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. Here we describe novel functions for bradykinin with therapeutic relevance.

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...
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 CO2 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 × 105 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% CO2.

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 × 105 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% CO2. Cultures were grown for 10 days with one passage prior to neuronal 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 embryonal carcinoma cells were grown and differentiated as described previously (19, 21). In brief, for the induction of neuronal differentiation, 1 µM all-trans-retinoic acid was added to 5 × 105 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 mM 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-
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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 added at 1:500 dilutions. After washing with PBS, DAPI solution (0.3 μg/ml) was used as a nuclear stain. Coverslips 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 × 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 × 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 neuronal 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 Cytlogic 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 ± 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 Cytlogic 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× 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-
teration of the specificity of the amplicon produced in each reaction. A distinct peak indicated that a single DNA sequence 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.

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 $2^{-\Delta\Delta C_{T}}$ 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 the 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 ($F_{\text{max}}$) and 10 mM EGTA ($F_{\text{min}}$) to provide an estimation of the absolute change in the intracellular calcium concentration using the following equation: $[Ca^{2+}]_{i} = K_{d} (F - F_{\text{min}})/(F_{\text{max}} - F)$; 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—2 °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 substrates at 4 °C. B2BkR sense ($5^{\prime}$-GGACCTCCTACACACACAGGCAAC-3') and antisense ($5^{'-}GGACAACGGGTCTCC-AGTG-3'$) probes were generated by linearization and in vitro transcription of pBluescript II KS-B2BkR (NM_009747.2) with XbaI/T3 and Xhol/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
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. 1A). 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 $\beta$3-tubulin, and astrocyte-specific s100$\beta$ were expressed at high levels in differentiated cells (Fig. 1B). Cells elongated in a radial pattern with intense staining for GFAP and nestin. Network-forming differentiated cells were located most distally (Fig. 1C). Double-immunostaining against MAP-2 and B2BkR on day 7 of differentiation revealed that the B2BkR are expressed in mature neurons. 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.

**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$\beta$), and neurons ($\beta$3-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.
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. 2A). Chronic treatment of differentiating rat neurospheres with HOE-140, a specific antagonist of the B2BkR, significantly decreased the gene expression of B2BkR (Fig. 2B). 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. 2C). 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. 2D). 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. 2E).

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 1695 ± 1100 nM (p = 0.0371) and from 1320 ± 126 to 703 ± 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 2023 ± 304 nM were reduced to 243 ± 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
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**FIGURE 3.** Effects of chronic B2BkR blockade on neurotransmitter-induced \([\text{Ca}^{2+}]\), transients in differentiated rat neurospheres. A, representative images following stimulation by 100 \(\mu M\) ATP, 100 \(\mu M\) UTP, 1 \(\mu M\) Bk, 100 \(\mu M\) muscarine, or 100 \(\mu M\) glutamate in cells differentiated for 7 days in the absence or presence of 1 \(\mu M\) HOE-140. HOE-140 was removed from cell cultures by medium change and washing the cell layers five times 1 h before 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 \([\text{Ca}^{2+}]\), transients. Arrows indicate the time point of agonist application (\(F_0\) values represent basal \([\text{Ca}^{2+}]\), levels of nonstimulated cells). C, mean values of \([\text{Ca}^{2+}]\), peak amplitudes in differentiated neurospheres pre-treated or not with 1 \(\mu M\) 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 \(\mu M\) 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 Cylogic software. Cell death measured by the TUNEL assay was not significantly altered in the presence of 1 \(\mu M\) HOE-140 (\(n = 2\)). Scale bars = 20 \(\mu M\). B, immunodetection of BrdU (0.2 \(\mu M\)) after a 14-h pulse in differentiated neurospheres in the presence of 1 \(\mu M\) Bk in the absence or presence of 1 \(\mu M\) 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 \(\mu M\).

**Kinin-B2 receptors** followed by wash-out of the antagonist prior to calcium measurements (control = \(Δ956 ± 109 \text{nM}\); treated with HOE-140 = \(Δ407 ± 59 \text{nM}\)) (\(p = 0.0058\)). The observed changes did not affect all signaling systems as no significant changes in glutamate-induced \([\text{Ca}^{2+}]\), peak amplitudes in differentiated neurospheres pre-treated or not with 1 \(\mu M\) 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, \(p = 0.1688\)).

**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 \(\mu M\) HOE-140 (Fig. 4) and analyzed by TUNEL staining. Flow cytometry analysis revealed that the chronic treatment with 1 \(\mu M\) HOE-140 did not affect the number of TUNEL\(^+\) cells when compared with control experiments (5.1 \(±\) 0.3% TUNEL\(^+\) cells) (Fig. 4A). The effect of B2BkR blockade on cell proliferation was analyzed by the BrdU incorporation assay (Fig. 4B). Approximately 11% of the cells on day 7 of differentiation were proliferative. Similar values were obtained in cells co-treated with 1 \(\mu M\) HOE-140 and 1 \(\mu M\) 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-glial 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;
NPCs presented a radial migration pattern closely linked to a gradient of maturation (Fig. 5). Fig. 5A 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.

### 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.** Relative expression mean ± S.D. of β3-tubulin, GFAP, and Nestin. 
- **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 presented are representative of at least two independent experiments (n = 3). 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.

### Table 1

| Treatment          | Mean ± S.D. | Median | Mean ± S.D. | Median | Mean ± S.D. | Median | Mean ± S.D. | Median |
|--------------------|-------------|--------|-------------|--------|-------------|--------|-------------|--------|
| **β3-Tubulin**     | 69.4±7.3    | 23     | 59.8±3.9    | 57     | 38.0±2.7    | 68     | 22.1±6.5    | 12     |
| **GFAP**           |             |        |             |        |             |        |             |        |
| **Nestin**         |             |        |             |        |             |        |             |        |
| **NSE**            | 87.3±6.0    | 39     | 78.6±4.1    | 71     | 20.8±1.2    | 42     | 26.3±7.3    | 11     |
| **β3-Tubulin**     | 34.5±5.1    | 11     | 28.3±0.2    | 10     | 18.7±5.3    | 136    | 11.8±5.2    | 13     |
| **GFAP**           |             |        |             |        |             |        |             |        |
| **Nestin**         |             |        |             |        |             |        |             |        |
| **β3-Tubulin**     |             |        |             |        |             |        |             |        |
| **GFAP**           |             |        |             |        |             |        |             |        |
| **Nestin**         |             |        |             |        |             |        |             |        |

**E.** Flow cytometry analysis of GFAP, β3-tubulin, neuronal specific enolase (NSE), and nestin expression in mouse neurospheres differentiated for 7 days in the presence of Bk, HOE-140 or captopril. 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 presented 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.
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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. 5B). 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. 5C). 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 (notch1 and Stat3), whereas in the presence of Bk a significant difference of NeuroD1 expression was obtained compared with the control group (Fig. 5D). Conversely, Ngn1 expression levels were decreased with HOE-140 treatment and notch1 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. 5E). 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. 5F).

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 stages 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 increased 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-
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Mice were differentiated in the presence of 1 μM Bk activation, neurospheres from the B2BkR knock-out mice 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 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. 8A). 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-Arg⁹]-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. 8B). 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<sup>−/−</sup> 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<sup>−/−</sup> mice to obtain neurospheres allowed further study of the process of cell differentiation and neural migration. To verify the homoygosis 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<sup>−/−</sup> 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<sup>−/−</sup> 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. 9A. In addition, immunocytochemical analysis revealed the same pattern characterized mainly by radial GFAP<sup>+</sup> cells and a low migration of β3-tubulin<sup>+</sup> cells in B2BkR<sup>−/−</sup> mice neurospheres (Fig. 9B). The immunostaining also reveals less β3-tubulin<sup>+</sup> cells (~72%) and a high content of GFAP<sup>+</sup> cells (47%) in B2BkR<sup>−/−</sup>.

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,
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. 10A), then increasing their expression pattern to the whole nervous system at days E11.5 (Fig. 10B) and E12.5 (Fig. 10C). Negative controls with B2BkR sense probes did not reveal any specific labeling (Fig. 10D). 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 /H92523-Tubulin in the telencephalon and cortex at several time points during WT and B2BkR−/− mice development (E9.5-adult) (Fig. 10E). The developing knock-out mice brains showed significantly less expression of β3-Tubulin from E11.5 until adulthood (*, p < 0.05; adult, p = 0.0083; 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. 10F). 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
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During mouse embryo development and neuronal marker expression in telencephalons 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).

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.

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.
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![Diagram of neural differentiation](image)

**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 neuronal 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 of 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 [Ca²⁺] 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 to 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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