem cells	(207)

NGF was shown to influence the migration of oligodendrocytes in the CNS (17) and of Schwann cells in the peripheral nervous system (PNS) (18), mediated through the p75NTR (19). Another effect of NGF is the induction of neurite outgrowth. NGF-producing NSC have longer neurites than naïve NSC (20), and this effect is triggered by the down-regulation of ATF5 transcription factor expression (21). Potential clinical applications of this trait are being investigated, for instance, the use of dorsal root ganglion cells together with NGF for regeneration of spinal ganglion neurons (22).

It has been demonstrated that NGF regulates the differentiation of NSC into mature neural phenotypes, a trait inhibited by EGF (15). NGF signaling leads to differentiation into neurons and astrocytes, but not into oligodendrocytes (23-26). NSC isolated from specific regions were able to differentiate into glutamatergic and sensory neurons and also into nociceptors (13,27,28). Differentiation is induced by NGF by down-regulation of ATF5 (21) and upregulation of TIMP-2 metalloproteinase inhibitor expression (29).

It has been suggested that NGF also plays a role in apoptosis, mediated by the p75NTR (12,30). Although NGF has been more associated with TrkA-mediated neuroprotection and cell survival, in both CNS (13) and PNS (27), TrkA is also involved in apoptosis at low concentrations of NGF (100 fg/ml) (31). P3IK/Akt and mitogen-activated protein kinase (MAPK) pathways were shown to be involved in neuronal survival (32).

Brain-Derived Neurotrophic Factor (BDNF)

BDNF is another member of the neurotrophin family essential for developmental events of the nervous system, including proliferation, migration, differentiation, survival, apoptosis, and synaptic plasticity (33). BDNF-mediated effects are controversially discussed (34). It has been suggested that BDNF promotes only survival of neurons from the rat SVZ (subventricular zone) (35). However, it also enhanced both survival and differentiation of postnatal hippocampal stem cells (36). Our group has observed that BDNF alone has no effect on rat telencephalon-derived NSC proliferation and differentiation (Fig. 2).

BDNF exerts its effects by TrkB and p75NTR activation, the latter is known to have a role in postmitotic neural survival (37–39). Young et al. have demonstrated p75NTR expression defines a population of cells in the SVZ that persists in adulthood and is able to respond to stimulation by neurotrophins. The results of this work suggest that p75NTR is a specific postnatal marker that can be useful for identification and purification of those cells by flow cytometry. TrkB seems to be involved in proliferative mechanisms, while BDNF-induced neurogenesis occurs via p75NTR activation alone, independently from TrkB (40-42). BDNF has been so far described as the only neurotrophin able to affect dendritic development of SVZ-derived neurons via its high affinity receptor TrkB (43).

It has been proposed that TrkB and p75NTR can affect each other (44). Alternative TrkB mRNA splicing originates eight receptor isoforms, which form heterodimers with full-length receptors or competitively bind to available ligands. Truncated Trk receptors can inhibit full-length Trk receptors either by acting as dominant negative receptors or by forming nonfunctional heterodimers (45,46).

Delayed differentiation of NSC caused by inhibition of nitric oxide (NO) production was shown to be reversed by BDNF (unpublished data), probably by upregulation of p75NTR expression (47,48). Moreover, NO inhibits cell proliferation (49), but this effect is also abolished by BDNF (50), indicating that p75NTR is also involved in the regulation of cell proliferation.

Takahashi et al. demonstrated that hippocampus-derived stem cell clones did not reveal any response following stimulation with neurotrophins; however, following cell exposure to retinoic acid (RA) the expression of all neurotrophins receptors became

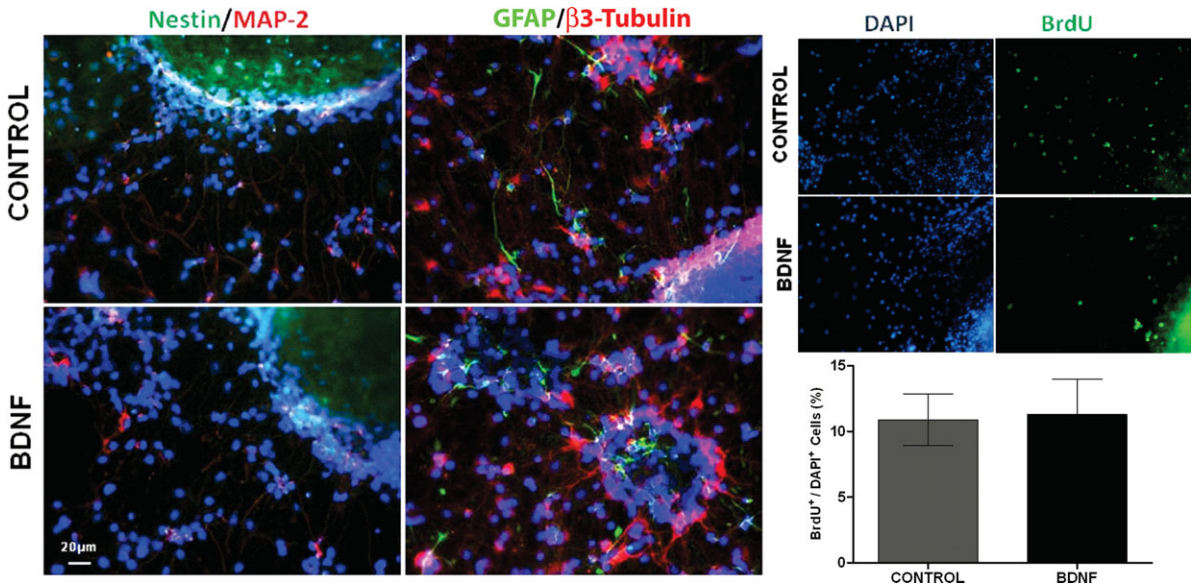

Figure 2. BDNF-mediated effects on proliferation and differentiation of rat telencephalon-derived NSC by imaging cytometry technique. (**A**). Immunostaining for Nestin, MAP-2, β3-Tubulin, and GFAP of neurospheres on day 7 of differentiation in the presence or absence (control) of 20 ηg/ml BDNF. Briefly, cells plated onto coverslips were blocked for 1 h with 3% FBS in PBS/0.1% Triton X-100, followed by a 2 h incubation with primary antibodies against β3-Tubulin (Sigma-Aldrich, 2G10 monoclonal), Nestin (Millipore, rat-401 monoclonal), and GFAP (DAKO, 6F2 monoclonal) at 1:500 dilution. NSC were washed with PBS and Alexa 555 and Alexa 488 (Molecular Probes, clone not informed) at 1:500 dilutions were added for 1 h. Cell nuclei were counterstained with DAPI. Coverslips were mounted and analyzed under a fluorescence microscope (Axiovert 200, Zeiss). Scale bars = 20 μm. (**B**). Immunodetection of BrdU incorporation after a 12 h pulse of 0.2 mM BrdU (Sigma-Aldrich) in neurospheres on day 7 of differentiation in the presence or absence (control) of 20 ηg/ml BDNF. Cells were fixed with ice-cold methanol for 10 min, washed with PBS and incubated for 30 min in 1.5 M HCl. Following the washing step, cells were incubated for 2 h with rat anti-BrdU (Abcam; 1:200 dilution, ICR1 monoclonal). Cells were again washed with PBS followed by addition of Alexa fluor 488 secondary antibodies (Molecular Probes, clone not informed) at 1:500 dilution. After washing with PBS, DAPI solution (Sigma-Aldrich; 0.3 μg/ml) was used as a nuclear stain. Coverslips were mounted and analyzed under a fluorescence microscope (Axiovert 200, Zeiss). BrdU incorporating nuclei are shown in green. The percentages of BrdU-positive cells were calculated as the ratio of immunolabeled cells over the total number of DAPI-stained cells. Scale bar = 20 μm.

upregulated. Treatment with BDNF or neurotrophin-3 (NT-3) after exposition to RA led to augmented expression of mature neuronal markers (51). In neuroblastoma cell lines and sympathetic neurons from newborn rats, expression of TrkB and subsequent dependence on BDNF, actions were induced by RA (52,53).

Taken together, these results show that BDNF-induced neurogenesis is determined by the interaction of TrkB isoforms and p75NTR with others factors, such as NO and RA.

Neurotrophin-3 (NT-3)

NT-3 was identified and cloned in 1990. Its primary structure is very similar to NGF and BDNF (54,55), and it binds to TrkC and p75NTR. Unlike other neurotrophins, NT-3 binds to TrkA and TrkB, although with lower affinity than its original ligands, NGF and BDNF (12,56). The effect of NT-3 on cell fate determination depends on the expression of these receptors (12). In vitro studies demonstrated that exogenous NT-3 increased proliferation of neural crest and somite derived NSC as well as of cells cultured on NT-3 impregnated scaffolds (26,57,58). Cells overexpressing NT-3 were also shown to proliferate faster in vitro (59). Effects of NT-3 on proliferation were dose-dependent. Low doses promoted cell proliferation (1-20 ηg/ml) while higher doses (50 ηg/ml) actually lowered it (60).

NT-3 also affects migration and neurite outgrowth. Cells overexpressing NT-3 migrated more than nontransfected ones in rat injured spinal cord (61). NSC overexpressing NT-3 displayed longer neurites in vitro (62) and promoted neurite outgrowth in vivo (63).

NSC expressing NT-3 revealed a significant increase in the number of MAP2-positive cells after 14 and 56 days in culture (62). Interestingly, NSC expressing TrkC treated with NT-3 showed a higher MAP2-positive population than those treated with NT-3 or TrkC alone (64). Co-transplantation of Schwann cells expressing NT-3 or NSC resulted in an increase in neuronal population in rat injured spinal cord (65). Besides the role in proliferation and differentiation events, NT-3 is strongly involved in neuronal specification (27,66,67). Engraftment of NSC expressing NT-3 in infarcted brains increased the number of cholinergic, GABAergic, and glutamatergic neurons (68); moreover, co-transplantation of NSC and Schwan cells expressing NT-3 promoted differentiation into serotoninergic neurons in rat injured spinal cord (63). NT-3 also participates in the differentiation of oligodendrocytes (23,57,69,70) from cortical multipotent cells, but not of primary culture of cortical oligodendrocyte progenitors, which differentiate only in response to stimulation by platelet-derived growth factor (PDGF) (71).

Different mechanisms are involved in NT-3-induced differentiation. NT-3, as well as transforming growth factor

(TGF)-β1 and FGF2, upregulates norepinephrine transporter expression, promoting differentiation into noradrenergic neurons (72,73). MAPK, together with PI3K/Akt pathways, is also activated by NT-3, inducing neuronal differentiation of the NSC (74,75). Although some evidence points at NT-3 as a promoter of NMDA-induced cell death (76), its survival-promoting effects are more remarkable. NSC expressing NT-3 had a higher viability than the control group (62), and the same effect was observed in vivo, when were rats subjected to axotomization of Clarke's nucleus axons (77).

Epidermal Growth Factor (EGF)

The discovery of EGF by Stanley Cohen initiated a new era in the research field of growth and differentiation (Nobel Prize in Physiology and Medicine in 1986). Cohen isolated and characterized the EGF receptor (78,79). EGF binds to the EGF receptor (EGFR), which in turn promotes enzymatic activity (79). The EGFR, also named ErbB-1, belongs to a family of four structurally related receptor tyrosine kinases. EGF has several important roles during development of the nervous system, including induction of proliferation and migration of NSC. There is a consensus that EGF is not mitogenic at the beginning of neural development, since its expression becomes detectable only at later stages (80,81). Therefore, exogenous FGF2, but not EGF, stimulated the proliferation of mouse neuroepithelial cells from embryonic day 10 (E10) as well as of rat cortical cells obtained on E13. On the other hand, EGF is critical for the proliferation of EGF-responsive NSC isolated from the E14.5 subgranular zone (SGZ) (82). In other words, FGF2-responsive NSC divide symmetrically for self-renewal and proliferation, and asymmetrically, yielding EGF-responsive cells (83,84). EGF-dependent SVZ precursor expansion measured by using the neurosphere assay is lost when the EGFR is inhibited, and the constitutive expression of active receptors is sufficient to rescue the proliferation of NSC induced by hypoxic/ischemic brain injury. These results reveal the EGFR as a key regulator of the expansion of SVZ precursors in response to brain injury (85,86).

It is well known that the EGF/EGFR promotes phosphoinositide 3-kinase (PI3K) and extracellular-signal-regulated kinase1/2 (ERK1/2) pathway activation, resulting in NSC proliferation (87-89). Recent studies demonstrate that EGF activates adenylate cyclase and inhibits cAMP-specific phosphodiesterase, leading to intracellular cAMP accumulation and subsequent PKA activation which, in turn, stimulates CREB (90,91). This transcription factor is required for EGF-induced cell proliferation in cultured adult NSC of the SVZ (92). Another recent study showed that EGFR-mediated signaling promotes Sox2 expression, which binds to the EGFR promoter and directly upregulates EGFR expression by a positive feedback loop in NSC of the mouse embryonic cortices (E18.5). Knockdown of Sox2 down-regulates EGFR expression and attenuates colony formation of NSC, whereas overexpression of Sox2 augments EGFR expression and promotes progenitor cells self-renewal (88). Moreover, Pax6 is also induced in regulation of EGF-induced proliferation of SVZ-derived NSC. Expression of EGFR in neurospheres from Pax6 mutant mice (E18.5), was down-regulated in vitro and in vivo, as determined by flow cytometry (93). EGFR has also been associated with the maintenance of multipotency. Flow cytometry analysis revealed that, independently from age or region of the brain, most cells overexpressing EGFR are multipotent precursor cells. However, these cells did not show higher neurogenic capabilities, indicating that EGFR is not directly linked to differentiation (94).

EGFR signaling plays an important role in migration of adult and embryonic neural precursors (94-96). This is associated with increased phosphorylation of Akt and focal adhesion kinase (97). Overexpression of EGF promotes radial migration toward the cortical plate (95), and ventrolateral migration in the lateral cortical stream (96) of fetal telencephalon. Interestingly, EGFR overexpression in nonmigratory cortical nerve/glial antigen 2 ($NG2^+$) cells converts these cells into a migratory phenotype in vitro and in vivo (98). EGF-evoked effects have been associated with the progression from transit-amplifying precursor cells to neuroblasts. $EGFR^+$ cells, purified by flow cytometry, demonstrated functional voltage-dependent Ca^{2+} channels and later on differentiated into neuroblasts (99). EGFR activity has also been connected with enhanced gliogenesis, increasing the number of newborn glia and decreasing the number of newborn neurons in vivo and in vitro (100). First of all, EGF infusion induces vast proliferation and migration of SVZ progenitors; however, seven days later, most labeled cells derived from SVZ primary precursors (type B1 cells) gave rise to the oligodendrocyte lineage, including $NG2^+$ progenitors, and premyelinating and myelinating oligodendrocytes. SVZ B1 cells also originated a population of $S100\beta^+/GFAP^+$ cells in the striatum and septum, but neuronal differentiation was not observed (101). Reduced EGFR signaling in progenitor cells of the adult SVZ attenuates the production of oligodendrocytes, whereas EGFR overexpression expanded the oligodendrocyte population (102,103). EGF induced proliferation and migration of isolated SVZ B cells which, in turn, gave rise to migratory cells expressing Olig2/NG2, but not to neuronal phenotypes. Upon EGF removal, Olig2/NG2 migratory cells stopped migrating and originated to cells expressing an oligodendrocyte- specific marker (104).

Fibroblast Growth Factor (FGF)

The nervous system has a limited capacity for self-repair. Thus, efforts have been made to improve the repair process by transplanting exogenous cells into sites of injury. In this context, FGFs can be used to maintain and expand embryonic stem cells (ESC) and NSC and to guide differentiation into specific neuronal cell subtypes in vitro. Therefore FGF plays a major role in such cell replacement therapies (105,106). The FGF family comprises 22 ligands and 4 receptors, of which FGFR-1, 2 and 3 influence neurogenesis. Co-activation of FGFR-1 and 3 promoted symmetrical divisions of NSC, whereas inactivation of either of them resulted in asymmetrical divisions and neurogenesis. Developmental upregulation of FGFR2 expression correlated with a shift of NSC into a multi-potential state (107).

Signaling pathways involved in the maintenance of human ESC pluripotency are not fully understood. Leukemia inhibitory factor (LIF) signaling, which is essential for mouse ESC self-renewal, is not active in undifferentiated human ESC (108,109) and activin signaling is not sufficient to sustain long-term growth of them in a chemically defined medium (106). In this context, recent studies have shown that addition of FGF2, in combination with activin, maintains long-term expression of pluripotency markers in human ESC. In addition, inhibition of the FGF signaling pathway causes human ESC differentiation (106).

Exogenous FGF2 alone improved the commitment of mouse and human ESC to a neural fate and generated NSC (105,110). These cells proliferate in response to FGF2 and can differentiate into neurons, astrocytes, and oligodendrocytes (111,112). This acquired multipotency results from the induction of multiple genes by FGF2, like Olig2 and EGFR, making the cells responsive to EGF and increasing their proliferative capacity (95,113). The addition of high concentrations of both FGF2 and EGF is a standard procedure to expand NSC and progenitor cells as floating neurospheres or adherent cultures (105,114). The determination of EGF- and FGF2-induced proliferation of NSC can be analyzed by imaging and flow cytometry techniques, as shown in Figure 3. NSC proliferation was assessed using BrdU (5-bromo-2'-deoxyuridine, an analogue of thymidine) incorporation. The percentage of $BrdU^+$ cells increased from 8.4% to 35.8% in the presence of EGF and FGF2 (Fig. 3A). EdU (5-ethynyl-2'-deoxyuridine), another thymidine analogue, has advantages over BrdU, since EDU does not result in epitope destruction permitting co-staining by antibodies. It allows the identification of proliferative neural progenitor cells, as shown in Figure 3B.

FGF2 promotes proliferation of NSC by activation of MAPK/ERK pathway, upregulation of cyclin D2, and downregulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$ expression (107,115). FGF2 signaling is mediated through increased expression of β-catenin, nuclear translocation, and phosphorylation of GSK-3β and tyrosine phosphorylation of β-catenin. Overexpression of β-catenin, in the presence of FGF2, keeps NSC in a proliferative state and, in the absence of FGF2, enhances neuronal differentiation (116). Although FGF2 generally acts as a mitogen for NSC, in granule cell precursors, obtained from the developing cerebellum, FGF2 strongly inhibits the proliferative response to Sonic hedgehog by the activation of ERK and c-Jun N-terminal kinases (JNK). FGF2 also promotes granule cell differentiation in vitro and in vivo (117). Schwindt et al. reported that short-term removal of EGF and FGF2 from the medium promotes neurogenesis and neurite extension in human and rat neural progenitor cells (118,119).

FGFs are essential for regular neurogenesis in the brain and spinal cord, and the development of multiple neuronal lineages in the embryo. Mice lacking FGF2 have neuronal deficits in the spinal cord and cerebral cortex (50% reduction in the number of cortical neurons at birth), and phenotypically anomalous neurons in the hippocampus (120). In vitro, FGFs have been used to stimulate and guide the differentiation of mouse (FGF2 and FGF4) and human (FGF2, FGF8, and FGF20) stem cells. FGF4 has similar effects of FGF2 in NSC obtained from embryo and adult mouse (121,122). Furthermore, the addition of FGF4 increased the number of NSC generated from ES cells (122,123). Finally, FGF-4 has also been suggested to be a potent mitogen for NSC (122). FGF4 is produced in an autocrine fashion by undifferentiated mouse ESC (124). If left unchecked, this factor acts in the block self-renewal and promotes commitment to mesodermal or neural lineages. On the other hand, without FGF4/ERK1/2 input, commitment of ESC to any lineage does not occur and alterations in expression of pluripotency markers Oct4, Rex1, and Nanog are not observed (125). Bone morphogenetic protein (BMP) and BMP signaling inhibitors can act downstream of FGF4 to promote non-neural and neural fates, respectively (125,126). LIF/Stat3 inhibits lineage commitment and intervenes downstream of Erk 1/2 to override the autoinductive capacity of FGF4 (125). Another important study with mouse NSC was done by Palmer et al. (127), showing that FGF2 is critical for neuronal generation from adult NSC derived from non-neurogenic regions. Several studies also showed that mouse NSC, isolated from neocortex or dorsal spinal cord, which do not generate the oligodendrocyte lineage in vivo, can be isolated by flow cytometry and induced in vitro by FGF2 to express Olig2 and NG2 and then give rise to oligodendrocytes (113,128,129).

In human ESC, FGFs can also guide the differentiation in vitro. First, FGF signaling through FGFR1 was demonstrated to be required for olfactory bulb morphogenesis (130). A second study demonstrated that 100% of FGF8-treated aggregates (0% of untreated controls) co-expressed Tbx21/Reelin/Tbr1, which is characteristic of neuronal projections in the olfactory bulb, suggesting that FGF8 is sufficient to induce differentiation of olfactory bulb neurons from telencephalic progenitors (131). However, another study showed that FGF2 provoked human fetal forebrain-derived NSC to express the motor neuron marker Hb9, which is blocked by specific inhibition of FGFR. Thus, treatment with FGF2 within a specific time window generates cholinergic neurons with spinal motor neuron properties (131). The FGFR1c ligand FGF20 has potent effects in generating large numbers of dopaminergic neurons from hESC co-cultured with mouse stromal cells (132). These effects in neural cell type specification make FGFs useful for stem cell-based therapies of neurologic disorders.

Brain morphogenesis comprises essential steps, like migration of newborn neurons, glial cells and NSC, formation of neural circuits, and repair of injuries. In this way, some in vitro studies have been performed demonstrating the effects of FGF2 in these processes. For instance, the reduction in Neurogenin expression in cultured NSC can be partially restored by a brief exposure to FGF2 during the early phase of differentiation, resulting in increased migration and survival of neurons after transplantation (133). Another study revealed similar results demonstrating that FGF2 overexpression significantly enhances the migratory capacity of grafted NSC in complex three-dimensional structures, such as cortical slices (134).

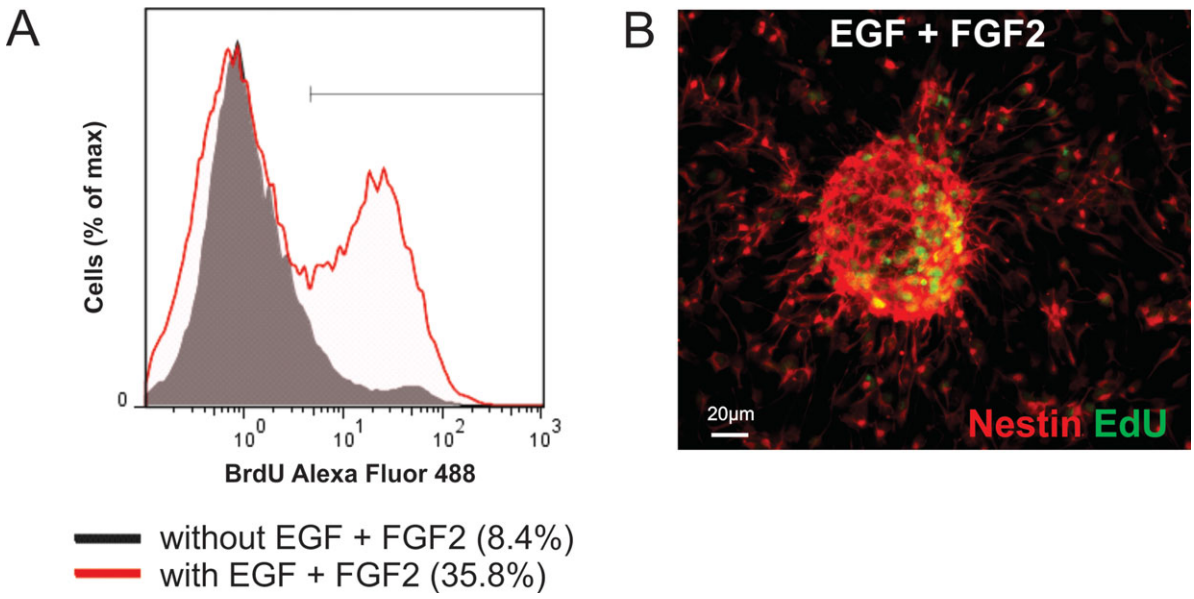

Figure 3. Determination of EGF and FGF2-induced proliferation of mouse telencephalon-derived NSC by flow cytometry and immunostaining. (**A**). BrdU incorporation was used to measure cell proliferation. Undifferentiated cells were stimulated to proliferate with EGF and FGF2 (both 20 ηg/ml) for a 24 h period compared to proliferation rates of unstimulated cells, followed by a 2 h incubation in the presence of BrdU (100 μM). An increase in the percentage of $BrdU^+$ cells (35.8%) was observed in the presence of growth factors when compared with unstimulated cells cultured in the absence of these factors (8.4%). Protocol for BrdU labeling: cells were washed with PBS, fixed with 70% ethanol for 4 h at 4°C, and then incubated with 2 M HCl for 30 min at room temperature. Following another washing step, the preparation was treated with 0.1 M sodium tetraborate (pH 8.5) for 5 min and then washed again. The preparation was then incubated with primary anti-BrdU antibody (Axyll, ICR1 monoclonal) at 1:100 dilution in PBS containing 2% FBS at room temperature for 2 h, followed by washing with PBS; addition of a secondary antibody solution (Alexa Fluor 488, Molecular Probes, clone not informed) at 1:500 dilution in PBS containing 2% FBS at room temperature and incubation for 1 hour protected from light. After a final washing step, cells were resuspended in PBS and analyzed by flow cytometry in agreement with the MiFlowCyt standards (208). Further details are provided in the Supplementary Material. (**B**). Immunostaining of undifferentiated neurospheres for Nestin and EdU was used to assess cell proliferation. Cells were stimulated to proliferate with EGF and FGF2 (both 20 ηg/ml) over a 24h period followed by a 14h incubation in the presence of EdU (100 μM, Life Technologies) showing several progenitor cells proliferating in the presence of these factors. Proliferation was assessed by Click-iT EdU Imaging Kits (Life Technologies) according to the manufacturer's procedure (209). The preparation was then incubated with primary anti-Nestin antibody (Millipore, rat-401 monoclonal) at 1:500 dilution in PBS containing 2% FBS at room temperature, for 2 h, followed by washing with PBS and incubation for 1 h with of a secondary antibody solution (Alexa Fluor 555, Molecular Probes, clone not informed) at 1:500 dilution in PBS containing 2% FBS at room temperature and protected from the light. After a final washing step, slides were mounted with coverslips and analyzed under a fluorescence microscope (Axiovert 200, Zeiss) ($\times$100 magnification). Scale bar = 20 μm.

The balance between apoptosis and cell survival is tightly controlled during brain maturation, as well as during neurogenesis in vitro (135). In vitro studies showed that FGF1, FGF2, and FGF4 are survival factors for neuronal cells isolated from distinct regions of the brain in the embryo (122,136). FGF5 has also survival effects in cultured embryonic spinal motor neurons (137). However, FGF2 induces a switch in death receptor signaling, thereby upregulating TNF-α-mediated death and down-regulating the Fas-death pathway in both progenitor and primary hippocampal cells (138). Lastly, among the molecules implicated in the maintenance of NSC and neural differentiation, FGFs may have the most widespread roles in generating the cellular diversity and morphological complexity of the nervous system (105).

Glial Cell Line-Derived Neurotrophic Factor (GDNF)

Neurotrophic factors are essential for differentiation and neuron survival and maintenance of its phenotype. GDNF was found in culture supernatants of the B49 glial cell line, and it was first related with survival promotion of cultured rat mesencephalic dopaminergic neurons (139,140). It has been demonstrated that GDNF increases the differentiation of NSC into dopaminergic neurons. The treatment of NSC with 25 ηg/ml of GDNF for 5 days increased the population of dopaminergic neurons from 2.9% to 50%, as demonstrated by flow cytometry (141).

The neurotrophic and neuroprotective effects of GDNF have been broadly described and are mainly exerted through cell survival, involving complex interactions between multiple signaling cascades (142), like the activation of PI3K/Akt and MAPK/ERK pathways (143,144). The antiapoptotic effect of Akt is triggered by downstream targets, including Bad (Bcl-2-associated death promoter), FKHR-1 (forkhead transcription factor 1), and NF-κB (145). Neuronal survival is also supported by the activation of the MAPK/ERK pathway (146,147). Nicole et al. (143) demonstrated that, after GDNF treatment, cortical neurons and astrocytes displayed activation of the MAPK pathway, which is responsible for the regulation of cell proliferation and differentiation. Recently, studies of GDNF signaling and function in adult brain were made using genetic animal models with deficiency in the GDNF-depend-

ent pathways. Experiments with a conditional GDNF null mouse model allowed one to demonstrate a major physiological neuroprotective effect of GDNF and its absolute requirement for survival of dopaminergic and noradrenergic neurons in adult brain (148).

Storch et al. reported that dopaminergic neurons can be obtained from long-term cultures of human fetal mesencephalic precursor cells by incubation in differentiation medium containing interleukins, LIF, and GDNF. The resulting neurons were immunoreactive for tyrosine hydroxylase and exhibited morphological and functional properties of dopaminergic neurons in vitro (149). Addition of GDNF to E12 mouse ventral mesencephalon-derived neurospheres resulted in significantly higher cell numbers expressing early dopaminergic markers, Nurr1 and Ptx3 (150). In the presence of NT-3 and GDNF, ESC cultures did not augment proliferation, however, the number of neurons in the cultures was increased 7 days after plating. Pretreatment of ESC with GDNF also reduced the vulnerability of ESC-derived neurons to NMDA-induced death (151). Cell transplantation has been shown to be an effective therapy for CNS disorders in animal models. During the early phases of the implantation process, cells are exposed to an environment that causes hypoxia-ischemia damage, which may induce cell death. Optimization of cell transplantation efficacy depends critically on improving grafted cell survival. Wang et al. investigated the neuroprotective effects of GDNF on NSC survival, both in vivo and in vitro. NSC pretreated with GDNF for 3 days were subjected to oxygen-glucose deprivation (OGD) (152). GDNF was shown to increase NSC survival and also to reduce the number of apoptotic cells significantly, as compared to cells treated with saline. Pretreatment of NSC with GDNF also increased cell survival after transplantation into the striatum of a Parkinson Disease (PD) rat model (152). The results reported by Lei et al. partially elucidated the mechanisms involved in PD, as well as the GDNF protective effects upon ventral midbrain dopaminergic neurons. They showed that Nurr1, a critical transcription factor, is essential for the regulation of expression of a set of genes involved in dopamine metabolism (tyrosine hydroxylase, vesicular monoamine transporter (Vmat2), dopamine transporter, and aromatic L-amino acid decarboxylase). Nurr1 cross-talks with Pitx3, which is involved in the development and maintenance of dopaminergic neurons of the substantia nigra compacta (SNc) and the ventral tegmental area (VTA) (153).

The beneficial effects of GDNF were also demonstrated in a rat model of stroke. The injection of stem cells into the tail vein has been demonstrated to increase the expression of GDNF in the ischemic boundary zone (154). Studies from Lee et al. (155), using GDNF-secreting human NSC, resulted in an improvement of motor performance and an increase in survival of transplanted NSC in a mouse model for intracerebral hemorrhage (ICH). Adult mouse striatum was lesioned with bacterial collagenase to induce the ICH model. In GDNF grafted ICH brain, they found a significant increase in the concentration of antiapoptotic and cell survival-promoting factors (Bcl2, Akt, ERK-MAPK), together with a marked reduction in proapoptotic proteins (p53, Caspase 9 and 3, Bax) when compared with the control group (155). Rats subjected to middle cerebral occlusion and reperfusion and then treated with GDNF-secreting rat NSC revealed improved neurological function and increased expression of synaptophysin and postsynaptic density-95 (PSD-95) proteins. Interestingly, in the GDNF-treated group, the number of NSC was augmented, and cell survival was also positively affected by GDNF, as detected by a decrease in TUNEL labeling and caspase-3 expression. The neurotrophic factors BDNF and NT-3 were also detected in the GDNF-treated group. Transplanted NSC in the control group (naive) also promoted improvements, as GDNF-secreting NSC do, but the neuroprotective effects of GDNF-NSC were more drastic than those observed of control NSC (156).

Genetically modified human NSC secreting GDNF were transplanted unilaterally into the spinal cord of a transgenic mutant superoxide dismutase (SOD1 G93A) rat model for amyotrophic lateral sclerosis (ALS). GDNF promoted a remarkable preservation of motor neurons at early and end stages of the disease, but enhanced neuronal survival did not improve ipsilateral limb use, suggesting that additional strategies should be used for maintenance of neuromuscular connections and functional recovery (157). A transgenic mouse model for Huntington's disease, N171-82Q was transplanted with GDNF-secreting NSC derived from mouse striatum. GDNF expressing NSC transplanted mice were able to maintain motor function and showed increased striatal neuronal survival.

Transplantation studies with GDNF-modified human amniotic fluid-derived mesenchymal stem cells (AFMSC) confirmed the ability of GDNF to promote peripheral nerve regeneration. GDNF-modified AFMSC promoted improvement in muscle action potential ratio and motor function. The administration of AFMSC alone also resulted in the same effect, but it was more moderate. Moreover, early regeneration markers, such as neurofilament, had increased expression. In addition, Schwann cell apoptosis was reduced, supporting a neuroregenerative environment promoted by AFMSC and GDNF (158). GDNF-transfected NSC were able to promote sciatic nerve regeneration in rats when seeded in a poly (D,L-lactide) conduit. Thicker myelin sheaths, a higher number of myelinated axons, and a larger area of nerve regeneration were found after GDNF overexpression. Another important aspect for nerve regeneration is the vascularization rescue. The number of blood vessels was significantly increased in the group receiving GDNF-transfected NSC. The regeneration capacity of rat sciatic nerve in the presence of GDNF-transfected NSC was confirmed by histology, functional gait, and electrophysiology (159).

In summary, GDNF promoted neuronal regeneration and survival in vitro (140,160) and can be useful for improving clinical outcome in various animal models of neurological disorders, such as Parkinson disease, spinal cord injury, and ischemia. However, underlying mechanisms for induction of neurogenesis and neuroprotection are not yet elucidated.

Platelet-Derived Growth Factor (PDGF)

PDGF was discovered in the early of 1970s by Russel Ross et al., when they investigated the role played by the smooth muscle cells (SMCs) in the formation of atherosclerotic lesions. It was demonstrated that, in the absence of the endothelium, SMCs migrated and proliferated to form an initial atherosclerotic lesion. These results led Ross to believe that, in any way, the removal of the endothelium facilitated the penetration of certain plasma factors, having an effect on SMCs. Further studies with animals indicated platelets as the source of such factors and the putative factor was named PDGF (161). The PDGF ligand family includes four members (PDGF-A–D). PDGFs are disulfide-linked homo- and heterodimers: PDGF-AA, -AB, -BB, -CC, and -DD. PDGF-A and -B are secreted as active ligands, while C and D ligands, produced as latent factors, are activated under enzymatic cleavage of their N-terminal portion. These PDGF ligands exert their cellular effects by binding to structurally related tyrosine kinase receptors, PDGFR-α and PDGFR-β (162).

Although PDGF has been initially discovered in the platelets, nowadays it is well known that a plethora of cells is able to synthesize, store, and release PDGF, and of particular importance are the effects of this growth factor on embryogenesis and normal CNS development. A growing body of evidence suggests that proliferation, migration, differentiation, and survival processes of NSC are regulated by PDGF and their receptors.

Forsberg-Nilsson et al. demonstrated that cultured NSC from the rat embryonic cortex migrate to take their final position when stimulated by PGDF, which is blocked by incubation with PDGF specific antibodies. This finding suggests a role for PDGF in cell migration in the developing cortex (163). Mice neurospheres lacking PDGFRβ show reduced capacity of migration. Moreover, when PDGFR inhibitor STI571 was added to culture medium, the effects of FGF2 on control neurospheres were blocked. FGF2 increases the activity of the PDGFRB promoter as well as the expression and phosphorylation of PDGFRβ. These data indicate the presence of a cross-talking between PDGF and FGF2, in which the effects of FGF2 in migration and neural differentiation of cells is potentiated by activation of the PDGFRβ (164).

It has been reported that NSC from the embryonic rat cortex proliferate even after removal of growth factors, such as FGF2. Taking into account that these progenitors express PDGF receptors, and produce PDGF-BB during early NSC differentiation, and that cell numbers are reduced in cultures treated with PDGF receptor inhibitors, one may conclude that PDGF is important to maintain progenitor cell division (165). Effects of platelet microparticles on mouse neurospheres are suggested to be mediated by ERK and Akt, both involved in cell proliferation. Since such effects are partially abolished when cells are incubated with an antibody against PDGF, we can once more conclude that cell proliferation is to some extent controlled by PDGF and their receptors (166). The key role of PDGF in cell proliferation is also reinforced by the fact that PDGF-B overexpression causes both GFAP-expressing astrocytes and Nestin-expressing CNS progenitors to proliferate in culture. Furthermore, gene transfer of PDGF in neural progenitors and astrocytes induces the formation of oligodendrogliomas and either oligodendrogliomas or mixed oligoastrocytomas, respectively (167). Similar results are obtained following PDGF treatment of NSC from the adult brain SVZ specifically labeled with PDGFRα. Cell proliferation is induced, leading to hyperplasias resembling gliomas (168).

NSC from the embryonic rat cortex pretreated with PDGF do not complete neuronal differentiation, that is, although they are positive for the neuronal marker β3-Tubulin, they are almost completely devoid of neurites. The same pretreatment does not alter the proportion of both GFAP-positive cells (marker for glial cells) and cells expressing neuronal markers. It should be stressed that only a few oligondendrocytes are detected in comparison with astrocytes, and the latter ones show an immature morphology. However, when NSC cultures treated with PDGF were exposed to additional differentiation factors, like B27, CTNF, and NT3, however, the differentiation proceeded into neurons, astrocytes, and oligodendrocytes. As mentioned above, these cells produce PDGF-BB and, when this action was inhibited, neurons and oligodendrocytes differentiate more rapidly. This points PDGF as an inducer of partial differentiation of NSC (165). Neurospheres from PDGFRβ knockout mice are less responsive to PDGF and, consequently, have a lower differentiation into neurons (164). Hayon et al. also observed that platelet microparticle increases the differentiation of NSC to neurons and glia, which is blocked by specific antibody against PDGFR (166). NSC specifically labeled with PDGFα from the adult brain SVZ act as progenitors of neurons and oligodendrocytes, but not neurogenesis. This suggests that PDGF helps to balance differentiation between neurons and oligodendrocytes (168).

Recent studies demonstrate that PDGF does not only act as a mitogen for progenitors but, also, it reduces apoptosis rates. For instance, it has been reported that PDGF treatment of NSC derived from rat, reduced the number of apoptotic nuclei by half when compared with control measurements (169). Kwon also evaluated the antiapoptotic effect of PDGF by analyzing the presence of phosphatidylserine in the outer surface of NSC by staining with annexin V (phosphatidylserine, almost exclusively located on the inner side of the plasma membrane, appears in the outer surface of the cells at the beginning of apoptosis). It was observed that PDGF treatment abolish staining for annexin V, indicating a decrease in the number of apoptotic cells (170). Taken together, all these works suggest that PDGF is important in the early phase of differentiation process, increasing the number of progenitors and immature neurons, functioning as a mitogen and a protective factor against apoptosis.

Insulin-Like Growth Factor (IGF)

IGFs are polypeptide hormones with potent anabolic and mitogenic effects that control cell proliferation, survival, and differentiation. These factors act by binding to cell-surface heterotetrameric tyrosine kinase receptors and activating multiple intracellular signaling cascades. Two subtypes of IGF receptors have been identified: (I) the IGF-1 receptor

(IGF1R), to which IGF-1 preferentially binds instead of insulin; (II) the IGF-2 receptor (IGF2F), also called mannose-6-phosphate receptor, is devoid of signal transduction capacity, interacting mainly with IGF2, and preventing this from competing with IGF-1 by IGF1R (171).

Recent studies demonstrate that IGF-1 and its receptor play an important role in growth and differentiation of NSC. This is supported by the fact that IGF-binding protein-3, to which IGF-1 is always bound, inhibits the growth of rat NSC and promotes neurogenesis, as indicated by decreasing Nestin expression (172). Choi et al. also demonstrated, via flow cytometry analysis with antibodies against the neuronal markers β3-Tubulin and NeuN, that IGF-1 alone or in combination with other growth factors is able to stimulate the proliferation and differentiation of rat NSC (26). Surprising effects were also obtained in vivo, when animals had been subjected to a peripheral infusion of IGF-1. Such treatment led NSC derived from hippocampus to proliferate and differentiate selectively into neurons (151). On the other hand, multipotent adult rat hippocampus-derived NSC can be stimulated by IGF-1 to differentiate into oligodendrocytes (173). These works suggest an important role of IGF-1 in cell fate determination. IGF-1 may even play a neuroprotective role, in addition to its role as endogenous diffusible factors that mediate postischemic neural progenitor proliferation (174). The suggested role for IGF-1 as a key element in NSC growth is reinforced by the observation that neither EGF nor FGF2 are able to induce proliferation of mouse striatal NSC in the absence of IGF-1 (175).

Although little is known about the mechanisms by which IGF-1 mediates proliferation of NSC, a body of evidence points at participation of Akt. IGF-1 treatment of NSC increased the phosphorylation of Akt, but not of Erk. Moreover, the addition of U0126, a specific inhibitor of Akt, abolished cell survival induced by IGF-1. The same result is not obtained when an inhibitor of Erk is added, confirming once more that IGF-1 effects are mediated by Akt (176). As already mentioned, IGF-1 is also able to induce survival of NSC. This may, to certain extent, be accounted by a protection against apoptosis, as recently published by Gualco et al. The authors have shown that the expression of Survinin, an antiapoptotic protein, is IGF1R-dependent. In contrast to wild-type animals, the embryos of knock-out IGF1R animals with low Survinin levels, revealed increased numbers of apoptotic neurons in a earlier differentiation phenotype and reduced NSC proliferation rates (177).

Novel Proteins Implicated in Neural Stem and Progenitor Cell Proliferation and Phenotypic Characterization

In addition to many well-characterized antigens expressed by NSC (7), recent studies have identified novel markers specifically expressed by neural stem and progenitor cells (Table 1). The population expressing these markers can be screened by multiplex flow cytometry together with cell cycle analysis and proliferation, using BrdU, EdU, propidium iodide (PI), or 5-fluoruracil as DNA stains (178,179). As a result of such assays, implications of these novel markers in proliferation and differentiation of NSC have been suggested as follows. CD44 is a membrane glycoprotein expressed by NSC with importance in adhesion and proliferation. Zhang et al. demonstrated that EGF plays a role induces up-regulation of CD44 expression (180) and later Pollard et al. demonstrated that this increase can also occur after treatment with FGF2 (181). The MAPK/ERK pathway is involved in this effect, since its inhibition reverses EGF-induced upregulation of CD44 expression (182). CD44v6, an alternative splicing form of CD44, also participates in the regulation of cell proliferation, acting as a co-receptor for growth factors (183). Like CD44, expression of this isoform is also subject to upregulation by both IGF and PDGF (184). CD133, also known as Prominin1, is expressed in NSC, but is not known to be present in progenitor cells already committed to a defined neural fate (185). Combined treatment of brain tumor stem cells with EGF and FGF2 augments the expression of CD133, forming aggregates of stem and progenitor cells known as gliospheres, with more than 50% of cells expressing CD133 (186). GD3 is a ganglioside present in the membrane of neural cells. Its expression in NSC is not affected by exposure of cells to EGF (187), nor to PDGF (188). C17.2 immortalized murine NSC expressing recombinant GD3 synthase had its EGF-dependent activation of the Ras-MAPK pathway repressed (189). GD3 synthase-transfected PC12 pheochromocytoma cells exhibited constant activation of the TrkA receptor, independently from the presence of NGF. The increased expression of GD1b and GT1b gangliosides results in conformational changes of the receptor, leading to its dimerization and activation (190). Other gangliosides, like GD2, are also expressed by NSC and can be used as a phenotypic marker to identify these cells (191). Id1 is a nuclear factor that acts as inhibitor of cell differentiation. Treatment of rat SVZ causes an up-regulation of Id1 (192). Expression of Id1 is also increased in FGF15 null mice dorsolateral midbrain (193). Neuroblastoma cells treated with FGF2 showed induced expression of both Id1 mRNA and protein (194). Other markers for NSC or differentiated neural progenies have been recently characterized (Table 1). However, the effects of neurotrophins and growth factors on some of these markers have so far, not been studied.

Conclusion

The regulation of NSC migration, proliferation, differentiation, and cell death is extremely complex. Among a diversity of agents involved in the modulation of these processes, neurotrophins and growth factors play an important role. These molecules affect not only stem cells, but also committed progenitors, as reviewed in Figure 1. Imaging and flow cytometry analysis, combined with other techniques, have been important to uncover these roles, by allowing the characterization of distinct cell populations, according to the expression of neural phenotype-specific markers (Table 1, Fig. 1). A better understanding of the mechanisms underlying the regulation of proliferation, differentiation, and cell death, brings new advances in the neurogenesis field and cell therapy.

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Cell Biology:

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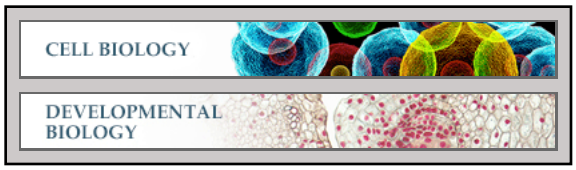

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Interactions between the NO-Citrulline Cycle and Brain-derived Neurotrophic Factor in Differentiation of Neural Stem Cells*

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Background: NO and BDNF are responsible for numerous functions in the CNS; however, joint actions exerted by these factors have not been studied.
Results: BDNF reversed the block on neural differentiation caused by insufficient NO signaling.
Conclusion: The NO-citrulline cycle and BDNF through up-regulation of p75 expression interact for restoring normal NO signaling and promoting neural differentiation.
Significance: New insights are provided for BDNF and NO-citrulline cycle actions in neurogenesis.

The diffusible messenger NO plays multiple roles in neuroprotection, neurodegeneration, and brain plasticity. Argininosuccinate synthase (AS) is a ubiquitous enzyme in mammals and the key enzyme of the NO-citrulline cycle, because it provides the substrate L-arginine for subsequent NO synthesis by inducible, endothelial, and neuronal NO synthase (NOS). Here, we provide evidence for the participation of AS and of the NO-citrulline cycle in the progress of differentiation of neural stem cells (NSC) into neurons, astrocytes, and oligodendrocytes. AS expression and activity and neuronal NOS expression, as well as L-arginine and NO_x production, increased along neural differentiation, whereas endothelial NOS expression was augmented in conditions of chronic NOS inhibition during differentiation, indicating that this NOS isoform is amenable to modulation by extracellular cues. AS and NOS inhibition caused a delay in the progress of neural differentiation, as suggested by the decreased percentage of terminally differentiated cells. On the other hand, BDNF reversed the delay of neural differentiation of NSC caused by inhibition of NO_x production. A likely cause is the lack of NO, which up-regulated p75 neurotrophin receptor expression, a receptor required for BDNF-induced differentiation of NSC. We conclude that the NO-citrulline cycle acts together with BDNF for maintaining the progress of neural differentiation.

The gaseous messenger NO has been widely studied because of its significant importance for human cell physiology. Understanding the role of NO in the development of CNS has been a complex task because the analysis of NO accumulation in cell lines does not mimic the large diversity of neural phenotypes in the brain (1). The CNS develops from a specific set of precursor cells that divide in order to form the neural epithelium, migrate to appropriate niches, and differentiate into glia or various neuronal phenotypes. Neural stem cells (NSC)[4] can be grown *in vitro* as neurospheres and still retain their multipotent capacity (2–5). Under controlled experimental conditions, NSC proliferate, migrate, differentiate, and form neural networks, closely reflecting conditions of cortical development. NSC are useful for studying brain development and provide promising tools for cell therapy of neurodevelopmental and neurodegenerative diseases (reviewed in Ref. 5). Here, we have used neurospheres obtained from embryonic rat telencephalon (embryonic day 14) to investigate the roles of NO and enzymes of the NO-citrulline cycle in neural differentiation.

NO is a molecule generated from L-arginine by the action of the enzyme NO synthase (NOS). There are three NOS isoforms: NOS1 or neuronal NOS (nNOS), which is primarily found in the brain, although its expression is also observed in skeletal muscle and other tissues; NOS2 or inducible NOS; and NOS3 or endothelial NOS (eNOS), which was first observed in endothelial cells but is also present in other cell types (reviewed in Ref. 6). L-Arginine is the only substrate of NOS for NO synthesis. This semiessential amino acid can be directly obtained from the breakdown of dietary proteins, but it is also *de novo* synthesized from L-citrulline (reviewed in Ref. 7).

L-Citrulline is metabolized to L-arginine involving the enzymes argininosuccinate synthase (AS, EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1). AS is responsible for the condensation reaction between L-citrulline and L-aspartate in

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[4] The abbreviations used are: NSC, neural stem cell(s); AS, argininosuccinate synthase; GFAP, glial fibrillary acidic protein; MAP-2, microtubule-associated protein 2; NOS, nitric-oxide synthase; eNOS, endothelial NOS; nNOS, neuronal NOS; L-NAME, L-N^{γ}-nitroarginine methyl ester; MDLA, α-methyl-DL-aspartic acid; 7-Ni, 7-nitroindazole; p75NTR, p75 neurotrophin receptor.

TABLE 1
Primers used for real-time PCR

Genes	Primers	
	Forward (5′–3′)	Reverse (5′–3′)
Nestin	TGGAGCGGGAGTTAGAGGCT	ACCTCTAAGCGACACTCCCGA
β3-tubulin	AGACCTACTGCATCGACAATGAAG	GCTCATGGTAGCAGACACAAGG
GFAP	AAGAGTGGTATCGGTCCAAGTTTG	CAGTTGGCGGCGATAGTCAT
MAP-2	GTTTACATTGTTCAGGACCTCATGG	TCGGTAAGAAAGCCAGTGTGGT
eNOS	AAAATGAGCAGAAGGCCA	TTTTGCTGCACTTTTCCTTTC
nNOS	CAGCCAAAGCAGAGATGA	ATTGAAGACGCGGTCATT
AS	TGCACTCTATGAGGACCGCTATC	CTAGGCACCTCTCTCGCCAGGCCT
BDNF	CAACATCGATGCCAGTTGCT	TCCGCAAGCTTCAACTCTCA
p75NTR	CGACCAGCAGACCCATACG	GGCTACTGTAGAGGTTGCCATCA
GAPDH	TGGCCTCCAAGGAGTAAGAAA	GGCCTCTCTCTTCCTCTCAGTATC

an ATP-dependent manner in order to form argininosuccinate, AMP, and pyrophosphate; argininosuccinate lyase catalyzes the conversion of argininosuccinate into fumarate and L-arginine, which is then metabolized by NOS into NO and L-citrulline, closing the NO-citrulline cycle (8). AS expression increases during brain development (9), suggesting a possible function for the enzyme in this process. In addition, AS activity has been described as a limiting step for the biosynthesis of NO in numerous tissues (10). Among the many functions of NO (11), this transcellular-signaling molecule regulates proliferation of NSC (12) by acting cytostatically on cell division, which is a prerequisite for cells to enter a program of differentiation (13). It is also known that in spinal cord development, interneurons express NOS during migration to their final destination (14). However, the exact functions of NO and the NO-citrulline cycle enzymes in brain development remain unknown.

We hereby show that AS and nNOS are differentially expressed along differentiation and that L-arginine as well as intracellular and extracellular NO levels, follow up the appearance of neural phenotypes. Moreover, we have observed that eNOS expression was induced by NOS inhibition, indicating that this isoform is amenable to modulation by extracellular cues. Inhibition of NOS and AS enzymatic activities prevented final differentiation, indicating the importance of correct working of the NO-citrulline cycle for the progress of neural differentiation. Nevertheless, no neuroanatomical alterations were detected in adult nNOS or eNOS knock-out mice (15, 16). This discrepancy might be explained by the existence of physiological and biochemical compensation mechanisms. Based on previous observations that BDNF and NO play similar biological activities in the brain (17, 18), and NO inhibits BDNF release (19), we have now investigated the role of BDNF in reverting effects caused by interruption of the NO-citrulline cycle.

Here we show that BDNF reverses the block of neural differentiation caused by insufficient NO signaling. We suggest that BDNF-mediated effects may be mediated by the p75 neurotrophin receptor (p75NTR), whose expression was up-regulated as consequence of inhibition of NO production. Taken together, we present evidence that the NO-citrulline cycle and BDNF interact for promoting neural differentiation.

EXPERIMENTAL PROCEDURES

Isolation, Culture, and Differentiation of NSC—NSC were obtained by dissection of embryonic telencephalons (embryonic day 14) of Wistar rats or telencephalons (embryonic day 13) of C57BL/6 mice representing similar stages of neuronal development. The animals were housed in the animal facility of the Instituto de Química of the Universidade de São Paulo and sacrificed in a CO_2 gas chamber using protocols reviewed and approved by the local ethics committee. Telencephalons were dissected under a stereo microscope in aseptic conditions followed by incubation with trypsin for 10 min at 37 °C. Then an equal volume of FBS was added for inactivation of trypsin, and the cells were mechanically dissociated in order to obtain a single cell suspension. Cell viability was evaluated by trypan blue staining (Invitrogen). Cells were plated at a density of 2×10^5 cells/ml in culture medium containing 2% (v/v) B-27 (Life Technologies), 98% (v/v) DMEM/Ham's F-12 medium, 20 ng/ml EGF (Sigma-Aldrich), 20 ng/ml FGF-2 (Sigma-Aldrich), 5 μg/ml heparin (Sigma-Aldrich), and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and cultured at 37 °C in a water-saturated atmosphere and 5% of CO_2. For induction of neural differentiation, neurospheres were plated onto adherent poly-L-lysine- and laminin-precoated cell culture grade dishes and cultured in the absence of EGF and FGF-2. The medium was changed every 2 days. Under these experimental conditions, enrichment of neurons and glias in the culture was confirmed by immunofluorescence staining against β3-tubulin and glial fibrillary acidic protein (GFAP) on day 7 of differentiation (20). On day 14, peak values of expression of proteins characteristic for mature neurons, such as microtubule-associated protein 2 (MAP-2), and for astrocytes, such as GFAP, were observed, whereas expression of Nestin, a marker protein for NSC and neural progenitor cells, was decreased, being in agreement with the conversion of undifferentiated cells into defined neural phenotypes (20). For studying the role of NO-citrulline cycle during differentiation, NSC were also cultured in the presence of substrates of NOS or AS (1 mM L-arginine or 1 mM L-citrulline), or 1 mM L-N^{γ}-nitroarginine methyl ester (L-NAME), 1 mM α-methyl-DL-aspartic acid (MDLA), or 1 μM 7-nitroindazole (7-Ni), inhibiting all isoforms of NOS, AS, or nNOS, respectively. In other experiments, NSC were cultured in the presence of 1 mM L-NAME and 20 ng/ml BDNF or 1 mM MDLA and 20 ng/ml BDNF, concomitantly, or with 20 ng/ml BDNF alone. The drugs were newly supplied every day along differentiation.

Real Time Polymerase Chain Reaction—Total RNA was extracted from NSC using TRIzol (Invitrogen). All samples were further treated with amplification grade DNase I (Sigma-Aldrich). Reverse transcription for cDNA synthesis was carried out with a thermal cycler using the SuperScript III first

strand synthesis system according to the manufacturer's protocol (Invitrogen) in the presence of specific primers listed in Table 1. The transcription rates of selected mRNAs were measured by real time PCR using the ABI Step One Plus instrument (Life Technologies). Real time PCR was performed in 25 μl of buffer reaction containing 1 μl of cDNA, SYBR Green Master Mix (Life Technologies), and 5 pmol of each sequence-specific primers (Table 1). Thermal cycling conditions consisted of a preincubation step for 2 min at 50 °C, then denaturation for 10 min at 95 °C followed by 40 cycles for denaturation for 15 s at 95 °C, and annealing/extension for 1 min at 60 °C. The comparative $2^{-\Delta\Delta CT}$ method was employed for relative quantification of gene expression as described previously (21) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression as an internal standard for normalization.

AS Activity Assay—AS activity was determined based on accumulation of the product pyrophosphate as inorganic phosphate following cleavage by pyrophosphatase. After lysis of NSC by heat shock, 50 μg of total proteins were used for measuring enzymatic activity of AS. Samples were added to the reaction buffer (20 mM Tris-HCl, pH 7.8, 2 mM ATP, 2 mM citrulline, 2 mM aspartate, 6 mM $MgCl_2$, 20 mM KCl, and 0.2 units of pyrophosphatase) to a final volume of 0.2 ml. Reactions were incubated at 37 °C in 96-well microtiter plates and stopped after 30 min by the addition of an equal volume of molybdate buffer (10 mM ascorbic acid, 2.5 mM ammonium molybdate, 2% (v/v) sulfuric acid). Accumulation of phosphate was determined spectrophotometrically at 650 nm, and concentrations were extrapolated from a standard curve of inorganic phosphate (22).

Determination of L-Arginine Concentration—The methodology used to measure total L-arginine levels is described elsewhere (23). Both extra- and intracellular media of NSC were collected for determination of L-arginine concentration by reversed phase high performance liquid chromatography (HP 1100 series HPLC) with amino acid detection analysis following sample separation on a C18 analytical column (250-mm length, 4.6-mm diameter, 5-μm particle size; Merck).

Chemiluminescence Assay for Detection and Quantification of Nitric Oxide Products—NO_x (nitrate, nitrite, nitrosothiol, nitrosamines, and iron-nitrosyl complexes) concentration in extracellular and intracellular media of NSC were determined using a chemiluminescence Sievers nitric oxide analyzer (NOA280i; GE Analytical Instruments) according to the procedure optimized by Feelisch *et al.* (24). Intracellular media were obtained following cell lysis with radioimmune precipitation assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM diethylene triamine pentaacetic acid (DTPA), and 10 mM *N*-ethylmaleimide) and centrifugation as previously described (25). Extra- and intracellular media of NSC were directly injected into a vessel containing a saturated solution of vanadium (III) chloride in 1 M HCl at 90 °C. Under these conditions, all nitric oxide-derived products (NO_x) were reduced and compared with those of standard nitrate solutions (24).

Flow Cytometry Analysis of Neural Marker Proteins and p75NTR Expression—Flow cytometry procedures were in agreement with previously published protocols (5, 20). NSC were detached from the flasks using trypsin, then centrifuged for 5 min at 200 × *g*, and dissociated to provide a single cell suspension. The cells were fixed for 20 min in ice-cold 1% (v/v) formaldehyde in PBS, washed with PBS supplemented with 2% (v/v) FBS, and incubated for 2 h with primary antibodies specific for neural markers β3-tubulin (Sigma-Aldrich), GFAP (DAKO), and Nestin (Millipore) at 1:500 dilutions in 0.05%

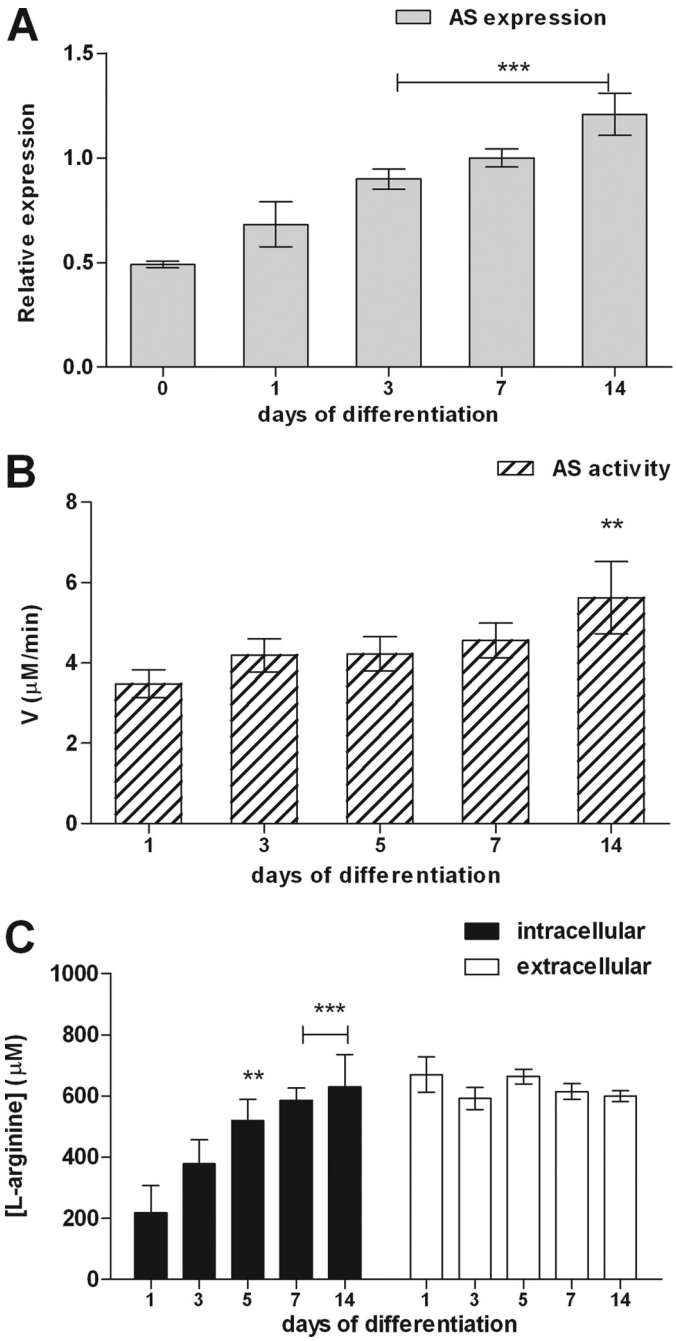

FIGURE 1. **Expression and activity of AS along neural differentiation.** *A*, AS gene expression in neurospheres was determined by real time PCR. Normalization of expression levels was done by comparison with GAPDH RNA transcription levels as internal standard for gene expression. *B*, determination of AS enzymatic activity of neurospheres along differentiation using a colorimetric assay. *C*, quantification of intra- and extracellular L-arginine levels during neurosphere differentiation. Cell culture supernatants and intracellular contents were collected and analyzed for L-arginine by HPLC. The experimental data are presented as the mean values ± S.E. **, $p < 0.01$; ***, $p < 0.001$, compared with control data collected on day 0 or 1 of differentiation.

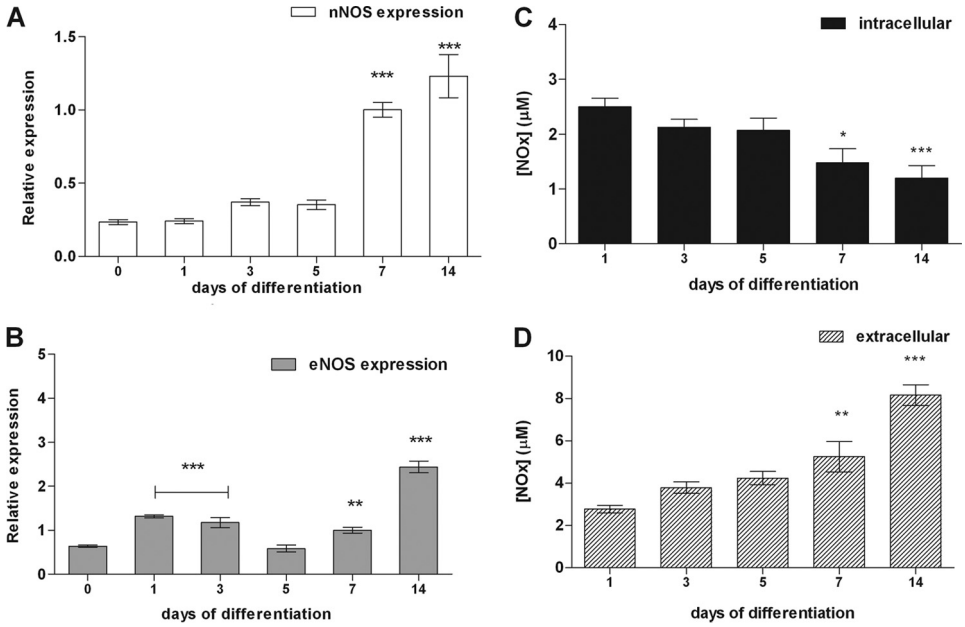

FIGURE 2. **Gene expression of eNOS and nNOS and NO metabolite (NO_x) production along neurosphere differentiation.** *A* and *B*, nNOS (*A*) and eNOS (*B*) gene expression changes in neurospheres cultures from days 0–14 following induction to neural differentiation were analyzed by real time PCR. Normalization of expression levels was done by comparison to GAPDH gene expression. The data are shown as the mean values ± S.E. of three independent experiments. *C* and *D*, cells in differentiation were lysed with radioimmune precipitation assay buffer for measuring NO_x contents in intracellular (*C*) and extracellular media (*D*) by a chemiluminescence assay. The culture medium of neurospheres was changed 1 day before the collection of intra- and extracellular media. The values of concentration of NO_x in the basal media used for measurement of nitric oxide were subtracted from the samples collected during NSC differentiation. In addition, we have normalized the production of these metabolites to the protein concentration of the culture. NO concentrations are expressed as mean values ± S.E. of six independent experiments. *, $p < 0.01$; **, $p < 0.01$; ***, $p < 0.001$, compared to control data collected on days 0 or 1 of differentiation.

(v/v) Triton X-100. For determination of p75NTR expression, NSC were incubated with a primary antibody anti-NGFR p75 C-20 (Santa Cruz) at 1 μg/1 $\times$ 10^6 cells in PBS supplemented with 2% (v/v) FBS. Following a washing step with PBS, cells were incubated with 1:500 Alexa Fluor 488$^-$ or 555$^-$ conjugated secondary antibodies (Invitrogen) and then analyzed with a flow cytometer (Fc500; Beckman Coulter).

An argon laser line was used for fluorescence excitation (emission wavelengths of FL1, 525 nm, and FL2, 575 nm, were defined by band pass filter). Thirty-thousand events were acquired per sample with fluorescence emission values measured in logarithmic scales. Background fluorescence was determined using unlabeled cells and cells labeled with secondary antibody alone and used to set gating parameters between stained and unstained cell populations. Forward and side light scatter gates were set to exclude cell aggregates and small debris. The data were analyzed using the Flowjo 7.6.4 software (Ashland, OR).

Immunofluorescence Staining Assay—Imunocytochemistry assays were performed according to Ref. 26. Briefly, the cells were blocked for 1 h with 3% (v/v) FBS in PBS, 0.1% (v/v) Triton X-100, followed by a 2-h incubation with primary antibodies against β3-tubulin (Sigma-Aldrich), Nestin (Millipore), and GFAP (DAKO) at 1:500 dilution. NSC were washed with PBS and anti-mouse Alexa 555-conjugated, or anti-rabbit Alexa 488-conjugated secondary antibodies (Invitrogen) at 1:500 dilution were added. After washing with PBS, DAPI solution (Sigma-Aldrich; 0.3 μg/ml) was used as a nuclear stain. Coverslips were mounted, and slides were analyzed under a fluorescence microscope (Axiovert 200, Zeiss, Jena, Germany).

BrdU Incorporation Assay—Cell proliferation was measured following incubation with 0.2 μM 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) for 12 h. The cells were fixed with ice-cold methanol for 10 min, washed with PBS, and incubated for 30 min in 1.5 M HCl. After washing with PBS, they were incubated for 2 h with rat anti-BrdU antibodies (Abcam; 1:200 dilution). Alexa Fluor-488 secondary antibodies (Invitrogen) were used at 1:500 dilution. After another washing step, DAPI solution (Sigma-Aldrich; 0.3 μg/ml) was used as a nuclear stain. Slides were mounted and analyzed under a fluorescence microscope (Axiovert 200, Zeiss). The percentages of BrdU-positive cells were calculated as the ratio of immunolabeled cells over the total number of DAPI-stained cells.

Determination of Protein Expression Levels by Western Blotting—Following lysis of NSC in radioimmune precipitation assay buffer, samples were incubated on ice for a period of 20 mins and centrifuged for 5 min, at 21,000 $\times$ *g* and the supernatant were stored at −80 °C. After quantification of protein concentration, 50 μg of each protein sample was separaed by SDS-PAGE (10%). Proteins were transferred onto nitrocellulose membranes in 0.38 M Tris-HCl, 0.18 M glycine, and 20% (v/v) methanol under constant voltage of 30V for 12 h. The membrane was incubated in 5% (w/v) BSA dissolved in TBS-Tween (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl and 0.05% (v/v) Tween 20). After three washes with TBS-Tween-20, membranes were incubated for 2 h with primary antibodies against eNOS, β3-tubulin, and GFAP. Next, the membrane was incubated with the secondary antibody conjugated to alkaline phosphatase (Promega Corp., Madison, WI) and revealed with alkaline phosphatase solution (5 M NaCl, 1 M Tris-HCl, pH 9.5, 1 M $MgCl_2$ in the presence of

0.02% (w/v) 5-bromo-4-chloro-3-indolyl phosphate and 0.03% (w/v) nitro blue tetrazolium).

Statistical Analysis—Comparisons between experimental data were made by one- or two-way analysis of variance following the Bonferroni post-test using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). The criteria for statistical significance were set at $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***).

RESULTS

Expression and Activity of AS during Neural Differentiation—The role of AS as step-limiting enzyme in the synthesis of substrate for NOS (L-arginine) was studied along differentiation of NSC. Real time PCR experiments revealed increasing AS expression during differentiation. Peak values were observed on day 14 when cells were completely differentiated (2.5-fold increase compared to expression on day 0) (Fig. 1*A*). In agreement with these results, AS enzymatic activity also augmented during differentiation with peak values of 5.62 ± 0.90 μM/min compared to 3.48 ± 0.34 μM/min at the onset of differentiation (day 1) (Fig. 1*B*). Augmented expression and enzymatic activity of AS was accompanied by an increase in intracellular levels of L-arginine from 217.8 ± 88.9 μM in undifferentiated cells reaching maximal levels of 630.5 ± 104.6 μM on day 14 of differentiation, whereas extracellular L-arginine levels remained constant along the course of differentiation (Fig. 1*C*).

Detection of nNOS and eNOS Expression and NO Production during Neural Differentiation—Gene expression of nNOS and eNOS and NO production were detected in undifferentiated NSC and along differentiation into neural phenotypes (Fig. 2). However, inducible NOS could not be detected by Western blot analysis during NSC differentiation (data not shown), in agreement with previous findings (27) excluding inducible NOS as participant in NO production during NSC differentiation. Increased expression of nNOS (Fig. 2*A*) accompanies rising levels of the neural markers β3-tubulin and GFAP during ongoing differentiation (5, 26). Different from nNOS, the eNOS expression pattern was partially uniform throughout the days of differentiation, with the highest values on day 14 (Fig. 2*B*).

NO is a gaseous molecule with short half-life, because it is quickly converted into nitrate, nitrite, nitrosothiols, nitrosamines, and nitrosylated compounds (NO_x) (24). Intra- and extracellular NO_x levels in NSC at different days of differentiation were measured in gaseous phase by a chemiluminescence assay (28). Intracellular NO concentrations decreased from 6.9 ± 0.7 μM on the onset of differentiation to 2.3 ± 0.3 μM on day 14 (Fig. 2*C*), while extracellular NO_x concentration ranged from 1.6 ± 0.4 on day 1 to 3.4 ± 0.2 μM on day 14 of differentiation (Fig. 2*D*).

Involvement of the NO-Citrulline Cycle in the Progress of Neural Differentiation—The role of the NO-citrulline cycle in neural differentiation was assessed by treating NSC with L-citrulline or L-arginine, which are AS and NOS substrates, respectively, until day 7 of differentiation. Expression levels of neural markers were compared to those of neurospheres differentiated in the absence of these compounds. L-Arginine promoted NSC differentiation, as shown by 25 and 35% increases in β3-tubulin and GFAP expression, respectively. Chronic

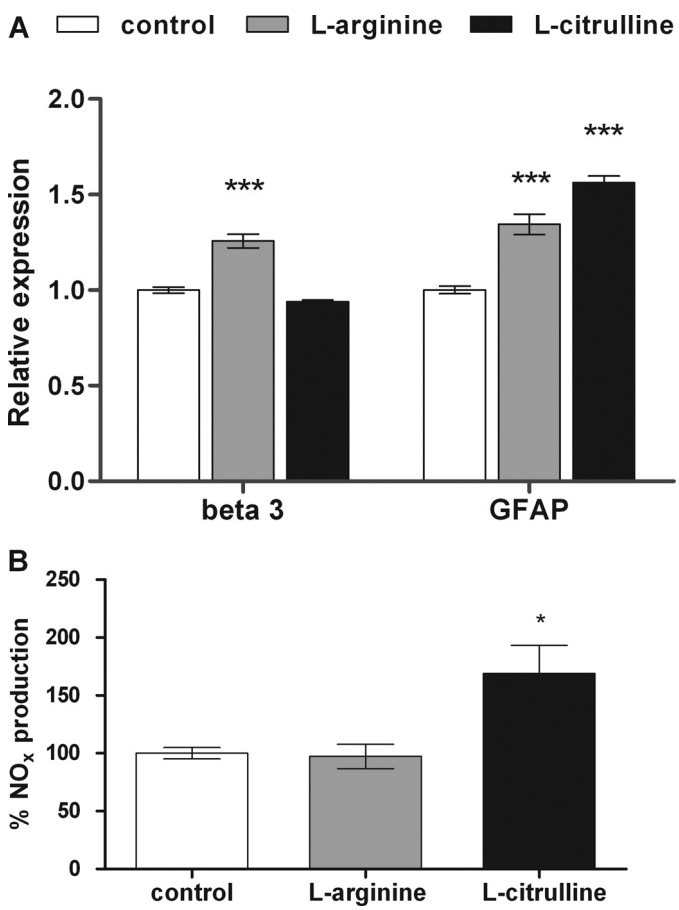

FIGURE 3. **Interference of NO-citrulline cycle intermediates with neurosphere differentiation.** *A*, relative gene expression of specific markers for differentiating neurons (β3-tubulin abbreviated as beta) and glia (GFAP) of L-arginine- or L-citrulline-treated neurospheres on day 7 of differentiation were determined by real time PCR. Neurospheres were maintained in culture until day 7 of differentiation in the presence of 1 mM L-arginine, a natural NOS substrate, or 1 mM L-citrulline, an AS substrate, which were newly supplied every day. The culture medium was changed every 2 days. Nontreated differentiated cells were used as control. *B*, NO production of neurospheres differentiated in the absence or presence of L-arginine- or L-citrulline on day 7 was measured by a chemiluminescence assay as described under "Experimental Procedures." NO production of nontreated cells was considered as 100%. The shown data are mean values ± S.E. *, $p < 0.05$; ***, $p < 0.001$, compared to control data.

treatment of NSC with L-citrulline augmented GFAP expression by 56%, whereas β3-tubulin expression did not change (Fig. 3*A*). In addition, total NO_x production by L-arginine- and L-citrulline-treated NSC was measured on day 7 of differentiation. Increases of 70% in NO_x levels were detected in NSC differentiated in the presence of L-citrulline, whereas NO levels of L-arginine-treated differentiated NSC were equal to those of control cells (Fig. 3*B*). Treatment of cells with inhibitors of AS and NOS enzymes resulted in inhibition of the progress of neural differentiation, measured as alterations in neural marker expression. Both L-NAME- and MDLA-treated NSC along differentiation revealed about 30 (Fig. 4*A*) and 6 times increased Nestin expression (Fig. 4*B*), respectively, when compared to cells differentiated in the absence of these drugs. Expression levels of neuronal β3-tubulin and glial GFAP in differentiated cells were drastically reduced by both treatments, whereas expression rates of MAP-2 were not affected. Flow cytometry analysis revealed changes in the percentage of Nestin-, β3-tu-

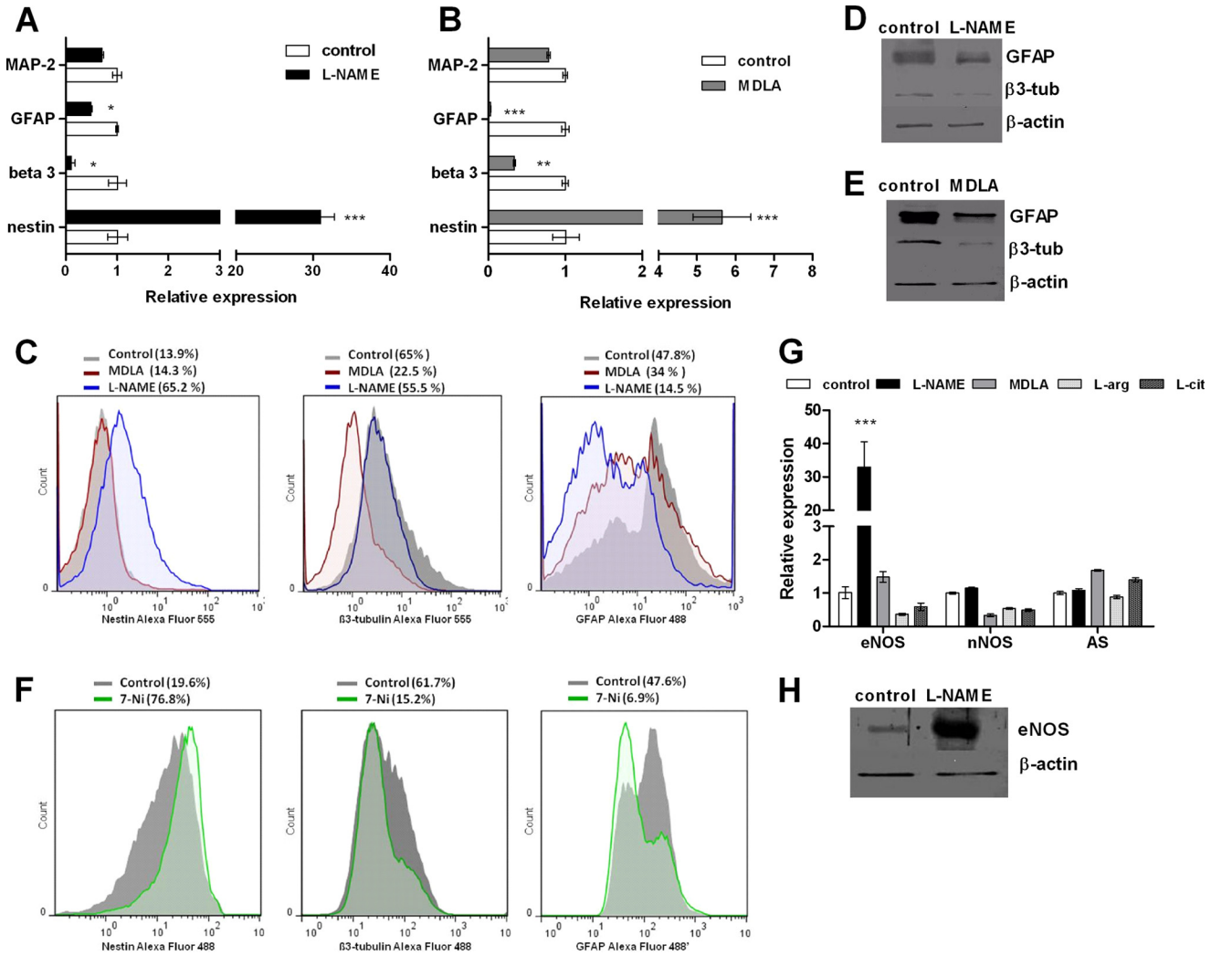

FIGURE 4. **Interference of NO inhibition with neurosphere differentiation.** *A* and *B*, gene expression levels of specific markers for mature neurons (MAP-2), glia (GFAP), differentiating neurons (β3-tubulin), and progenitor cells (nestin) of L-NAME-treated (*A*) and MDLA-treated (*B*) neurospheres on day 7 of differentiation were determined by real time PCR. Neurospheres were maintained in culture until day 7 of differentiation in the absence or presence of 1 mM L-NAME, a nonselective antagonist of NOS, or 1 mM MDLA, an inhibitor of AS, which were newly supplied every day. The medium was changed every 2 days. Cells differentiated in the absence of these compounds were used as control. *C*, flow cytometry analysis of Nestin, β3-tubulin, and GFAP expression in neurospheres differentiated for 7 days in the absence or presence of 1 mM MDLA or 1 mM L-NAME. Representative histograms compare expression levels of neural markers in differentiated neurospheres (*gray*) with neurospheres treated with MDLA (*red*) or L-NAME (*blue*). *D* and *E*, immunoblots of protein extracts from NSC on day 7 of differentiation cultures in the absence or presence of 1 mM L-NAME (*D*) or 1 mM MDLA (*E*) were probed for GFAP and β3-tubulin expression levels. *F*, flow cytometry analysis of Nestin, β3-tubulin, and GFAP expression in murine neurospheres differentiated for 7 days in the presence of 1 μM 7-Ni. Representative histograms compare expression levels of neural markers in untreated neurospheres (*gray*) and treated with 7-Ni (*green*). *G*, regulation of AS, eNOS and nNOS expression in differentiated neurospheres (day 7) in the presence of activators and inhibitors of the NO-citrulline cycle. *H*, Western blot analysis was performed to confirm increased eNOS expression in L-NAME-treated neurospheres. The data shown are representative for at least two independent experiments. The data are presented as the mean values ± S.E. ***, $p < 0.001$. *β-tub*, β-tubulin, *L-cit*, L-citrulline.

bulin-, and GFAP-positive cells from the group treated with L-NAME and MDLA when compared to cells differentiated in the absence of these inhibitors. Expression of nestin was detected in 13.9% of untreated neurospheres and in 14.3 and 65.2% of MDLA and L-NAME-treated neurospheres, respectively, on day 7 of differentiation. A slight decrease in β3-tubulin expression (from 65 to 55.5%) and a marked reduction of GFAP-expressing cells (from 47.8 to 14.5%) were observed following chronic treatment with L-NAME along differentiation. The presence of MDLA during differentiation led to a pronounced reduction of β3-tubulin-expressing cells (from 65 to 22.5%), whereas GFAP expression only slightly decreased (from 47.8 to 34%) (Fig. 4*C*). Western blot analysis of β3-tubulin and GFAP also confirmed the decreased expression of neural markers in cells differentiated in the presence of L-NAME (Fig. 4*D*) or MDLA (Fig. 4*E*). Because L-NAME inhibits both NOS isoforms, we have used 7-Ni to identify which isoform is involved in the delay on neural differentiation caused by deficiency in the NO production. The results of flow cytometry analysis showed that nNOS inhibition during differentiation caused changes in the percentage of Nestin-, β3-tubulin-, and GFAP-positive cells, similarly to those observed in the presence of MDLA and L-NAME. The chronic treatment of neurospheres with 7-Ni led to an increase of Nestin-expressing cells (from 19.6 to 76.8%), a decrease in β3-tubulin expression (from 61.7 to 15.2%), and a reduction in GFAP-expressing cells compared to untreated neurospheres (from 47.6 to 6.9%) (Fig. 4*F*).

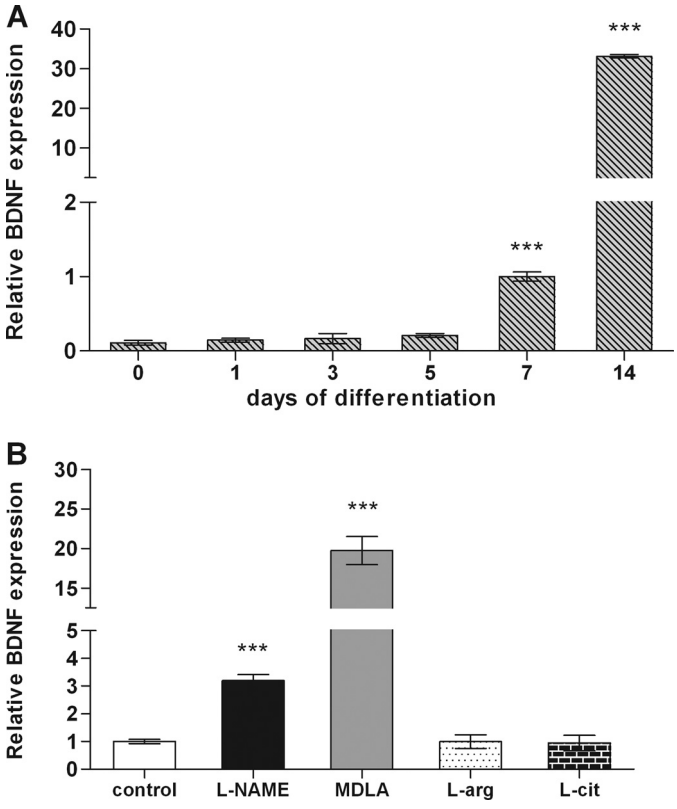

FIGURE 5. **Differential BDNF expression during neurosphere differentiation.** For real time PCR experiments, neurospheres were cultured in the absence or presence of 1 mM of L-NAME, 1 mM MDLA, 1 mM L-arginine, or 1 mM L-citrulline and collected on different days of differentiation on day 7 of differentiation. *A*, BDNF gene expression along neurospheres differentiation. *B*, BDNF expression changes in neurospheres treated during 7 days of differentiation with inhibitors or substrates of NO-citrulline cycle enzymes. The obtained data are shown as the mean values ± S.E. of three independent experiments. ***, $p < 0.001$. *L-arg*, L-arginine; *L-cit*, L-citrulline.

Gene Expression of AS, nNOS, and eNOS in Neurospheres Treated with Substrates and Inhibitors of the NO-Citrulline Cycle—Gene expression of AS, nNOS and eNOS was studied in neurospheres treated with substrates and inhibitors of enzymes of the NO-citrulline cycle. Cells treated during differentiation with the NOS inhibitor L-NAME for a period of 7 days revealed about 30 times elevated eNOS expression compared to untreated cells, whereas nNOS and AS expression did not change under these conditions (Fig. 4, *G* and *H*). Exposure of cells to the NOS substrate L-arginine, the AS substrate L-citrulline, or MDLA, a competitive inhibitor of AS, during differentiation, did not affect the expression of any of the analyzed enzymes of the NO-citrulline cycle (Fig. 4*G*).

Up-regulation of Gene Expression of BDNF in Differentiating Neurospheres and in Neurospheres Treated with Inhibitors of the NO-Citrulline Cycle—Evidence from *in vitro* studies suggests crucial functions for BDNF in neurogenesis (29). Real time PCR analysis in neurospheres during differentiation revealed that gene expression of BDNF was up-regulated beginning from day 7 of differentiation. Peak values of BDNF expression were reached on day 14 with a 30-fold increase compared to expression levels on day 7 (Fig. 5*A*). Treatment of cells with the NOS inhibitor L-NAME and the AS antagonist MDLA during differentiation induced BDNF expression by factors of 3- and 20-fold, respectively, whereas chronic exposure of differentiating cells to L-arginine or L-citrulline did not evoke any changes in BDNF expression (Fig. 5*B*).

Effects of BDNF on Neurosphere Differentiation—In the present work, BDNF by itself did not affect NSC differentiation; *i.e.*, neurogenesis was not promoted by this factor such as reported in previous studies (30). However, we have verified that BDNF reversed the delay in NSC differentiation caused by inhibition of NO production. Immunofluorescence studies revealed reduced expression of GFAP and β3-tubulin in neurospheres treated along differentiation with MDLA or L-NAME (Fig. 6*A*). The concomitant treatment of NSC with BDNF and L-NAME or BDNF and MDLA did not affect the progress of neural differentiation when compared to control cells differentiated in the absence of these compounds. In agreement, real time PCR studies confirmed increased nestin and decreased GFAP and β3-tubulin expression in neurospheres differentiated in the presence of L-NAME and MDLA. Expression levels of β3-tubulin in cells differentiated in the presence of BDNF or of both BDNF and L-NAME were identical to those observed in NSC differentiated without any of these compounds (Fig. 6*B*). MAP-2 expression levels were not affected in any of the mentioned experimental conditions (Fig. 6).

Effects of Endogenous NO and BDNF on Cell Proliferation and Migration—Effects of L-NAME, MDLA, and BDNF on cell proliferation were evaluated. To this end, neurospheres were differentiated for 7 days in the presence of one of those. L-NAME and MDLA induced proliferation rates were about 50% higher than those of untreated control neurospheres, whereas BDNF-treated neurospheres revealed proliferation levels similar to those of untreated cells (Fig. 7*A*). Co-treatment with BDNF and inhibitors of NO production (L-NAME or MDLA) (31, 32) re-established normal proliferation levels. Neurosphere migration, a prerequisite for neurogenesis, was also inhibited by L-NAME and MDLA, and such as in the proliferation assay, BDNF reversed this effect caused by inhibition of NO production (Fig. 8).

Involvement of p75NTR in the Effect of BDNF on Differentiating Neurospheres—Experimental evidence indicates that many growth factors including BDNF play an important role in regulating proliferation and differentiation of NSC. However, the effects of BDNF on neurogenesis have not yet been fully elucidated (30, 33). In this context, it is known that p75NTR defines the population of NSC responsive to BDNF (34). Therefore, we have measured the relative expression of p75NTR in untreated and treated NSC on day 7 of differentiation. In fact, treatment with MDLA or L-NAME increased p75NTR gene expression 2- and 3-fold, respectively (Fig. 9*A*). Moreover, flow cytometry analysis revealed a 31% increase of p75NTR-expressing cells when compared to untreated cells, providing a mechanism for BDNF-driven reversion of inhibition of neural differentiation as consequence of impaired NO production (Fig. 9*B*).

DISCUSSION

Evidence collected in many studies indicates crucial effects of NO signaling in promotion of neural differentiation (reviewed in Ref. 35); however, excessive levels of NO can be deleterious to

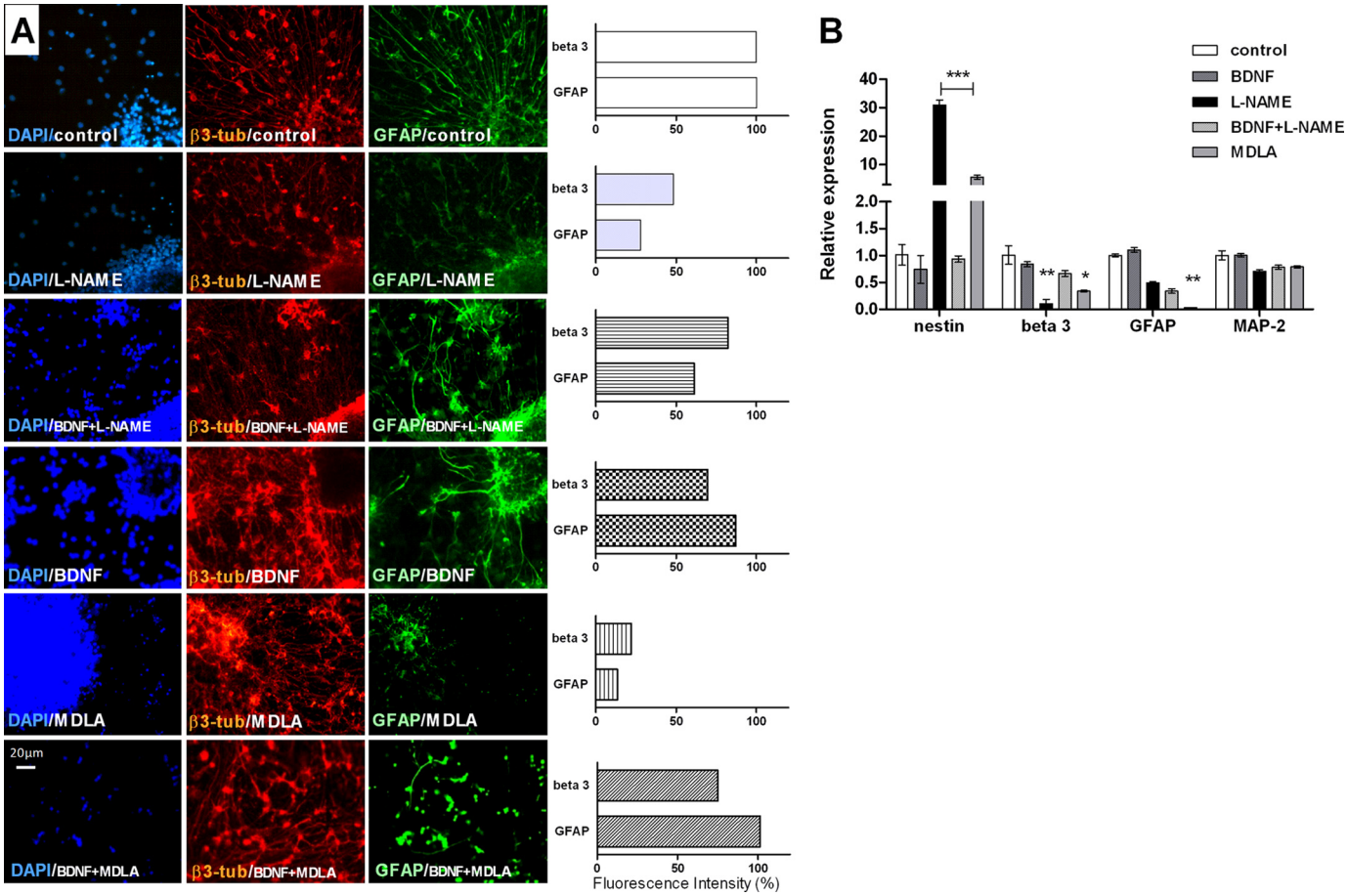

FIGURE 6. **BDNF-mediated reversion of neurosphere differentiation caused by inhibitors of NO-citrulline cycle.** *A*, immunostaining of neurospheres differentiated in the presence of 1 mM L-NAME, 1 mM MDLA, 20 ng/ml BDNF, 20 ng/ml BDNF and 1 mM L-NAME, or 20 ng/ml BDNF and 1 mM MDLA, for β3-tubulin (β-tub) and GFAP expression. *Scale bar*, 20 μm. The data were analyzed using the NIS Elements software (Nikon) and represented as the ratio of β3-tubulin or GFAP fluorescence intensity over DAPI fluorescence intensity. *B*, analysis of gene expression of specific markers for mature neurons (MAP-2), glia (GFAP), differentiating neurons (β3-tubulin, beta 3), and progenitor cells (Nestin) on day 7 of neurosphere differentiation by real time PCR. The data are shown as the mean values ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with control experiments obtained with cells differentiated without any of these compounds.

the organism. In the NO-citrulline cycle, NOS is the enzyme that synthesizes NO from L-arginine, whereas AS is a step-limiting enzyme in the supply of this substrate for NOS. Therefore, AS has been considered as such important as NOS in the process of NO formation, because the regulation of L-arginine supply may be of pivotal importance for a delicate balance of NO benefiting physiological actions and avoiding the induction of pathological events. In the present work, we have addressed the role of the NO-citrulline cycle in regulating neurogenesis and gliogenesis.

We show here that nNOS expression progressively increased throughout NSC differentiation, in agreement with previous studies describing the pattern of nNOS gene expression in developing rat cerebral cortex (36). We also observed that the enzymatic activity of AS, as well as its expression, increased during differentiation, augmenting the concentration of intracellular L-arginine, concomitantly with the onset of *in vitro* differentiation of NSC into neural phenotypes. These data are in line with the work of Husson *et al.* (9) showing differential gene expression of this enzyme in the brain of adult and fetal rats and suggesting the physiological significance of AS during brain development.

Expression of eNOS did not uniformly increase during differentiation, such as observed for AS and nNOS. Peak expression of eNOS was reached on day 14 together with maximal immunostaining for MAP-2 and GFAP. Expression of eNOS was induced by chronic inhibition of NOS, indicating that this isoform is subject to modulation by extracellular signals. High concentrations of nNOS are present in the CNS; thus, other mechanisms may exist for compensation of interruption of NO production including up-regulation of eNOS activity (37). Expression of eNOS is relatively uniform throughout the brain development of ewe fetuses, whereas peak expression is observed during pregnancy. It is known that both isoforms of NOS are expressed in the developing and mature brain; however, eNOS expression does not present a differential pattern of expression such as observed for rising nNOS expression accompanying the increasing complexity of neural development (38).

Several studies have attempted to identify factors that regulate NSC proliferation and subsequently lineage specifications. We hypothesized that NO production is a determinant for the progress of NSC differentiation and neural phenotype determination. NO can be directly and rapidly synthesized in response to growth

factor stimulation and other extracellular signals and then rearrange gene expression. We observed that inhibition of NOS and AS activity prevented the progress of differentiation.

Moreover, our results revealed that extracellular production of NO_x increased, whereas intracellular NO_x levels diminished during neural maturation. The synthesized NO diffuses into neighboring cells without the need for packaging, vesicle secretion, or membrane receptors and can interact with a variety of intracellular proteins. It is a gaseous molecule with an extremely short half-life being rapidly converted into other products such as nitrite or nitrate or incorporated into proteins by nitrosylation (24). Opposing changes of quantities of NO metabolites in intra- and extracellular environments throughout differentiation suggest differences in expression patterns of proteins that are nitrosylated along neural differentiation. NO can exert its effects on neuronal function through modification of sulfhydryl groups such as *S*-nitrosylation (39, 40). For example, the impaired dendrite outgrowth of $nNOS^{-/-}$ mice was explained by the absence of nitrosylation of the collapsin response mediator protein (41, 42). Target proteins for *S*-nitrosylation as studied in brain lysates, include metabolic enzymes, ion channels such as NMDA-glutamate receptors and structural proteins such as neurofilament heavy chain (NF-H) and β3-tubulin (43).

We have also investigated which is the most important NOS isoform for neural differentiation, using a selective inhibitor of nNOS. The obtained results indicate that nNOS inhibition alone was sufficient to block the progress of neural differentiation, suggesting key functions for nNOS in NO production during CNS formation.

Specific blockade of nNOS during neural differentiation resulted in increased expression of the NSC and progenitor antigen Nestin and decreased expression of neuron-specific β3-tubulin and glia-specific GFAP. MAP-2 expression levels were not affected in any of the mentioned experimental conditions, suggesting that NO signaling is essential for the initial progress of neural fate determination and less important for final neuronal maturation. An underlying mechanism for the observed effects during the onset of differentiation might be the failure of transition into cytostasis in the presence of L-NAME or MDLA. Inhibition of AS in cultured cells blocked production of L-arginine and consequently NO formation (44). NOS inhibition maintained NSC in a proliferative state, thereby suppressing neuronal differentiation; furthermore, L-NAME administration into lateral ventricle of adult mice significantly increased the number of proliferating cells (45). Thus, besides being important for triggering neural differentiation, NO plays an important signaling role in proliferation. As further evidence for NO as an essential factor in neural development, the addition of the substrates of NOS and AS, L-arginine and L-citrulline, respectively, to the culture medium during differentiation resulted in an increase of the number of cells expressing neural marker proteins, despite an increase in NO_x production was only observed in cultures that had received L-citrulline, a phenomenon that can be explained by the "L-arginine paradox" (46). The underlying mechanism implies the existence of separate intracellular pools of L-arginine directed to different path-

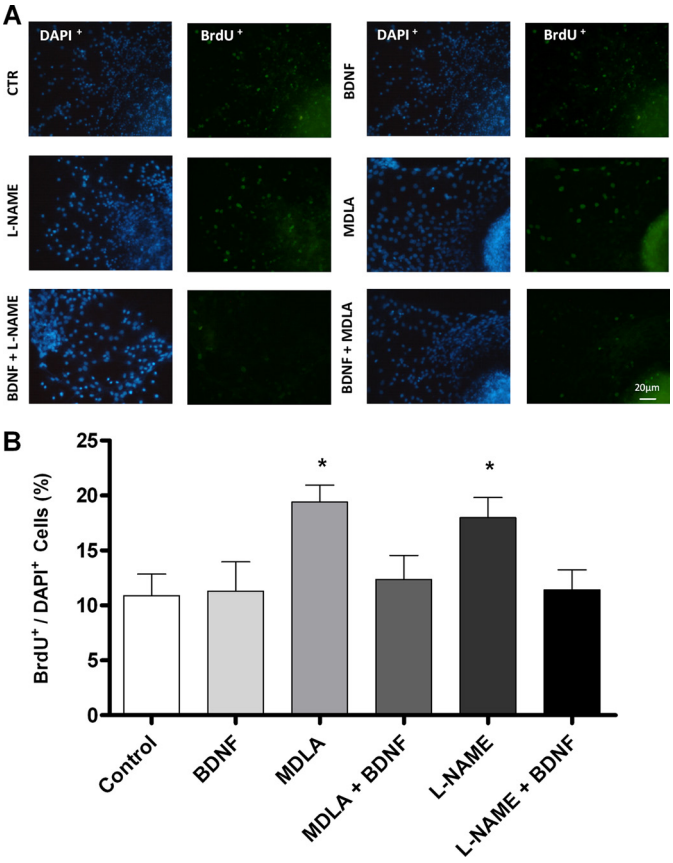

FIGURE 7. **Effects of inhibition of NO production and of BDNF on neural progenitor cell proliferation.** *A*, immunodetection of BrdU incorporation following a 12-h pulse in differentiating neurospheres on day 7 in the presence of 1 mM L-NAME, 1 mM MDLA, 20 ng/ml BDNF, 20 ng/ml BDNF and 1 mM MDLA, or 20 ng/ml BDNF and 1 mM L-NAME. BrdU incorporating nuclei are shown in *green*. *Scale bar*, 20 μm. *B*, quantification of proliferation in different conditions of treatment was performed by determining the ratio of BrdU+ over DAPI+ cells. Six fields were analyzed for each treatment by using the NIS Elements software (Nikon). *, $p < 0.05$, compared with untreated control cells). *CTR*, control.

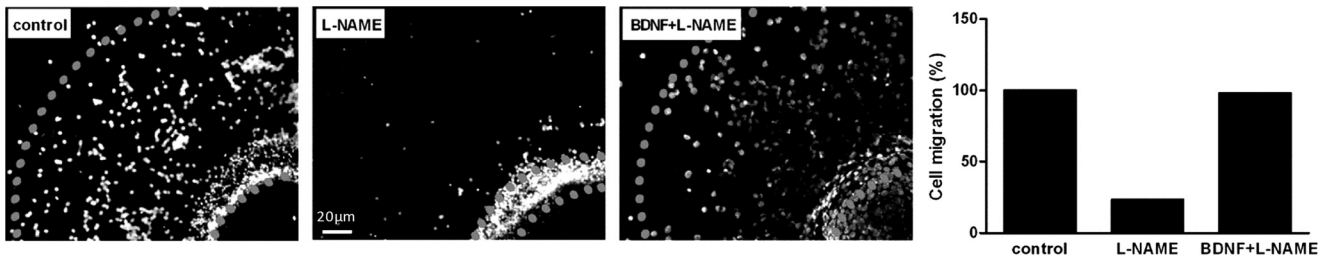

FIGURE 8. **Interference of inhibited NO production with neural stem cell migration.** Neurospheres were differentiated for 7 days in the presence or absence of 1 mM L-NAME or 1 mM L-NAME and 20 ng/ml BDNF. The cells were visualized by cell nuclei staining, and the distances of migration were determined by using the NIS Elements software (Nikon).

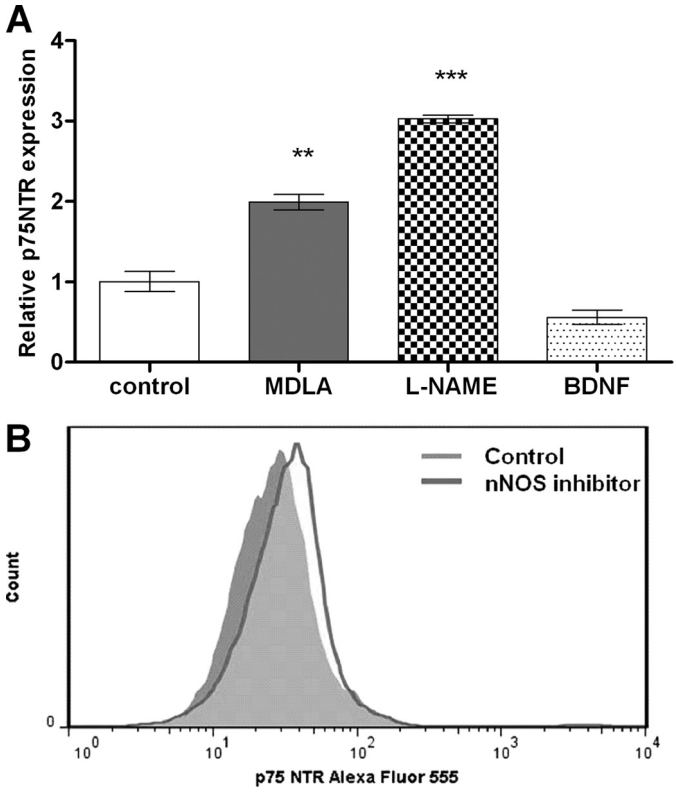

FIGURE 9. **Modulation of p75 neurotrophin receptor expression in neurospheres.** The cells were culture in the presence of 1 mM MDLA, 1 mM L-NAME, 20 ng/ml BDNF, or 1 μM 7-Ni. *A*, neurospheres were collected on day 7 of differentiation for RNA extraction, and p75NTR expression was analyzed by real time PCR. *B*, flow cytometry analysis of p75NTR expression in murine neurospheres differentiated for 7 days in the absence or presence of the nNOS inhibitor. The data are shown as mean values ± S.E. of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$, compared with untreated control cells.

ways. This L-arginine is synthesized in the NO-citrulline cycle by L-citrulline recycling (44). The enhancement of NO_x production by exogenous L-citrulline can therefore be attributed to the capacity of NSC to efficiently regenerate L-arginine from L-citrulline. Besides providing a source for NO production, L-arginine is a basic amino acid that has versatile metabolic roles, being involved in the generation of a wide range of biologically active intermediates such as NO, polyamines, creatine, and L-amino acids (47). As an alternative to NO production, L-arginine can be deviated to polyamines synthesis (reviewed in Ref. 7).

Agmatine derived from L-arginine decarboxylation (48) is a central neuromodulator with high affinity for α2-adrenoceptors and imidazoline-binding sites, in addition to blocking calcium influx, particularly by inhibiting receptors of the NMDA class (49). Moreover, it has been shown that agmatine increases neurogenesis by recruiting NSC in the hippocampus of adult mice because of blockade of NMDA receptors (50). Furthermore, treatment with drugs with marked affinity to imidazoline-binding sites led to increased levels of GFAP immunostaining together with increased density of imidazoline-binding sites in rat brain (51). Thus, L-arginine, even without altering NO concentration, increased GFAP and β3-tubulin expression, possibly because of induction of polyamine synthesis, such as agmatine. At the same time L-citrulline only affected GFAP expression. Having in mind that NO-induced effects depend on the dose and local of production of this gaseous messenger (7, 42), the incapability of L-citrulline in promoting neurogenesis can be explained by the lack of NO production in most of the cells of the heterogeneous population of differentiating NSC. Consequently, NO is only produced in a subset of cells expressing L-citrulline transporters (51), by yet unknown mechanisms

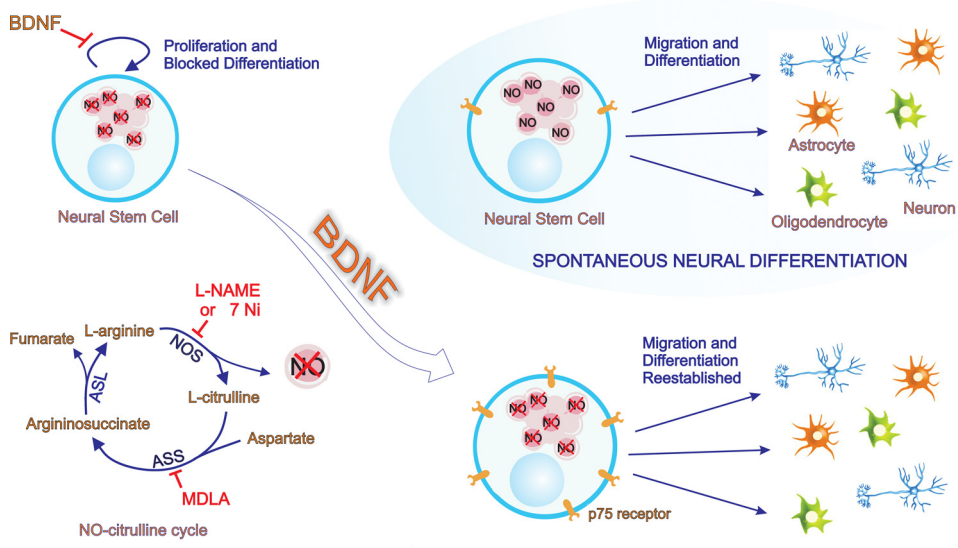

FIGURE 10. **BDNF restores neural differentiation inhibited by lack of nitric oxide.** NO signaling is essential for the spontaneous progress of neural fate determination. After plating, neural stem cells spontaneously differentiate into neurons, astrocytes, and oligodendrocytes. However, when formation of endogenous NO is interrupted by inhibitors of the NO-citrulline cycle (L-NAME, 7-Ni, and MDLA inhibiting all isoforms of NOS, AS, and nNOS, respectively), the progress of neural differentiation is blocked. In these conditions, NSC remain in a proliferative state; they do not migrate, nor do they differentiate into neural cells. BDNF does not affect neural differentiation in the absence of the above-cited inhibitors, but it re-establishes migration and differentiation of NSC when the NO-citrulline cycle is blocked. The addition of BDNF to NSC cultures treated with L-NAME, 7-Ni, or MDLA decreases proliferation and promotes migration and neural differentiation. These BDNF-induced effects are suggested to result from an increase in p75NTR expression induced by lack of NO. The p75NTR is required for BDNF-induced differentiation of neural stem cells, pointing at a novel mechanism for joint actions between the NO-citrulline cycle and BDNF for the maintenance of normal neurodevelopment processes.

supporting gliogenesis but not neurogenesis. Further studies will be necessary to validate such a hypothesis. Cheng *et al.* (45) have demonstrated that regulation of proliferation and/or differentiation fate occurred under the control of BDNF, acting in a positive feedback loop with NO for the correct neuronal phenotype choice. Moreover, there are very similar biological activities of NO and this other diffusible factor, BDNF (17, 18), implying potential interaction and association between these neurotransmitter. It is known that endogenous NO regulates BDNF production, supporting the hypothesis that BDNF and NO influence each other and may function as trans-synaptic signaling molecules in the brain (52). We have observed that BDNF expression increased along with neural differentiation of NSC and neuronal maturation, similarly to the pattern of nNOS expression.

Because BDNF signaling is associated with neurogenesis, we have investigated the possible interference with BDNF production in conditions of chronic exposure of NSC to L-NAME and MDLA along differentiation. Corroborating such hypothesis, we hereby show that the blocked endogenous NO production in culture as consequence of NOS or AS inhibition led to augmented BDNF gene expression. As further support for our experimental data, cerebroventricular administration of a NOS inhibitor increased BDNF content in the neocortex (52).

As an underlying mechanism, NSC-induced BDNF expression may be an attempt to compensate missing signaling caused by the lack of NO. However, neither induction of endogenous BDNF production nor induction of eNOS expression as a consequence of missing NO availability were enough to restore normal signaling *in vitro*. We hypothesized that endogenous paracrine effects of this neuron-derived factor might not be sufficient for restoring the normal progress of differentiation, because just few neurons, being able to secrete this factor, had been originated from differentiating NSC (53). As expected, the addition of 20 ng/ml BDNF to the culture medium reversed the block on neural differentiation caused by insufficient NO signaling. In addition to inducing neural differentiation, NO plays a role as negative regulator of precursor proliferation (35). As further evidence of re-establishing missing NO signaling by BDNF treatment, inhibition of proliferation caused by L-NAME and MDLA was re-established in the presence of the neurotrophic factor. On the other hand, treatment of differentiating neurospheres with BDNF in the absence of any inhibitor of NO production did not induce any changes in the progress of NSC differentiation and cell fate determination when compared with control differentiation assays performed without any of these compounds.

BDNF exerts its signaling by acting through two receptors: the high affinity trkB and the low affinity tyrosine receptor p75NTR, which does not have any enzymatic activity. The p75NTR is required for BDNF-induced blockade of proliferation and induction of differentiation of NSC, even in NSC populations that did not express trkB, suggesting that BDNF induced neurogenesis via p75NTR activation alone (34, 53, 54). NSC cultures obtained from embryonic rat telencephalon are not responsive to BDNF, indicating a low expression of p75NTR. However, our data show that the lack of NO_x production following treatment with L-NAME, MDLA, or 7-Ni, induced p75NTR expression; subsequently NSC became BDNF-responsive. These data are in line with previous studies that relate inhibition of NO expression to the up-regulation of p75NTR expression (55, 56).

In summary, we have shown for the first time that AS and NO are directly involved in the progress of NSC differentiation by using an *in vitro* system, which reflects differentiation conditions occurring in the developing cortex (5). Moreover, our results indicate that the cross-talk between BDNF- and NO_x-mediated signaling represents a mechanism by which NSC regulate their maintenance as precursor cells and subsequent neural differentiation (Fig. 10). Thus, this work provides new insights for understanding the involvement of the NO-citrulline cycle in regulation of NSC proliferation and differentiation, underlying the complex process of brain development. Further studies may reveal the roles of NO and BDNF in the maintenance of neurogenesis throughout postnatal life.

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REVIEW ARTICLE

Perspectives of purinergic signaling in stem cell differentiation and tissue regeneration

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Abstract Replacement of lost or dysfunctional tissues by stem cells has recently raised many investigations on therapeutic applications. Purinergic signaling has been shown to regulate proliferation, differentiation, cell death, and successful engraftment of stem cells originated from diverse origins. Adenosine triphosphate release occurs in a controlled way by exocytosis, transporters, and lysosomes or in large amounts from damaged cells, which is then subsequently degraded into adenosine. Paracrine and autocrine mechanisms induced by immune responses present critical factors for the success of stem cell therapy. While P1 receptors generally exert beneficial effects including anti-inflammatory activity, P2 receptor-mediated actions depend on the subtype of stimulated receptors and localization of tissue repair. Pro-inflammatory actions and excitatory tissue damages mainly result from P2X7 receptor activation, while other purinergic receptor subtypes participate in proliferation and differentiation, thereby providing adequate niches for stem cell engraftment and novel mechanisms for cell therapy and endogenous tissue repair. Therapeutic applications based on regulation of purinergic signaling are foreseen for kidney and heart muscle regeneration, Clara-like cell replacement for pulmonary and bronchial epithelial cells as well as for induction of neurogenesis in case of neurodegenerative diseases.

Keywords ATP · Adenosine nucleotides · Purinergic signaling · Tissue injury · Differentiation · Immune system

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An overview of purinergic signaling

Receptors for purines and pyrimidines are classified based on their agonist specificity. P1 receptors subtypes are selective for adenosine and are classical 7-transmembrane metabotropic receptors coupled to several families of Gi, Go, and Gs proteins. There are four types of adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) differing in their pharmacological and functional properties [1]. P2 receptors are divided into P2X and P2Y subtypes based on their structural characteristics. P2X receptors are ATP-activated, ligand-gated cationic ($Na^+/K^+/Ca^{2+}$) channels [2, 3], assembled in trimeric form from P2X1 to P2X7 subunits [1, 3]. Metabotropic P2Y purinoceptors expressed by mammalians are divided into $P2Y_{1,2,4,6,11,12,13,14}$ subtypes based on phylogenetic similarity and are stimulated by ATP, ADP, UTP, UDP, or UDP glucose [1]. Purinergic receptors are expressed by almost every cell type and are one of the first expressed neurotransmitter receptors in development [4–6]. The extracellular nucleotide/nucleoside availability is controlled by a highly efficient enzymatic cascade, which includes the members of the ectonucleoside triphosphate diphosphohydrolase (E-NTPDases, NTPDase1–8), ectonucleotide pyrophosphatase/phosphodiesterase (E-NPPs), ecto-alkaline phosphatases, and ecto-5′-nucleotidase/CD73. These enzymes catalyze the complete nucleotide

hydrolysis (e.g., ATP) to nucleosides (e.g., adenosine) and represent a powerful tool for controlling the effects mediated by extracellular purines [7–9].

Stem cells and purinergic signaling

Replacement of lost or dysfunctional tissues has recently raised many investigations on possible therapeutic application of stem cells. An impressive number of clinical trials and animal studies have already been performed to determine the therapeutic potential of various stem cells models [10, 11]. The first isolation of embryonic stem (ES) cells from mouse goes back to 1981 followed by human ES cell isolation and culture in 1998 [12, 13]. Organ-specific stem cells were isolated from embryonic and adult tissues including brain, bone marrow, umbilical cord, skeletal and cardiac muscles, and adipose tissue [14]. Pluripotent ES cells are capable to originate any somatic cell type, while tissue-specific stem cells are mostly multipotent and subsequently originate cell types found in these specific tissues. Both, ES and tissue-specific stem cells can proliferate symmetrically replicating themselves for self-renewal or asymmetrically giving rise to a stem cell and another more differentiated cell type. The most promising and recently discovered stem cell model for basic research and even therapy is the induced pluripotent stem cell (iPS cell), reprogrammed in 2006 from differentiated mouse cells and in 2007 from human cells [15, 16]. The recent described capacity of genetically reprogrammed somatic cells towards pluripotent ones could bypass obstacles, such as the lack of histocompatibility and ethical concerns, by allowing the generation of autologous cells from the patient. This new pluripotent cell source initially obtained by overexpression of the genes Klf-4, Oct4, Sox2, and c-Myc responsible for pluripotency [reviewed by 17] has opened expectations for treatment of many diseases. Importantly, iPS cells derived from different species demonstrated the potential to differentiate into tissues derived from the three germ layers, such as known from ES cells. However, care must be taken on using these cells as well as ES cells for transplantation purposes due to their possible tumorigenic potential.

Therapeutic application of stem cells in patients is particularly promising for treatment of heart disease, where new cardiomyocytes could restore contractile function after myocardial infarction. Cell regeneration therapy could be also relevant for repair of pancreatic function in diabetes with the replacement of β insulin-secreting cells [18]. Further possible applications are foreseen for the treatment of the damaged neuronal system and neurodegenerative diseases. For instance, efforts are being made to replace dopaminergic neurons in Parkinson's disease [19] or to use the stem cell therapy to restore motorneuron function in patients suffering from spinal cord injuries [16]. However, the little obtained progress in many cases did not satisfy the high expectations made. Moreover, observed functional improvements observed in the treated tissues did not often result from the integration of stem cells into existing tissue architectures. It is evident that transplanted cells contribute to endogenous tissue repair through paracrine mechanisms more than by differentiating themselves. For instance, the success of neural progenitor cell (NPC) engrafting into the spinal cord of Sprague–Dawley rats, subjected to contusion at T8–T9 levels, was limited by allodynia due to the death of transplanted cells [7]. However, injection of conditioned media recovered from cultured stem cells promoted arteriogenesis and functional improvement when injected into the damaged heart [20]. Therefore, it has been postulated that trophic factors represent the principle mechanism responsible for tissue repair.

Usual strategies for cell replacement therapy are based on the isolation of a stem cell source from a donor or the patient, followed by induction to proliferate and/or differentiate into tissue types which shall be repaired. Cell death and rejection of transplanted cells are mostly due to immune responses and the absence of adequate stem cell niches at the localization of transplantation. Although mechanisms by which the local milieu influences stem cell differentiation and tissue engraftment need yet to be elucidated, it seems that the fate of bone marrow stem cells is determined by the environment in which they engraft rather than by an intrinsically programmed fate. As support for such hypothesis, positive inotropic (pharmacologic augmentation of contractility) or chronotropic stimuli (heart rate increase by exercise) promoted and intensified the differentiation of bone marrow-derived stem cells into cardiomyocyte phenotypes [21]. Furthermore, stem cells secret trophic and immunomodulatory factors controlling local and systematic inflammatory responses. Such factors, liberated by, i.e., bone marrow stem cells are therapeutically important, since they stimulate local tissue regeneration and/or recruitment of endogenous stem or progenitor cells. Moreover, some studies have demonstrated that mesenchymal stem cells (MSC) can diminish the apoptosis degree and infarct size of the damaged areas by secreting a wide range of cytoprotective molecules like vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor 1, stromal cell-derived factor-1, platelet-derived growth factor, interleukin-1 beta, or hepatocyte growth factor [22].

Other factors with such therapeutic potential are UTP, UDP, ADP, and adenosine acting through purinergic receptors. Nucleotides, released after tissue injury and cell death and hydrolyzed by ectonucleotidases, also regulate immune cell function induced by damage-associated molecular pattern molecules [23]. Moreover, ATP released from immune cells participates in autocrine as well as in paracrine feedback loops with regulatory functions during T-cell

activation in the immune synapse (junction between T cell and antigen-presenting cell) [24]. During the inflammatory process following cell transplantation and hindering repair, purines exert trophic functions and keep several immune functions under control, including the release of prostanoids, activation of matrix metalloproteinase-9, cytokines and chemokines, proliferation, differentiation/maturation and stimulation of immune cells, endothelial adhesion, free radical production, degranulation, phagocytosis, fusion, and cell death [25]. Depending on the involved purinergic receptor subtype, ATP often exerts proinflammatory effects while adenosine induces mainly anti-inflammatory effects [25]. Several studies demonstrated that the absence or inhibition of the P2X7 receptor (a mediator of the pro-inflammatory effects of ATP) results in less severe outcomes in chronic inflammatory diseases and enhanced functional recovery [23, 26, 27].

Besides importance of purinergic receptor agonists in differentiated immune cells, these compounds also modulate hematopoietic stem cell (HSC) self-renewal, expansion, and differentiation with implications not only in hematopoiesis, but also in tissue repair and regenerative medicine [28, 29]. For instance, ATP induces the proliferation of human HSC and contributed through P2X receptor activation during inflammation process [29, 30]. UTP also induces proliferation and migration of HSCs [30, 31] while adenosine potentiates the stimulatory effect of growth factors and cytokines on HSC proliferation and differentiation [8]. Moreover, human MSCs at early stages of culture (P0–P5) spontaneously release ATP reducing cell proliferation. Increased human MSC proliferation is induced by the unselective P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonate (PPADS) and by the selective P2Y1 receptor antagonist 2′-deoxy-N6-methyladenosine-3′,5′-bisphosphate (MRS 2179). In summary, ATP modulates HSC and MSC proliferation and likely acts as one of the early factors determining their cell fate [32]. Furthermore, nucleotides also contribute to inflammatory responses and cell fate decisions occurring in the brain. P2X7 receptors expressed by NPCs are responsible for cell death, being in agreement with observations that high levels of extracellular ATP in inflammatory central nervous system (CNS) lesions hinder successful NPC engraftment [33].

The extracellular nucleotide/nucleoside availability is controlled by a highly efficient enzymatic cascade, which includes the members of the E-NTPDases (NTPDase1–8), E-NPPs, ecto-alkaline phosphatases, and ecto-5′-nucleotidase/CD73. These enzymes are responsible for nucleotide hydrolysis (e.g. ATP) into nucleosides (e.g., adenosine) and represent a powerful mechanism for controlling the effects mediated by extracellular purines [9, 34]. Although purinergic signaling has been extensively studied, only few studies are found in the literature demonstrating the involvement of extracellular nucleotide metabolizing enzymes in stem cell biology. Expression and activities of members of ectonucleotidase families as well as purinergic receptor subtypes have been detected in different types of stem and progenitor cells. Recent works have identified the presence of NTPDase2 in adult mouse hippocampal progenitors [35] and in type B cells of the subventricular zone (SVZ) [36], two neurogenic regions of the adult mammalian brain. In accordance, neurospheres cultured from the adult mouse SVZ express NTPDase2, the tissue nonspecific isoform of alkaline phosphatase (TNAP) and functional P2 receptors in synergism with growth factors for enhancing cell proliferation [37]. In addition, deletion of TNAP expression or inhibition of its enzymatic activity in neural progenitors reduces cell proliferation and differentiation into neurons or oligodendrocytes [38]. These published data corroborate the importance of NTPDase2 and TNAP, two potential ATP scavengers, as novel markers for progenitor cells both in the adult and developing brain [39]. Reinforcing these results, spontaneous ATP release was observed in murine NPCs and, interestingly, purinergic receptors antagonists were able to suppress progenitor cell proliferation [40]. Moreover, neuronal differentiation was accompanied by a decrease in ATP release and a loss of functional P2Y receptors, suggesting that purine nucleotides act as proliferation-inducing factors for NPCs and downregulators of neuronal differentiation, once again pointing at the importance of purinergic signaling and involved enzymes for neurogenesis in the adult brain [40]. These data are in agreement with results of our laboratory [41], showing down-regulation of P2Y1 receptor expression and activity in differentiating P19 mouse embryonal carcinoma cells. This observation is in line with functions of the P2Y1 subtype in promoting proliferation of undifferentiated cells, but not induction of neuronal differentiation. Finally, the studies presented here demonstrate the potential participation of ectonucleotidases in the biology of stem or progenitor cells from different tissues. Initial results on roles of these ecto-enzymes will encourage more studies for better understanding of their importance in stem cell biology, differentiation, and tissue repair. In the following, we will discuss new trends of stem cell research related to purinergic signaling and the perspectives of using these discoveries as tools for future tissue repair in clinical trials as this new approach develops (see Fig. 1 for a scheme of the possible therapeutic use of purines in combination with stem cells).

Purinergic signaling and perspectives in tissue regeneration

Implications of the purinergic system in stem cell biology and tissue regeneration will be discussed with emphasis on

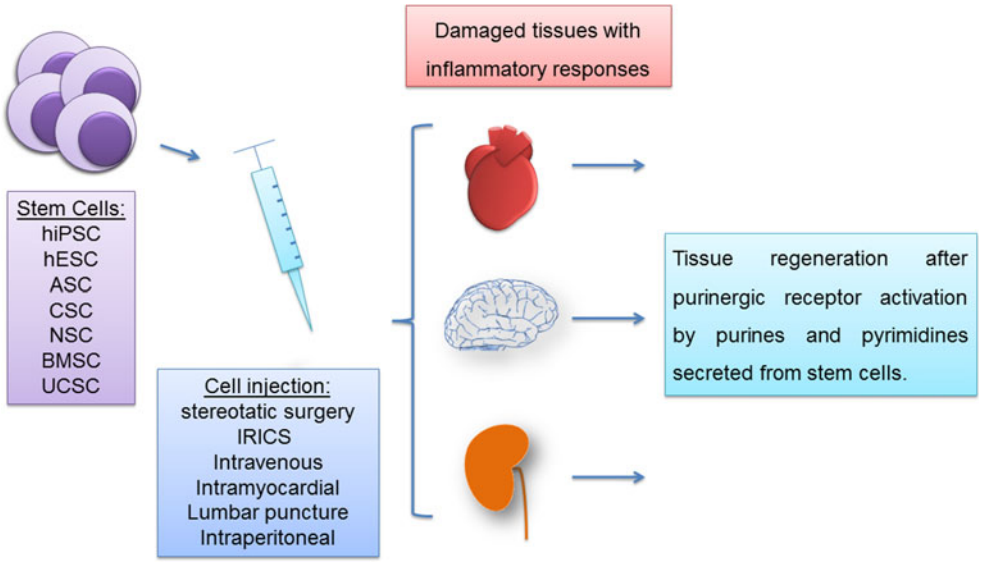

Fig. 1 Therapeutic potential of stem cells and supposed effects of purinergic signaling. Stem cells of diverse origins, such as from adipose, cardiac, and neural tissues can restore and regenerate damaged tissues by secreting paracrine factors including purines and pyrimidines. ATP and adenosine interfere with tissue reactions following transplantation of stem cells of various origins in different ways. (1) Nucleotides modulate the immune response and thereby reduce inflammation processes and the risk of transplant rejection and cell death. (2) Purines and pyrimidines promote proliferation and differentiation of transplanted and endogenous stem cells by providing adequate stem cell niches. (3) Purines and pyrimidines induce migration of endogenous stem cells to the site of injury and increase engraftment rates. Stem cell types with therapeutic applications are human induced-pluripotent stem cells (hiPSC), human embryonic stem cells (hESC), adipose stem cells (ASC), cardiac stem cells (CSC), neural stem cells (NSC), bone marrow stem cells (BMSC), and umbilical cord stem cells (UCSC) which are transplanted by using stereotaxic surgery (SS), intracoronary retrograde infusion through coronary sinus (IRICS) or intravenous, intramyocardial, or intraperitoneal injection or lumbar puncture

the recent hypothesis that paracrine effects present the most important mechanisms in this process. Since this idea is very recent, few data are available directly relating the purinergic system with stem cell differentiation and tissue regeneration; however, the authors of this review are confident that the present article will encourage research in order to better understand the participation of purinergic signaling in this context.

Heart injury

The heart is an organ composed basically of fibroblasts and cardiomyocytes, terminal-differentiated cells which give the heart the pumping ability. During ischemia and other injuries, the most affected cells are the cardiomyocytes because they die and a scar is formed due to the inability of renewing these cells. The scar stiffens the heart, decreasing its capability and efficiency in pumping the blood. Therefore, intense efforts are being made for the restoration of lost cells by cell therapy and maintenance of cardiac function in patients with heart injury. Many stem cells types have been studied in order to select the best model for cardiac cell therapy. ES cells, iPS cells as well as adult stem cells (bone marrow, adipose tissue-derived, and cardiac stem cells) are already tested in animal models and humans with often promising results [42, 43].

The most promising model is provided by cardiac stem cells (CSCs) that reside in small populations in the adult mammalian myocardium and have the potential to differentiate into cardiomyocytes and other cell types, such as endothelial and vascular smooth muscle cells [44–47]. However, differentiation of these cells is rare under physiological conditions [48]. For therapeutic purposes, CSCs can be generated by expanding autologous cells ex vivo or stimulating the regeneration capacity of these cells in vivo. Nevertheless, one of the biggest problems hindering the therapeutic use of stem cells lies still in the difficulty of keeping stem cells alive following transplantation. Cell death occurs before cells can engraft in their environment due to inflammation-signaling responses, or cells do not even identify the injured tissue site for engraftment. Therefore, signaling factors necessary for cell establishment at the location of transplantation are being investigated. Such paracrine factors include ATP and adenosine and their respective receptor subtypes. P2Y14 receptors expressed by bone marrow HSCs induce migration of these cells to the localization of injury followed by induction of differentiation at the site mediated by activation of other purinergic receptors [49] (Fig. 2). Adenosine plays many roles in the heart including regulation of growth, differentiation, angiogenesis, coronary blood flow, cardiac conduction and heart rate, substrate

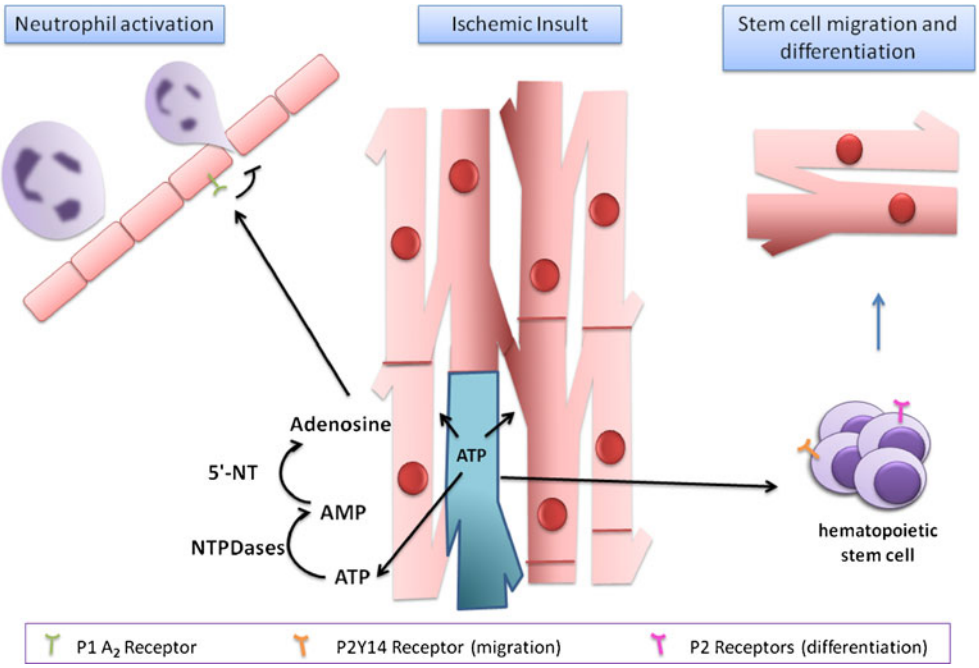

Fig. 2 ATP-and adenosine-induced actions following cardiac ischemic insult. After myocardial injury following an ischemic insult, dead cells release ATP into the extracellular space. The released ATP stimulates P2Y14 receptors expressed by hematopoietic stem cells and possibly purinergic receptors on cardiac stem cells. NTPDases (ecto-nucleoside triphosphate diphosphohydrolases) dephosphorylate ATP via ADP to AMP, and 5-nucleotidase (5′-NT) catalyzes the hydrolysis of AMP to adenosine inducing anti-inflammatory responses by activation of A_{2A} and A_{2B} receptors, blocking neutrophil activation and migration

metabolism, and sensitivity to adrenergic stimulation [50], and also functions as an endogenous determinant of ischemic tolerance [50]. The two A_2 receptor subtypes (A_{2A} and A_{2BA}) possess important anti-inflammatory and immunomodulatory functions, and probably control the impact of inflammatory processes during ischemic and post-ischemic damage. Vinten-Johansen and colleagues confirm protective functions of A_{2A} receptors in cardiac tissue by inhibition of neutrophil activation and neutrophil–vascular interactions as seen in Fig. 2 [51, 52].

Extracellular pyridoxal-5′-phosphate (PLP), a synthesis precursor of PPADS, is considered a P2 receptor antagonist. When this compound is used in the micromolar concentration range, it prevents ATP-induced calcium influx in isolated rat cardiomyocytes, inhibiting the positive inotropic effects of ATP on isolated perfused hearts and blocking ATP binding to the cardiac sarcolemma. Recent research suggests that at least part of the protective effect observed during reperfusion by PLP may be mediated through its inhibitory action on purinergic receptors. The possible receptors expressed in cardiomyocytes and subject to inhibition by PLP are P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 subtypes [53]. Taking together, the strategy of cell therapy following a heart attack could base on activation of P2Y14 purinergic receptors expressed by bone marrow stem cells which then would induce migration to the site of injury and thus could restore heart tissue before the formation of a scar. Furthermore, concomitant activation of A_2 receptors would decrease the damage caused by ischemia due to the anti-inflammatory activity of these receptors in preventing the activation of neutrophils which may cause further damage tissue. However, reservations remain regarding stimulation of P2Y receptors in cardiomyocytes due to their involvement in apoptosis induction. Taken together, fundamental roles exist for the purinergic system in cardiac protection and preconditioning suggesting possible applications together with stem cell therapy.

Bladder dysfunction and glomerular injury

Much effort has been spent for establishing a stem cell therapy for the regeneration of tissues, including nephron and bladder. The urinary system is composed basically by kidneys, ureters, bladder, and urethra, and disorders in any of these structures can cause much pain and suffering for the patient. Hemodialysis and implementation of tubes are usually used for the treatment of patients with urogenital diseases; however, unfortunately, there is no cure for many diseases. Different stem cell types have been tested for therapeutic applications with varying success. For therapy of bladder dysfunction, Nishijima et al. transplanted bone marrow stem cells by intrabladder injection resulting in restored bladder contraction in rats [54]. Huang et al. transplanted adipose-derived stem cells by intrabladder or intravenous injection resulting in improved tissue parameters and urodynamics in a rat model of overactive bladder [55]. Interestingly, De Coppi et al. showed that intrabladder transplantation of amniotic fluid or bone marrow stem cells promoted post-injury bladder remodeling by a paracrine mechanism [56]. According to Hallman et al., the repair of injured renal epithelium is thought to be mediated by surviving renal proximal tubular cells that must dedifferentiate to allow for proliferation and migration necessary for epithelial regeneration. ATP and its intracellular signaling

have also crucial functions in this regeneration process. Kartha et al. showed that adenine nucleotides stimulate migration of kidney epithelial cells in an in vitro culture resembling wounded kidney. In these experiments, cells were treated with 10 μM of different adenine nucleotides, and the number of cells that migrated into the leasioned area of 1 mm^2 in size was counted 24 h later [57]. Increases in migration were induced by cAMP, adenosine, AMP, and ATP suggesting purinergic receptors activation; however, P1 receptors may promote contrary functions in this context, as adenosine can induce apoptosis in glomerular mesangial cells causing glomerular injury [58]. Babelova et al. showed that the secretion of the pro-inflammatory master cytokine interleukin (IL)-1β during inflammatory renal injury interacts with purinergic P2X4/P2X7 receptors [59]. Moreover, P2X7 receptor expression in glomeruli was augmented tenfold in diabetic and hypertensive rat models when compared to that of healthy rat glomeruli [60]. Purinergic signaling has also been related to renal protection via A_{2a} adenosine receptor activation in conditions of reperfusion injury [61]. In summary, for treatment of renal epithelium injury, transplantation of bone marrow or adipose tissue stem cells are promising. Migration to the injured sites can be induced by injecting cAMP, adenosine, AMP, and ATP suggesting purinergic receptor activation. On the other site, P1 receptor inhibition is indicated due to the contribution of these receptors to apoptosis under these conditions.

Parkinson's disease

Probably most effort has been put into the study of the applicability of cellular therapy in the nervous system due to its enormous impact on patient's life and a lack of therapeutic strategies to cure neurodegenerative diseases and spinal cord injuries. We describe here some recent discoveries related to purinergic signaling with impact on tissue repair in the neuronal system.

Parkinson's disease (PD) is a neurodegenerative illness caused by death of dopaminergic neurons in the substantia nigra pars, but the underlying mechanisms of neuronal death remain largely unknown. Dopaminergic neurons are responsible for dopamine neurotransmitter secretion and control body movements. The absence of this molecule in patients with PD generates tremor rigidity, postural instability, and loss of motor coordination affecting writing capability among other disturbances. Increased survival is achieved by surgical therapies and medications, principally based on administration of L-DOPA, but its prolonged use may generate uncontrollable movements known as dyskinesia [63, 64]. Purinergic signaling has implications in PD, since treatment with ATP enhances the release of dopamine from dopaminergic neurons of the *substantia nigra*. However, at the same time, ATP release may activate P2X7 receptors expressed by neighboring cells thereby promoting cell death and contributing to an increase of the necrotic volume [62–65]. Furthermore, Feuvre et al. [66] provided evidence that P2X7 receptor activation following ATP release induces expression of proteins involved in the inflammatory response followed by liberation of cytokines. In addition, ATP together with glutamate released in neurodegenerative disorders may change intracellular Ca^{2+} homeostasis, mainly in neurons, with major importance for the disease progress [67].

Primary cultures of rat dopaminergic neurons express P2X1-7 and P2Y1 receptors together with D1 and D2 dopamine receptors [68]. P2Y receptor antagonists are potent neuroprotecting agents in the brain cortex, hippocampus, and cerebellum by modulating excessive neurotransmitter release in brain disorders [69, 70]; however, these effects would be undesirable in PD, since even PPADS blocking P2 receptors was shown to decrease dopamine secretion [71, 72]. A wide range of different strategies is under investigation for PD treatment, with a major focus research on stem cell therapy applications. Exogenous molecules are known to guide neural differentiation and are responsible for the high grade of phenotype specification, including induction of axonal growth and establishment of synaptic contact [73]. Milosevic et al. [74] detected P2Y4, P2Y6, and P2X4 receptor expression in cultured human NPCs from human fetal midbrain. UTP and UDP are known as agonists of the P2Y2/P2Y4 and P2Y6 receptors, respectively [75]. The treatment of hNPCs with UTP, in the presence of EGF and FGF2, increases cell proliferation. Moreover, UTP and UDP in the presence of specific culture medium enhance dopaminergic cell differentiation, and these effects are reduced by antagonists of P2 receptors.

Adenosine A_{2A} receptors are selectively located on striatopallidal neurons and are capable of forming functional heteromeric complexes with dopamine D2 and metabotropic glutamate mGlu5 receptors. A_{2A} receptor antagonists have emerged as an attractive nondopaminergic target to improve the motor deficits that characterize PD, based on the regional and unique cellular distribution of this receptor, being in agreement with data showing that A_{2A} receptor antagonists improve motor symptoms in animal models of Parkinson's disease and in initial clinical trials. Some experimental data also indicate that A_{2A} receptor antagonists do not induce neuroplasticity phenomena which complicate long-term dopaminergic treatments [76].

These data suggest the involvement of purinergic signaling in dopaminergic cell differentiation and possible applications for purinergic receptors in in vitro differenti-

ation cultures for posterior PD cell therapy [74]. However, more studies are needed to clarify whether extracellular nucleotides may contribute to favorable endogenous niches for stem cell transplantation or even recruit endogenous NPCs for dopaminergic differentiation.

Alzheimer's disease

The pathogenicity of Alzheimer's disease (AD) involves amyloids plaques and neurofibrillary tangle formation in the neuron extracellular medium. AD patients present an elevated production and secretion of the amyloid β peptide (Aβ) by neurons into the extracellular medium with progressive deposit of fibrils with high-grade toxicity, generating neuronal dysfunction and cell death [77–79]. This initial deposit triggers an inflammatory process with microglia and astrocyte recruitment to the injury site. Then, elevating cytokine secretion promotes Aβ internalization by neurons elevating neuronal damage [80–82]. ATP is released in high concentrations as result of cell death and enhances the local inflammatory effects besides increasing vulnerability of neurons by Aβ [61, 81]. Microglial cells recruited to the injury site showed elevated increased P2X7 receptor expression, as observed in animal models and human patients [83, 84]. P2X7 receptor activation by elevated ATP concentration promotes the secretion of the cytokines by microglial cells and activated oxygen species, increasing inflammation and stimulating Aβ-plaque formation, which also stimulates ATP liberation [82, 85, 86]. Furthermore, the P2Y1 subtype is expressed in AD typical structures such as Aβ plaques and neurofibrillary tangles, and receptor immunostaining was notably high in AD brain suggesting that P2Y1 receptors may participate in signaling events triggering neurodegenerative processes [61, 81, 82]. A_1 and A_{2A} adenosine receptor subtypes are expressed in the cortex, hippocampus, and microglia in the brain of patients suffering from AD. The A_{2A} receptor was suggested to contribute to memory deficits. The administration of caffeine, an antagonist of A_1 and A_{2A} receptors, promoted the protection against Aβ-induced neurotoxicity. Moreover, in vivo studies with A_{2A} antagonists resulted in reduced Aβ production and still protected against Aβ toxicity [87].

Hippocampus and the subventricular zone are the brain structures most affected in AD and are also the main sites of NPC localization. Increased NPC proliferation was observed in different illness stages; however, subsequent differentiation of these cells was not detected [88, 89]. NPCs implanted into the brain of a rat model of migrated to the disease site. Moreover, the presence of NPCs decreased microgliosis and the expression and secretion of pro-inflammatory cytokines, both characteristic conditions for AD. Elevated neuroprotection was also observed together with augmented expression of MAP-2, a marker protein for mature neurons. However, NPCs were nestin–positive and negative for expression of neuronal marker proteins in immunostaining assays, indicating that neuronal differentiation did not occur [90]. Secreted Aβ 1–42, a more toxic form of the amyloid peptide causing cell death, evoked a reduction of NPC proliferation [91]. Nowadays, acetylcholinesterase inhibitors are being used to enhance cholinergic function and induce a temporary cognition improvement. Implants of NPCs derived from the cholinergic regions of the forebrain, appear to be a valid approach for cell therapy. ATP, a natural cotransmitter of acetylcholine, may gain importance in this context for helping to reestablish defective cholinergic transmission.

Several cell lines and animal models are used to assess mechanisms of neural differentiation and the interrelationship of action of various metabotropic and ionotropic receptors in this process. Trujillo et al. [62] suggested the intrinsic regulation between purinergic, cholinergic, and kallikrein–kinin systems for phenotype determination during neural differentiation. Using P19 embryonal carcinoma cells as in vitro model for neuronal differentiation, our group observed that functional purinergic receptors are essential for cell differentiation into neurons with functional cholinergic receptors [41].

According to Delarasse et al. [92], activation of P2X7 receptors stimulates soluble amyloid precursor protein α release from mouse neuroblastoma cells. In view of that, a possible treatment for AD could include inhibition of P2 receptors to decrease inflammatory responses, together with NPC injection secreting factors for reduction of inflammatory responses. Further studies will also reveal whether stimulation with ATP will help restoring cholinergic functions.

Epilepsy

Epilepsy is a brain disturbance manifested by frequent seizures with constant neural activation. It may be accompanied by massive glial cell proliferation, initiating following neurodegenerative processes. Several anti-epileptic agents inhibit the ability of astrocytes in transmitting intracellular Ca^{2+} waves. In view of that, purinergic receptor antagonists should offer a novel treatment for blocking Ca^{2+} wave propagation stimulated by ATP [93]. As further proof for such mechanism, injection of high doses of ATP into rat cortex promoted an increase in seizure occurrence, which could be antagonized by suramin [94]. Hippocampi from chronic epileptic rats demonstrated elevated P2X7 receptor expression and abnormal responses to ATP, suggesting a possible participation of this system in the pathophysiology of epilepsy [95]. Potent drugs are administrated in high doses in rats and mice to promote sequential seizures and behavioral and electrographic

changes [62]. In a rat epilepsy model, kainate application elevated microglial purinergic receptor expression, mainly P2X7 and P2Y12 receptor subtypes. Both receptors are associated with the active state of microglia, inducing inflammatory responses and microglia migration, respectively [95]. In a temporal lobe epilepsy model induced by pilocarpine injection, P2X4 receptor expression was significantly reduced in pyramidal neurons reflecting a neuronal loss in a chronic status, while elevated P2X7 receptor expression was observed in glial cells suggesting again its participation in the inflammatory response [95]. Oses [96] observed a decrease in P2X receptor expression in rat hippocampus following convulsive periods which may be associated with progressing neurodegeneration and seizure worsening during epilepsy. However, adenosine acting through A1 receptors in an epilepsy model induced by pilocarpine promoted significant protection against seizures [95, 97]. As a possible mechanism, adenosine participates in cell proliferation regulation and apoptosis eliminating useless and damaged cells during repair, without the necessity of neurotoxic mediators or immunomodulators. Furthermore, adenosine can control astrocyte proliferation triggered by other purine nucleotides [98].

Cell transplantation strategies have been employed for the treatment of epileptic disorders, but the effect of exogenous neural stem cells is unknown. Chua et al. evaluated possible anti-epileptogenic effect of NSCs in adult rats with status epilepticus and showed that NSCs differentiate into inhibitory interneurons and decrease neuronal excitability, preventing spontaneous recurrent seizure formation in adult rats with pilocarpine-induced temporal lobe epilespy [99]. Therefore, a novel cellular source for the local therapeutic delivery of adenosine, a stem cell-based delivery system for adenosine, was generated by disruption of both alleles of adenosine kinase (AK) in mouse ES cells. These Ak−/− ES cells were differentiated into glial precursor cells and released significant amounts of adenosine. Rats with adenosine releasing Ak−/− ES cell-derived implants displayed transient protection against convulsive seizures and a profound reduction of after-discharge activity in EEG recordings, providing a proof-of-principle evidence that Ak−/− ES cell-derived brain implants suppress seizure activity by a paracrine mode of action [100]. In summary, stem cell therapy may be successful for epilepsy if transplanted NSCs feature a paracrine effect by releasing adenosine, which decreases the number of seizures, besides their ability to differentiate into inhibitory interneurons.

Trauma, ischemia, and hypoxia in the CNS

During several injury conditions such as trauma, ischemia, and hypoxia, ATP secretion is an important signaling molecule involved in repair of damaged tissue. After spinal cord injury, a large peritraumatic region sustains pathological processes to keep high ATP concentrations in the extracellular medium [61] involving P2X7 receptor activation and cell death as already discussed in this review. For instance, P2X7 receptors are expressed in neurons, astrocytes, and microglia of brain tissue suffering from ischemic conditions [82, 101]. Accordingly, administration of P2 receptor antagonists improved cell function and reduced cell death in the peritraumatic zone [81]. Moreover, after lesions in the peripheral nervous system. P2X3 receptor expression in intact neurons, suggesting a role for this receptor in post-traumatic repair [102]. Following trauma, astrocytes increased expression of P2X4 receptors thereby inducing trombospondin-1 secretion, which constitutes an extracellular molecule for synapse formation contributing to CNS remodeling [81, 103]. However, as already said, ATP also promotes neuronal apoptosis, necrosis and astrocytic death after traumatic events. P2 receptors promote the recruitment of microglial cells from distal areas to the traumatic core [104]. In vivo studies showed that P2X4 and P2Y12 receptors stimulated migration of microglial cells to the injury area after trauma, followed by expression of P2Y6 receptors favoring the secondary damage moment, the debris phagocytosis [82]. Experimental evidence indicates liberation of adenosine into the extracellular medium after tissue damage together with down-regulation of AK expression, leading to adenosine accumulation and neuroprotection following injury [105]. ATP in the extracellular medium may attract astrocytes and microglia to the site of injury in order to assist tissue repair [106]. In the normal adult brain, ATP secreted by astrocytes stimulates NPC proliferation and migration, while P2Y receptor antagonists reversed this effect by inhibiting proliferation. During the neurogenesis process, NPCs revealed NTPDase activity for controlling ATP concentration and subsequently directing neuronal and glial differentiation [40]. In summary, treatment of spinal cord injury and other traumatic and ischemic disorders of the CNS would benefit from P2 receptor inhibition in order to reduce cell death, followed by activation of P2X3 and P2X4 receptors for induction of synapse formation. Thereby, extracellular adenosine accumulation leads to neuroprotection during injury while ATP may attract astrocytes and microglia to the site of injury to assist tissue repair. In vitro NPC differentiation is directed by P2Y receptors; such mechanism should be further validated in animal models.

Skin injury

Skin is a stratified epithelium, where the epidermis is the outermost part of this tissue and dermis is innermost. Epidermis is mainly constituted by keratinocytes (90–95%)

and these cells are arranged in continuous layers from the inside towards outer layers: the basal layer, the stratum spinosum, the granular layer and stratum corneum. The epidermis is capable of self-renewal by presenting adult stem cells, which proliferate and can originate a new epidermis to cover all body surfaces. These stem cells are located in a portion of the follicle hair known as bulge migrating upwards to the proliferative basal layer. Keratinocytes migrate from the basal layer to the skin surface with concomitant differentiation [107–109]. In physiological and in pathological conditions, many kind of cells related to nervous and immune systems, can generate ATP extravasation and accumulation in the extracellular medium of keratinocytes. Two important functions are attributed to this nucleotide such as modulation of keratinocyte proliferation and differentiation [110]. Many studies have shown that these effects are mediated by ATP action through P2X and P2Y receptor subtypes, and it is known that the epidermis expresses P2X5, P2X7, P2Y1, and P2Y2 subtypes with diverse functions [111–113].

P2Y1 and P2Y2 receptors expressed in basal layers of the fetal and adult epidermis [111, 113] were immune colocalized with the cell proliferation markers Ki67 and PCNA (proliferation cell nuclear antigen) [111]. The P2Y1 receptor agonist 2-methylthio-ADP (P2Y1 agonist) and UTP activating P2Y2 receptors induced proliferation in cell cultures of basal keratinocytes [113]. P2Y1 and P2Y2 subtypes are coupled to Phospholipase C via $G_{q/11}$ proteins with generation of Inositol 3-phosphate and, in sequence, induce intracellular calcium mobilization [113] leading to Cl^- conductance and starting keratinocyte differentiation [112]. In an in vivo wound-healing model, the P2Y1 receptor is expressed in epidermal basal layers and the wound edge, while the P2Y2 subtype is expressed in basal and suprabasal layers, but is not expressed in the wound edge. Alterations of distribution patterns of purinergic receptors occur during phenotype changes as keratinocytes become migratory cells in the wound-healing process [110]. P2X5 receptors are expressed in undifferentiated basal and intermediate layers of fetal epidermis with high immunoreactivity for cytokeratin-10, an initial differentiation marker [111]. In wounded epidermis, keratinocytes of the wound edge increase P2X5 receptor expression [111]. P2X7 receptor expression was detected together with labeling for caspase-3 and TUNEL, markers for terminal differentiation and apoptosis, respectively, suggesting that this receptor eliminates not any more needed cells during final epidermis development [111]. Furthermore, the P2X7 subtype is also expressed in corneum stratum in adult epidermis suggesting its participation in apoptotic control [111, 113]. During wound healing processes, P2X7 receptor expression was not detected [111]. ATP is released by keratinocytes into the extracellular space by mechanical stress and external damage and achieves elevated extracellular, cytotoxic levels. Elevated ATP concentrations (300 μM) were applied together with UV radiation as external damage model in cultured human epidermal keratinocytes. Both situations augmented significantly P2X7 receptor and reduced P2Y2 receptor expression while P2X5 and P2Y1 subtype expression levels were not altered. These events associated with elevated extracellular ATP concentration result in skin inflammation, demonstrating the role of purinergic signaling in skin physiology and disease induction [112]. Purinergic signaling could promote skin injury therapy by selective activation of P2Y1 and P2Y2 receptors favoring the phenotype of migratory cells without induction of inflammatory responses.

Pulmonary epithelium injury

The airway epithelium is exposed to environmental pollutants, allergens and pathogens that might lead to tissue damage or the development of a variety of infectious and inflammatory diseases such as chronic bronchitis, chronic obstructive pulmonary disease, asthma, and fibrosis. In this context, stem and progenitor cells are involved in lung regeneration. They are located within the basal layer of the upper airways, within or near pulmonary neuroendocrine cell rests, at the bronchoalveolar junction, and within the epithelial surface [114–116]. The airway epithelium represents the first barrier to inhaled particles and pathogens and because of this, it suffers constant damages. Thus, the mechanism of the repair of damaged epithelium has been widely studied. Epithelial progenitors termed Clara cells (transit-amplifying cells) are broadly distributed and after injury differentiate into ciliated cells [117, 118]. In addition to Clara cells, bronchiolar airways have also rare stem cells that contribute to repair of the tissue [119]. Both Clara and stem cells present the $CD45^{neg}$ $CD31^{neg}$ $CD34^{neg}$ $Scal^{low}$ phenotype. However, it is possible to distinguish between the two cell types based on high (AF^{high}) and low autofluorescence (AF^{low}), respectively [120]. Clara-like cells are another cell type that exhibits many features of pluripotent stem cells and apparently contributes to epithelial regeneration [120–122]. They can be discriminated from Clara cells by their resistance to naphthalene and their close association with pulmonary neuroepithelial bodies (NEBs) [123, 124]. ATP released from secretory vesicles of rodent NEBs [125] in response to depolarization in lung slices promotes paracrine effects on surrounding Clara-like cells by activation of P2Y2 receptors. Considering the stem cell-like characteristics of Clara-like cells, this purinergic signaling might be of great importance for airway epithelial repair after injury [123].

Furthermore, ATP regulates diverse processes involved in host defense such as anion transport, ciliary function and mucin expression and is also suggested to function in wound repair [126–128]. ATP-mediated P2 purinergic receptor

activation promotes bronchial epithelial migration and epithelial repair. This is suggested to occur after activation of dual oxidase 1 mediated by release of ATP during injury [123]. In addition, adenosine also stimulates cell migration, proliferation, and angiogenesis [129, 130]. Experimental evidence suggests that adenosine evokes wound closure via A_{2A} receptor activation, since A_{2A} agonists promote early wound closure while A_{2A} antagonists impede the healing process [131]. The continuous denudation and repair of airway epithelium occurs especially in inflammatory airways diseases such as asthma [132]. Asthma is a chronic inflammatory airway disease orchestrated by eosinophils, mast cells, Th2 lymphocytes, and dendritic cells (DCs) [133]. ATP is reported to be important for the genesis and maintenance of this disease. For instance, ATP triggers and maintains asthmatic inflammation by activating DCs and enhancing its Th2-priming capacity [134, 135]. Another study demonstrated that this allergic inflammation in humans and mice is associated with the functional up-regulation of P2X7 receptor expression on immune cells (macrophages and eosinophils) and that P2X7 receptor signaling (e.g., via modulating of DC function) is involved in ATP-mediated pro-asthmatic effects [136]. P2X7 receptor −/− knock-out animals or animals treated with a selective P2X7 receptor antagonist showed a strong reduction in all cardinal features of acute allergic airway inflammation including airway eosinophilia, goblet cell hyperplasia, and bronchial hyperresponsiveness to methacholine [137]. Thus, P2X7 receptor antagonists might be a new therapeutic option for the treatment of severe asthma. Moreover, adenosine is also important in asthmatic inflammation. Inhaled adenosine induced bronchoconstriction in patients suffering from chronic asthma or obstructive pulmonary disorder (COPD), and adenosine receptor blockade prevented this bronchoconstriction [138]. Adenosine-mediated effects through A_{2B} and A_3 receptor activation play key roles in mast cells producing pro-inflammatory mediators (histamine, IL-8, and degranulation) [139, 140]. Therefore, CVT-6883, an A_{2B} receptor antagonist, is being evaluated in phase I clinical studies for the management of asthma and COPD in human patients. Mobilization of hematopoietic progenitor cells from the bone marrow comprises also a feature of asthmatic inflammation [141–143]. However, in the airway, these progenitor cells have the potential to generate in situ mature inflammatory cells, principally eosinophils [142, 144]. Moreover, it has been suggested that purinergic signaling in HSCs is important for genesis of asthma. Some studies indicate that this allergy is transferable and curable with allogeneic hematopoietic cell transplantation, but more studies are still necessary [144, 145]. In summary, for pulmonary epithelium repair, promotion of P2 purinergic receptor-mediated effects inducing bronchial epithelial migration and epithelial repair would be a valid strategy, while adenosine stimulates migration, proliferation and angiogenesis. Hematopoietic progenitor cells from the bone marrow have the potential to generate in situ mature inflammatory cells; therefore, it would be necessary to inhibit this effect while the epithelium is regenerating.

Table 1 Functions of purinergic receptor in stem cells and tissue repair

Tissue/cell	Purinergic receptors	Action
Kidney epithelial cells	↑P2R	Induction of cell migration in wounded kidney
Kidney	↑P1 A2aR	Protection during reperfusion (ischemia)
Heart	↑P1 A2A and A2BR	Anti-inflammatory function (ischemia)
Heart	↓P2Y1, P2Y2, P2Y4, P2Y6, P2Y11R	Protection during reperfusion (ischemia)
Substantia nigra	↑P2X7R	Induction of cell death (Parkinson's Disease)
Brain cortex, hippocampus and cerebellum	↑P2YR	Modulation of neurotransmitter release (healthy tissue)
Human neural progenitor cell	↑P2Y4, P2Y6R	Induction of proliferation/dopaminergic differentiation of NPCs
Brain	↑P2X7R	Cytokine secretion by microglial (increasing inflammation)
Brain	↓A1, A2AR	Protection against Aβ plaque-mediated neurotoxicity (Alzheimer's disease)
Skin	↑P2Y1, P2Y2R	Induction of proliferation / migration of basal keratinocytes (wounded tissue)
Epidermis	↑P2X5R	Induction of differentiation to keratinocytes (wounded tissue)
Epithelial pulmonary cells	↑P2Y2R	Activation of Clara-like cells for tissue repair (tissue damage)
Bronchial epithelial cells	↑P1 A2AR	Activation of cell migration and wound repair

↑ upregulation and ↓ downregulation of purinergic receptor expression

Conclusions

Stem cell transplantation and engraftment depends on the secretion of anti-inflammatory molecules, in addition to extrinsic and endogenous factors promoting differentiation into distinct cell types depending on the injury site. While adenosine receptors often, but not every time, exert beneficial effects in providing adequate stem cell niches, functions of P2Y and P2X receptors depend very much on the tissue and the expression pattern of these receptors (see Table 1). Therapeutic applications based on activation of purinergic signaling are foreseen for kidney and heart muscle regeneration, while other disease conditions will yet need further investigation. While nucleotides have been shown to promote differentiation of dopaminergic neurons destroyed in Parkinson's disease, other neuronal diseases involve excitatory cell damage mostly due to P2X7 receptor action. Therapeutic inhibition of such receptor activity would be required for improving disease conditions. Finally, the need of P2Y2 and A_{2A} receptor activation during Clara-like cell differentiation into pulmonary and bronchial epithelial cells just corroborates the fact that purinergic signaling is well involved in tissue repair, specially mediated by stem cells. More work need to be done for elucidation of crucial concepts which could revolutionize cell therapy.

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Trends in Stem Cell Proliferation and Cancer Research

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Chapter 1
An Introduction to Proliferation and Migration of Stem and Cancer Cells

Micheli Mainardi Pillat, Talita Glaser, Telma Tiemi Schwindt and Henning Ulrich

Abstract Throughout life, complex genetic systems regulate the balance between cell birth and death in response to growth and death signals. During early development, only a few cells abandon the cycle, but in several adult tissues, the cells normally do not proliferate, except during healing processes, which are supported by stem cells. However, in some adult tissues, cells continuously divide as a strategy for constant tissue renewal. In this context, cancer occurs when the control of growth and death is defective, driving the cells to an erroneous escape from death and causing intense cell proliferation. In the same way, the mechanisms and processes that coordinate cell migration are related to cell–cell contact and are important for homeostasis and constitution of the organism. Moreover, migration is a normal event during embryo development and tissue regeneration, however, when regulation of migration fails, it can lead to a diverse number of pathologies including cancer. This chapter shall introduce the reader to following specialized topics on proliferation mechanisms, written by experts in the field.

Keywords Stem cells • Cells proliferation • Cancer • Migration

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1.1 Proliferation

1.1.1 Cell Cycle

Cell cycle control must be extremely accurate, because it manages one of the most important processes in nature, the birth of a new cell. Its understanding is so important that the authors of the initial experiments elucidating the main regulators of cell cycle in eukaryotes, Leland H. Hartwell, Tim Hunt and Sir Paul Nurse, were awarded with the Nobel Prize in Physiology or Medicine in 2001. Basically, the cell cycle is divided into **four phases** (shown in Fig. 1.1). Two of these phases are responsible for the execution of the basic events in cell division: duplication of its genetic material (the synthetic phase or S phase) and partition of the cellular components into two identical daughter cells (mitosis or M phase). The other two phases of the cycle are gap periods (G1 and G2 phases), when cells prepare themselves for the subsequent initiation of the S and M phases. Cells may exit the cycle and stay in a non-dividing state known as quiescent state called G0. Stem cells are often found in this state.

The passage from one phase to the next is regulated by **cyclins** and **cyclin-dependent kinases** (CDKs; Fig. 1.1). Cyclins are proteins synthesized and degraded throughout the cell cycle at specific checkpoints. They gain their regulatory role by activating CDKs. On the other hand, CDKs are serine/threonine kinases that form active heterodimeric complexes after binding to cyclins. There are several cyclins and CDKs involved in cell cycle control: 3 interphase CDKs (CDK2, CDK4 and CDK6); 1 mitotic CDK (CDK1, also known as cell division control protein 2 or

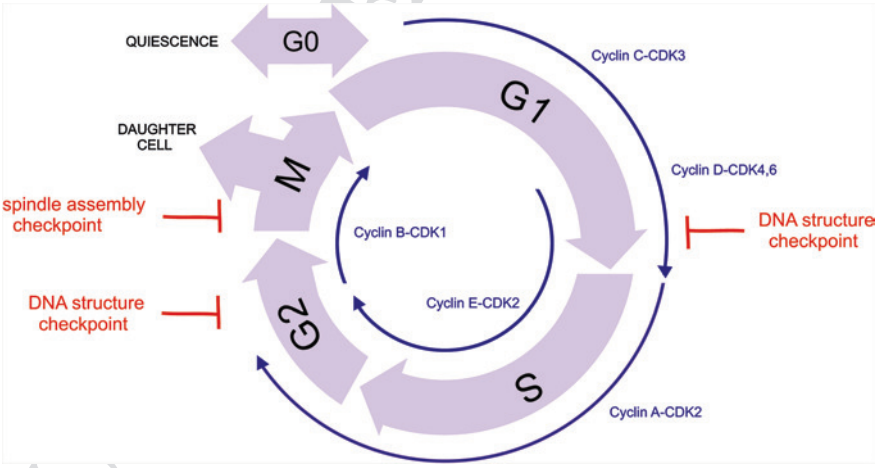

Fig. 1.1 Cell cycle: phases, cyclins, cyclin-dependent kinase (CDKs) activities and checkpoints. Phases of cell cycle: *G1* gap 1 phase, *S* synthesis phase, period of DNA replication, *G2* gap 2 phase, *M* mitosis phase, period of chromosome separation and cytokinesis, *G0* resting phase. Cyclin-CDK activities during individual phases. Three major checkpoints are shown

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CDC2); 10 cyclins belonging to four different classes (the A-, B-, D- and E-type). The cyclin-CDK complexes regulate the activities of several proteins involved in DNA replication and mitosis by phosphorylating them, activating some and inhibiting others in a coordinated manner. It is also important to emphasize here that tumor associated mutations frequently deregulate some cyclin-CDK complexes (Morgan 1997; Malumbres and Barbacid 2009; Deckbar et al. 2011).

The high fidelity of cell division is due to **checkpoint** surveillance mechanisms (Fig. 1.1). It ensures that one phase of cell cycle is not initiated until the previous process has been successfully completed. There are checkpoints at S and M phase initiation and during the M phase (anaphase, telophase and cytokinesis onset). DNA damage, for example, activates checkpoint mechanisms to target the cyclin-CDK complexes, interrupting cell cycle progression to provide extra time for damage removal. Mitotic spindle checkpoints, for example, are activated indirectly by sensing the consequences of the damage, such as incorrect alignment at the equatorial plane or impaired formation of the spindle fibers (Hartwell and Weinert 1989; Malumbres and Barbacid 2001; Deckbar et al. 2011).

All cells are able to enter **quiescent** state and to return to the cycle, except those in terminal differentiation, depending on extra- and intracellular scenarios that control this important decision. Quiescent and newly divided cells probably also suffer certain checkpoints before they return to cell cycle, for instance, verification of the homeostatic size of the cell and influence of the size of the tissue. These parameters are mainly regulated by extracellular signals, like the availability of nutrients and the intensity of growth and mitogenic stimuli. Conversely, cancer cells lose the normal control in replication and can proliferate in conditions that a normal cell could not, for example, in the presence of DNA damage and in the absence of mitogenic stimuli.

1.1.2 Cell Division Signaling in Cancer

Tumors display mutations in genes encoding proteins involved in the regulation of proliferation, survival and apoptosis. To emphasize the role of these genes in cancer development, they are termed proto-oncogenes or tumor-suppressor genes, two broad classes of genes implicated in carcinogenesis (Table 1.1). Proteins encoded by **proto-oncogenes** are components of intracellular networks involved in survival, proliferative and anti-apoptotic processes. Proto-oncogenes change to oncogenes by mutations that generate hyperactive proteins and control-resistant proteins. On the other hand, proteins encoded by **tumor-suppressor genes** have anti-proliferative, pro-apoptotic and pro-differentiation roles. In this case, carcinogenesis and tumor growth can only occur if a mutation in a tumor suppressor gene occurs, leading to inactive forms of the protein. BMI-1 (B lymphoma Mo-MLV insertion region—it belongs to the polycomb group of epigenetic chromatin modifier), for example, is encoded by a proto-oncogene, whose expression is deregulated in certain types of cancer (Haupt et al. 1991; Cui et al. 2007). BMI-1 is required for the proliferation of leukemia initiating cancer stem cells, and it is also highly expressed in cancer neural stem cells and in medulloblastomas arising from

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Table 1.1 Examples of proto-oncogenes and tumor suppressor genes. Mutations in these genes may cause cancer

Proto-oncogenes		Tumor suppressor genes	
Erb2	EGF receptor	PTEN	PIP-3 phosphatase
Ras	Small G-protein	Rb	Retinoblastoma tumor suppressor protein
Akt	Ser/thr kinase	APC	Inhibitor of β-catenin signaling
Src	Tyr kinase	ARF	Indirect inhibitor of cell cycle
SKP2	S-phase kinase-associated protein 2	p53	Transcription factor
Jun/Fos	Formation of the AP-1 early response transcription factor	p16	Cyclin-dependent kinase inhibitor 2A
Myc	Transcription factor		
BMI-1	Belongs to the polycomb group of epigenetic chromatin modifier		

cerebellar granule precursor cells (Lessard and Sauvageau 2003; Leung et al. 2004; Grinstein and Wernet 2007).

1.1.3 Stem Cell Proliferation and Cancer Induction

Adult stem cells are important for tissue homeostasis and regeneration. They have the ability to self-renew indefinitely and to give rise to transit amplifying (TA) cells, capable of differentiating into tissue specific cells (Reya et al. 2001). Stem cells are usually quiescent, and this behavior has an important role in protecting them from exhausting their proliferative capacity, and in decreasing mutations that occur during DNA synthesis (Sang et al. 2008; Moore and Lyle 2011). However, stem cells can quickly proliferate in response to stressors. Hematopoietic stem cells, for example, proliferate in response to chemotherapy or bone marrow transplantation in order to rapidly originate progenitors, differentiated cells, and additional stem cells, which after that, return to quiescent state (Dixon and Rosendaal 1981).

Proliferation of stem cells is highly controlled to prevent cancer development, since they have the capacity to accumulate mutations over years or decades. It is expected that, as occurs to normal stem cells, cancer stem cells (CSCs) generate oncogenic TA cells, being capable to expand tumor mass and give rise to heterogeneous tumor populations (Reya et al. 2001; Moore and Lyle 2011). CSCs were discovered in acute myelogenous leukemia (Bonnet and Dick 1997) and, nowadays, proof for their existence has been provided for other neoplasias, such as breast, prostate, and colon tumors and glioblastomas (Al-Hajj et al. 2003, Singh et al. 2003; Collins et al. 2005). Quiescent CSCs are important targets for cancer therapy, since they are usually resistant to chemotherapy, due to their state of dormancy. In other words, CSCs do not die during continued therapy and contribute to cancer recurrence (Dick 2008; Li and Bhatia 2011). Expanding the knowledge of the mechanisms of stem cell quiescence and proliferation is not only important for the understanding normal stem cell function, but also for developing therapeutic approaches to exterminate quiescent CSCs.

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1.2 Cell Migration

1.2.1 Mechanisms

Besides connective tissues, cells are tied together by cell–cell contact, where cytoskeletal filaments are anchored transmitting stresses across the interior of the cells. There are 2 types of cell contact: adherens junctions, which anchorage actin filaments and desmosome junctions, which attach to intermediate filaments. Transmembrane adhesion proteins link cytoskeleton to extracellular structures, and are divided into 2 super families: cadherin, that attaches cell to cell; and integrin, that attaches cells to the matrix.

Cells must acquire a spatial asymmetry to enable them to migrate. First, there is a change to polarized morphology due to spatial or temporal stimulus gradients caused by microscopic nonuniformities or by kinetic fluctuations in receptor–ligand binding followed by a forward redistribution of chemosensory signaling receptors (Sullivan et al. 1984), integrin adhesion receptors (Maxfield 1993), and integrin cytoskeleton linkages.

During migration, protrusion of lamellipodia or filopodia membranes requires actin polymerization-generated force (Fig. 1.2), by the Brownian ratchet mechanism and/or the cortical expansion mechanism. Once the membrane protrusion has become adherent to the substrate, forward translocation of cell body may occur by myosin interactions with actin filaments, such as contraction of filaments connecting cell–substratum adhesion complexes with intracellular structures, or relative movement of adhesion complexes across cortical actin filament tracks. In both cases, the traction magnitude is greater than the rearward pull on the adhesion complexes. Detachment of the cell rear involves disruption of cell–substratum attachments, accelerated by myosin-mediated actin filament contraction pulling on

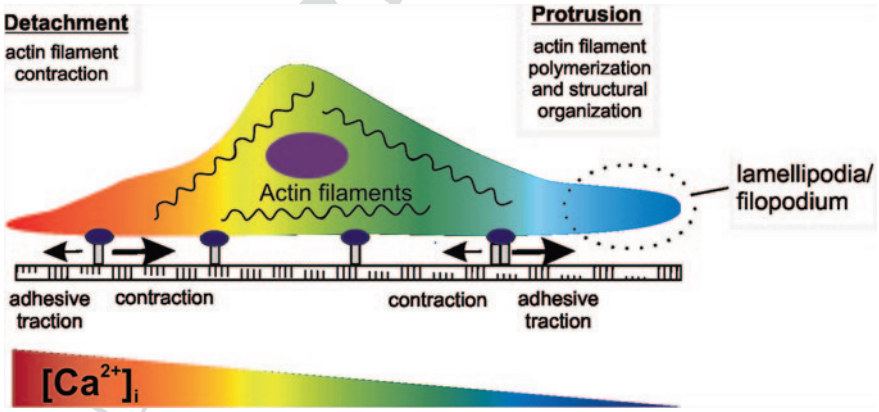

Fig. 1.2 Basic mechanism of cell migration. The actin-polymerization-dependent protrusion and firm attachment of lamellipodium. Contraction at the rear of the actin propels the body of the cell forward to relax some of the tension (traction). High intracellular calcium concentration ([Ca2+] i) at the cell rear contributes to the migration process

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adhesion complexes. Subsequently, the magnitude of traction is less than the contraction force in the rear.

Importantly spatial concentrations, or localized temporal concentration transients of second messengers, such as calcium and phosphoinositides, or even enzymes and motor proteins are distributed differentially across the cell length. As one simple regulation mechanismus, high calcium concentrations at the cell rear activate proteins that disrupt actin filament networks (Janmey 1994) and enhance myosin II contractile activity to promote the release of attachments there (Conrad et al. 1993; Maxfield 1993). Low calcium concentrations and high phosphoinositide levels at the cell front would activate proteins that cross-link actin filaments, facilitating membrane extension (Janmey 1994); myosin I activity at the cell front (Conrad et al. 1993) might additionally permit directed membrane-protein transport activity to promote formation of new attachments, or contraction to pull cell body structures forward (Sheetz 1994; Lauffenburger and Horwitz 1996).

1.2.2 Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) is the process that enables the derivation of a multitude of functionally specialized cells, tissues and organs from an initial small variety of pluripotent stem cells present in the developing embryo. EMT is most commonly observed in the wound healing response and in angiogenesis, where an endothelial-mesenchymal transition occurs. When epithelial cells in tissue culture undergo EMT, phenotypic alterations include is the loss of cell–cell contact and the change in expression or function of proteins involved in cell–cell adhesion, given that, in development, one of the primary functions of EMTs is to facilitate cell dispersion. Cell–cell adhesion is mediated by the cadherin-catenin based adherens junctions (AJ) keeping adjacent epithelial cells together. AJ also keep the cells of the epithelia in a nonmotile and non-proliferative state. It is comprised of a transmembrane-spanning receptor, E-cadherin that binds to another E-cadherin molecule in adjacent cells in a homophilic interaction. The intracellular domain of E-cadherin is linked to the actin cytoskeleton. E-cadherin levels on the plasma membrane can be regulated by epigenetic, transcriptional and post-translational mechanisms (Khew-Goodall and Wadham 2005).

Post-translational mechanisms shown to regulate cell–cell adhesion include tyrosine phosphorylation and relocalization of E-cadherin away from the plasma membrane. Moreover, tyrosine phosphorylation of cadherins or catenins leads to dissociation of the AJ to decrease cell–cell adhesion (Ayalon and Geiger 1997; Ozawa and Kemler 1998a, b; Roura et al. 1999). Therefore, regulation by phosphorylation would be expected to be highly reversible and lead to a transient loss of cell–cell adhesion, unless secondary events occur that either prevent dephosphorylation or increase turnover of the phosphorylated proteins.

A number of transcription factors capable of repressing E-cadherin transcription, namely Snail (Batlle et al. 2000), Slug (Bolos et al. 2003), ZEB1

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(Grooteclaes and Frisch 2000) and ZEB2 (Comijn et al. 2001), have been found to be upregulated during EMT. These transcription factors can repress certain epithelial genes such as E-cadherin and cytokeratin-8 while increasing the expression of mesenchymal genes. Furthermore, Snail (-/-) mice fail to complete gastrulation due to a defective EMT (Carver et al. 2001). The dual role of Snail, to shut down expression of the epithelial genes and turn on expression of mesenchymal genes, leads to the idea that it could be an EMT inducer.

1.2.3 Stem Cell Migration and Cancer

Cancer environment is comprised of tumor cells and a wide network of stromal and vascular cells participating in the cellular and molecular events necessary for invasion and metastasis. Tumor secretory factors can activate the migration of host cells, both near to and far from the primary tumor site, as well as promote the exodus of cells to distant tissues. Thus, the migration of stromal cells and tumor cells among specialized microenvironments takes place throughout tumor and metastatic progression, providing evidence for the systemic nature of a malignancy.

Mesenchymal stem cells (MSCs) are recruited from bone marrow to inflammation or damage areas by local endocrine signals, resulting in the formation of fibrous scars. Tumor tissue contains abundant growth factors, cytokines and matrix-remodeling proteins, explaining why tumors are linked to wounds that never heal. MSCs are reported to migrate to injury or tumor sites and to incorporate into tumor stroma, but the effects of the interactions between MSCs and tumor cells, as well as the mechanisms underlying these effects, remain unclear. Recent experiments revealed that MSCs promote tumor growth and metastasis. Reports suggest that MSCs are involved in tumor invasion and angiogenesis, immunosuppression and inhibition of apoptosis. Moreover, some studies reported that MSCs can differentiate into carcinoma-associated fibroblast (CAF)-like cells by prolonged exposure to tumor-conditioned medium and that these cells promote tumor growth (Shinagawa et al. 2010).

EMT in the adult is now recognized to be the forerunner to a number of pathological states. In the progression of epithelial tumors to metastatic disease, EMTs give rise to a cell type that is beyond recognition as an epithelial cell and confers it the ability to proliferate and invade the basement membrane and surrounding stroma. Epigenetic mechanisms include methylation of the E-cadherin gene, which has been observed in a number of human cancers (Khew-Goodall and Wadham 2005).

Ovarian cancer is the most lethal of all gynecological malignancies, and the identification of novel prognostic and therapeutic targets for ovarian cancer is crucial. It is believed that only a small subset of cancer cells is endowed with stem cell properties, which is responsible for tumor growth, metastatic progression and recurrence. NANOG is one of the key transcription factors essential for maintaining self-renewal and pluripotency in stem cells. Siu and coworkers demonstrated

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that NANOG was highly expressed in ovarian cancer cell lines with metastasis-associated property and in clinical samples of metastatic foci. Stable knockdown of NANOG expression prevented ovarian cancer cell proliferation, migration and invasion, which was accompanied by an increase in mRNA expression of E-cadherin, caveolin-1, FOXO1, FOXO3a, FOXJ1 and FOXB1. Conversely, ectopic NANOG overexpression enhanced ovarian cancer cell migration and invasion along with decreased E-cadherin, caveolin-1, FOXO1, FOXO3a, FOXJ1 and FOXB1 mRNA expression. Importantly, NANOG mediates cell migration and invasion and is involved in the regulation of E-cadherin and FOXJ1 expression (Siu et al. 2012).

The identification of the restrictive mechanisms that prevent the triggering of cellular transitions in adult organisms in cancerous tissues, may lead to the development of tools for therapeutic tissue repair and effective tumor suppression (Prindull and Zipori 2004).

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3.3 Artigo 3

ECTO-5'NT/CD73 OVEREXPRESSION REDUCES THE TUMOR GROWTH IN XENOGRAPH MEDULLOBLASTOMA MODEL BY INDUCE SENSITIZATION OF A_{2A} ADENOSINE RECEPTOR

Angélica Regina Cappellari; Micheli Mainardi Pillat; Hellio Danny Nóbrega de Souza; Liliana Hockenbach; Elizandra Braganhol, Francine Hehn de Oliveira; Ana Lúcia Abujamra; Rafael Roesler; Jean Sévigny; Henning Ulrich; Ana Maria Oliveira Battastini

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ABSTRACT

Ecto-5'nucleotidase/CD73 (CD73) is an important enzyme that converting AMP into adenosine in the extracellular medium. It is importantly described to influence the cancer metabolism in different kinds of tumors favoring the tumor progression and modulating invasiveness events. However, CD73 has been suggested as favorable prognostic marker to breast carcinoma attributing to this enzyme contradictory functions depending on the kind of tumor in which it is expressed. Medulloblastoma (MB) is the most common brain tumor that occurs at the cerebellum and affects mainly children with median age of 9 years old. Additionally it is considered a tumor highly aggressive. The present work was aimed to investigate the role of CD73 in MB cancer progression overexpressing this enzyme in D283 human MB cell line and evaluate its participation in the tumor growth in an *in vivo* xenograph model. Firstly we could observe that the CD73 overexpression successful where the D283hCD73, that received the sequence of this enzyme, presented the elevated mRNA expression as well a high protein expression accompanied by a prominent AMPase activity. D283ev, the transfection control was negative for CD73 expression. After this, the MB cell lines were implanted in a subcutaneous xenograph animal model and the data demonstrated that D283hCD73 promoted a reduction in the tumor growth when compared to control D283ev. The pathological analysis of tumor slices demonstrated that the samples presented characteristics of human MB. In addition, the analysis of P1 adenosine receptors expression were performed in Daoy and D283 MB cell lines, which expressed significantly levels of A1 and A2A adenosine receptors, respectively. In conclusion, this work presented that the modulation of CD73 expression in the tumor that did not express this enzyme can favoring the reduction of the tumoral growth in MB. Additionally, knowing that the main function of CD73 is adenosine production, we suggested that the sensitization of A_{2A} adenosine receptor might be involved in the reduction of tumor growth.

Key words: Ecto-5'-nucleotidase, CD73, adenosine, A_{2A}, medulloblastoma.