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TRANSLATIONAL AND CLINICAL RESEARCH

Isolation of Neural Crest Derived Chromaffin Progenitors from Adult Adrenal Medulla

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ABSTRACT

Chromaffin cells of the adrenal medulla are neural crest-derived cells of the sympathoadrenal lineage. Unlike the closely-related sympathetic neurons, a subpopulation of proliferation-competent cells exists even in the adult. Here, we describe the isolation, expansion, and *in vitro* characterization of proliferation-competent progenitor cells from the bovine adrenal medulla. Similar to neurospheres, these cells, when prevented from adherence to the culture dish, grew in spheres, which we named chromospheres. These chromospheres were devoid of genes specific for smooth muscle cells (MYH11) or endothelial cells (PECAM1). During sphere formation, markers for differentiated chromaffin cells, such as phenyletanolamine-N-methyl transferase, were down-regulated while neural progenitor markers nestin, vimentin, musashi 1 and nerve growth factor receptor, as well as markers of neural crest progenitor cells such as Sox1 and Sox9 were up-regulated. Clonal analysis and BrdU-incorporation analysis demonstrated the self-renewing capacity of chromosphere cells. Differentiation protocols using NGF and BMP4, or dexamethasone induced neuronal or endocrine differentiation, respectively. Electrophysiological analyses of neural cells derived from chromospheres revealed functional properties of mature nerve cells, such as tetrodotoxin-sensitive sodium channels and action potentials. Our study provides evidence that proliferation and differentiation competent chromaffin progenitor cells can be isolated from adult adrenal medulla that might harbour the potential for the treatment of neurodegenerative diseases, e.g. Parkinson's disease.

INTRODUCTION

Chromaffin cells in the adrenal medulla are neuroendocrine cells derived from neural crest. Together with the sympathetic neurons of the dorsal ganglia and the intermediate small intensely fluorescent cells, they constitute the sympathoadrenal lineage (SA) of neural crest derivates [1]. All these cells derive from common SA progenitor cells that migrate during early embryogenesis and acquire along their migratory route the specific characteristics of mature catecholamine-producing cells [1, 2]. However, unlike sympathetic neurons, the cells from adrenal medulla are able to proliferate throughout life [3-6]. In addition, chromaffin cells can be distinguished from mature sympathetic neurons by the expression of epinephrine-synthesizing

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enzyme phenylethanolamine-*N*-methyltransferase (PNMT) and the lack of neurofilament (NF) and neuritic processes *in vivo* [7]. Nevertheless, early postnatal rat adrenal chromaffin cells could be "transdifferentiated" into neuron-like cells with characteristic neurite outgrowth by exposure to nerve growth factor [8-10]. The existence of multipotential SA progenitor cells within the adrenal anlagen and the adult adrenal medulla has been suggested by the observation that in birds and mammals (including humans), adrenal chromaffin cells co-express catecholaminergic and neural markers [7, 11-14].

Due to their close relation to sympathetic neurons, their plasticity, and the restorative neurotrophic growth hormones secreted from chromaffin cells, autologous transplantation of adrenomedullary chromaffin cells has been suggested as an ideal therapy for neuron regeneration in neurodegenerative diseases such as Parkinson's disease and in pain therapy [15-17]. Indeed, improvement of clinical symptoms after adrenal medulla transplantation in a substantial number of Parkinson patients has been described worldwide; 390 patients received autologous adrenal autotransplants. With the previous protocols, however, long-term survival and functional efficacy of adrenal chromaffin cell grafts were insufficient in the brain [15, 17]. More recently, the use of fetal chromaffin cells, their isolation and differentiation for subsequent use in pain therapy, has been suggested, underlining the enormous therapeutic potential of these cells [14, 18]. Human fetal adrenal tissue, however, is limited, or, for ethical reasons, unavailable; however, the isolation and propagation of progenitors from the adult adrenal medulla is another strategy worth considering [19].

The purpose of the present study was to isolate chromaffin progenitor cells from the adult adrenal medulla. Elucidating the existence and characteristics of chromaffin progenitor cells in adult adrenal medulla should help in understanding the development and differentiation of the sympathoadrenal system. Furthermore, mastering the isolation of chromaffin progenitor cells and controlling their secretory capacity and survival will open new avenues for regenerative therapies.

MATERIALS AND METHODS

Cell Preparation

Adrenal glands from adult cattle were freshly obtained from the local slaughterhouse and transported to the laboratory in ice-cold PBS. Primary chromaffin cells were isolated as previously described [3, 20]. Following perfusion with 0.3% type II collagenase (Sigma-Aldrich, Saint Louis, MO, USA, http://www.sigmaaldrich.com/) and 30 units/ml DNase I (Sigma-Aldrich) injected through the central vein, the tissue was incubated at 37°C for 3 x 15 min. Cells from the adrenal medulla were separated by mechanical dissociation, sieved through 100 µm cell strainers and washed twice with PBS. Primary chromaffin cells were then cultured in DMEM/F12 (Gibco, Invitrogen, Carlsbad, CA, USA, http://www.invitrogen.com/) containing 10% steroid-free FBS (Charcoal/Dextran treated; Hyclone, South Logan, UT, USA, http://www.hyclone.com/) and 1% antibiotic-antimycotic solution (Gibco) in DMEM/F12 medium. The cells were kept at 37°C overnight in a humidified atmosphere (95% O_2, 5% CO_2). Chromaffin cells were then separated from non-chromaffin cells by differential plating [20-22]. This method takes advantage of the fact that chromaffin cells adhere to the plastic surface slower than most other cells in the suspension. The cell suspension was moved to a new flask 3 times every 1.5 hr, thereby removing most non-chromaffin cells. In our hands, the purity of the isolated medullary chromaffin cells is 97.5% [20]. The suspended chromaffin cells were then seeded at a density of 7000 cells/ml in the same medium in ultra low-attachment plates (Corning, Lowell, MA, USA, http://www.corning.com/) where they grew as nonadherent spheres. After 14 days of cultivation, chromospheres were collected for subsequent analysis using a 40 µm cell strainer.

Proliferation Analysis and Clonal Growth

The ability of chromosphere cells to self-renew was analyzed by culturing the cells at clonal density. Cells dissociated from chromospheres

were seeded into 96-well ultra low-attachment plates as single cell per well, using a FACS sorter (FACSAria, BD Biosciences, Erembodegem, Belgium, http://www.bdbiosciences.com/). The existence of only one cell per well was verified by microscopy; wells containing more than one cell were excluded from the analysis. After 2 weeks in culture, secondary chromosphere that had formed from single cells were counted by light microscopy (Axiovert200, Carl Zeiss, http://www.zeiss.com).

Electron Microscopy

For electron microscopical evaluation chromospheres were fixed in 2% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, postfixed for 90 min (2% OsO_4 in 0.1 M cacodylate buffer, pH 7.3), dehydrated in ethanol, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and were examined at 80 kV in an electron microscope (Philips CM-10, FEI Company, Hillsboro, OR, USA, http://www.research.philips.com/).

BrdU Incorporation and Immunostaining for Vimentin

To reveal proliferating progenitor cells within chromospheres double staining of 5-bromo-2'-deoxyuridine (BrdU) incorporation and vimentin was used. Two weeks old chromospheres were collected by centrifugation at 200 *g* for 8 min, and dissociated with Pasteur glass pipettes. The dissociated cells were cultured in 10% steroid-free FBS and 1% antibiotic-antimycotic in DMEM/F12 medium containing 10 µM BrdU (Sigma-Aldrich). After 6 hrs, 12 hrs, 24 hrs, 48 hrs or 72 hrs treatment, BrdU was removed by washing the cells twice with DMEM/F12. The cells were further cultured for 1 week in DMEM/F12 (10% FBS, 1% antibiotic-antimycotic) without BrdU. The newly formed chromospheres were collected, embedded in OCT medium (Tissue-Tek, Sakura, Zoeterwoude, Netherlands, http://www.sakura.eu/), frozen on dry ice, and 7 µm sections were cut. The cryosections were fixed in 4% PFA for 30 min at room temperature. After treating the sections with 0.3% Triton X100 in PBS for 30 min, autofluorescence was quenched with 0.1 M Glycine buffer for 30 min. The slides were then incubated with mouse monoclonal anti-vimentin (1:500, Abcam, Cambridge, UK, http://www.abcam.com/) overnight at 4 ºC. After washing, slides were incubated for 1 hr at room temperature with goat anti-mouse IgG conjugated with Alexa488 (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA, USA, http://www.jacksonimmuno.com/) together with DAPI (1:1000, 4',6-diamidino-2-phenylindole, Invitrogen). Slices were post-fixed with 4% PFA for 20 min at room temperature. After quenching with glycine buffer for 15 min, slides were incubated with 2 N HCl at 37ºC for 30 min to denature DNA. After washing with PBS, the slides were incubated with a direct anti-BrdU antibody conjugated to Rhodamine (1:10 in PBS containing 10% horse serum, MPI-CBG, Dresden, Germany, http://www.mpi-cbg.de/) for 2.5 hrs at room temperature. The slides were washed with PBS, mounted with fluorescent mounting medium (DakoCytomation, Glostrup, Denmark, http://www.dako.com/), and examined by microscopy (Axio Imager Z1, Carl Zeiss).

RT-PCR and Real-Time PCR Analysis

Harvested chromospheres or differentiated cells were collected and total RNA was isolated using RNeasy Plus Mini kits (Qiagen, Hilden, Germany, http://www.qiagen.com/) according to the manufacturer's instructions. Reverse transcription was performed with the reverse transcription buffer, RNase inhibitor, oligo$(dT)_{15}$ primer, dNTP mix, and M-MLV reverse transcriptase at 37°C for 60 min according to the manufacturer's specifications (Promega, Madison, WI, USA, http://www.promega.com/). The PCR reactions were carried out with initial denaturation at 94°C for 4 min; 25-35 cycles amplification at 94°C for 20 sec, annealing for 20 sec (temperatures in Table 1), and elongation at 72°C for 20 sec to 1 min (according to the expected product sizes), followed by final extension at 72°C for 4 min. Detailed information on all primers is listed in Table 1.

To generate the standard curve for real-time PCR quantification, DNA fragments were

amplified for nestin, TH (tyrosine hydroxylase), DDC (DOPA decarboxylase), DBH (dopamine ß-hydroxylase), PNMT, B3T (ß-III-tubulin) and GAPDH. PCR products were cloned into plasmid vectors (pCRII-TOPO) and transformed into competent *E. coli* using the TOPO TA Cloning kit (Invitrogen). Plasmids were purified using a Maxi Plasmid kit (Qiagen). Concentrations of plasmids were measured by spectroscopy and a linear regression standard curve was created by serial dilutions of plasmid. PCR reaction contained 2 µl of cDNA sample, 5 µl SYBR Green PCR master mix (Qiagen), 5 pmole of sense primer and 5 pmole of anti-sense primer, and proceeded on a Light Cycler 1.5 (Roche, Basel, Switzerland, http://www.roche.com/). The expression level of each gene was calculated by relative quantification to the expression of the reference gene GAPDH.

Single Cell RT-PCR

Chromospheres were mechanically dissociated into single cells and resuspended in DMEM/F12 medium. Cells were individually picked under the microscope by aspiration through a finely drawn capillary with 6 µl water, and were transferred to PCR tubes containing 0.5 µl (20 units) RNAase inhibitor and 0.5 µl water. Reverse transcription was performed at 37°C incubation overnight after adding 3 µl master mixture which contained 2 µl reverse transcription buffer, 0.25 unit M-MLV reverse polymerase, 0.25 µl oligo$(dT)_{15}$ primer mix and 0.5 µl dNTPs according to the manufacture's specifications (Promega). In the first PCR, GAPDH expression was tested; only GAPDH-positive samples were further analyzed for expression of nestin and PNMT. Intron-spanning primers were designed for all genes tested by using on-line software Primer3; their sequences and annealing temperatures are listed in Table 1. Due to very low amounts of cDNA and PCR products for nestin and PNMT in the first PCR, the following nested-PCR was performed with 2 µl template from the first PCR products. The specific primers for nested-PCR and their annealing temperatures are listed in Table 1. PCR reactions were carried out with initial denaturation at 94°C for 4 min; 40 cycles amplification at 94°C for 20 sec, annealing for 20 sec (temperatures in Table 1), and elongation at 72°C for 20 sec to 1 min (according to the expected product sizes), followed by final extension at 72°C for 4 min. Nested-PCR products (20 µl) were analyzed on 1.5% agarose gel containing ethidium bromide.

Western Blot Analysis

Cells were collected and lysed in ice-cold RIPA buffer (Sigma-Aldrich) containing 1% protease inhibitor cocktail (Sigma-Aldrich) on ice for 5 min. Cell lysates were then further disrupted with an ultrasonic homogenizer and centrifuged at 13,800 *g* at 4°C for 15 min. Protein concentrations in the supernatants were determined (BCA Protein Assay; Thermo Scientific, Rockford, IL, USA, http://www.piercenet.com) and the same amount of protein (30µg) from each extraction was separated by SDS-PAGE on 8% gels and transferred to PVDF membrane (Millipore, Danvers, MA, USA, http://www.millipore.com/). After blocking with 5% skim milk for 1 hr at room temperature, membranes were immunostained for vimentin using a mouse monoclonal antibody to vimentin (1: 1000, Abcam) in Tris buffered saline containing 5% skim milk overnight at 4 ºC, a horse-radish peroxidase-conjugated secondary anti mouse antibody (1 hr at room temperature) and developed with chemoluminescent substrate (Thermo Scientific). The signal was read with a luminescent analyzer (LAS-3000, Fujifilm, Tokyo, Japan, http://www.fujifilm.com/).

Cell Differentiation

Single cells from dissociated chromospheres were plated on poly-D-lysine-coated slides or in culture dishes (BD Biosciences). Cells were cultured in serum-free DMEM/F12 medium containing 20 ng/ml epidermal growth factor (EGF, Sigma-Aldrich), 20 ng/ml basic fibroblast growth factor (bFGF, Sigma-Aldrich), 10 ng/ml leukemia inhibitory factor (LIF, Sigma-Aldrich), 1% Insulin-Transferrin-Selenium-X (ITS-X, Gibco) and 1% antibiotics-antimycotic solution as the basal medium for cell differentiation experiments. Differentiation was induced by addition of 10 µM dexamethasone (Dex, Sigma-Aldrich), 20 ng/ml bone morphogenetic protein 4 (BMP4, R&D Systems, Minneapolis, MN, USA,

http://www.rndsystems.com/), or 20 ng/ml nerve growth factor (NGF, Sigma-Aldrich) to the basal medium. Within 24 hrs of plating, the majority of cells attached to the slide. Every second day, half of the medium was replaced by fresh medium up to 14 days after induction of differentiation.

Immunofluorescence Staining

After induction of differentiation for 14 days, cells were fixed on slides with 4% paraformaldehyde in PBS for 10 min at 4°C. After washing twice with PBS, they were incubated with mouse monoclonal anti-human B3T (1:500, Chemicon International, Billerica, MA, USA, http://www.chemicon.com/) and rabbit polyclonal anti-cow chromogranin A (CgA, 1:1000, Abcam) overnight in a humid atmosphere at 4°C. Slides were rinsed in three baths of PBS and incubated for 1 hr at room temperature with goat anti-mouse IgG conjugated with Rhodamine (1:200, Jackson ImmunoResearch Laboratories) and goat anti-rabbit IgG conjugated with FITC (1:200, Sigma-Aldrich). The slides were washed with PBS, mounted in mounting medium with DAPI (Vectashield, Vector Laboratories, Burlingame, CA, USA, http://www.vectorlabs.com/) and examined by laser confocal microscopy (FV-1000, Olympus, Tokyo, Japan, http://www.microscopy.olympus.eu/microscopes/). The images were analyzed using public software program ImageJ developed at the National Institutes of Health (Bethesda, MD, USA).

Electrophysiological recordings

Cells were investigated 4 to 14 days after differentiation using standard whole-cell patch-clamp techniques at room temperature. Recordings were made in the whole-cell voltage-clamp or current-clamp mode and data were recorded using an Axopatch 200B amplifier and Iso2 data acquisition software (Axon Instruments, Union City, CA, USA, http://www.moleculardevices.com/) essentially as described previously [23, 24]. Extracellular solution contained (in mM): 142 NaCl, 8.1 KCl, 1 $CaCl_2$, 6 $MgCl_2$, 10 HEPES, 10 D-Glucose (pH 7.4; 320 mOsm). Pipette solution contained (in mM): 153 KCl, 1 $MgCl_2$, 5 EGTA, 10 HEPES (pH 7.3; 305 mOsm). Using these solutions, borosilicate pipettes had resistances of 4-6 MΩ. Seal resistances in the whole cell mode were between 0.1 and 1 GΩ. Data were analyzed using Iso2, Prism4, Microsoft Excel 97 and Origin 5.0 software. Resting membrane potentials (RMP) were determined immediately after gaining whole-cell access. Action potentials were elicited by applying increasing depolarizing current pulses (5 pA current steps). Durations of action potentials were measured at half amplitude.

RESULTS

Formation of Chromospheres

To investigate whether chromaffin progenitors could be obtained from the adult adrenal medulla and expanded *in vitro* in a similar manner to central nervous system (CNS) precursors, single-cell suspensions of bovine chromaffin cells were grown in conditions that do not allow adherence to a substratum. Under these conditions most primary chromaffin cells died, but a small number survived and generated floating spherical colonies. We have termed these nonadherent colonies "chromospheres", as analogous to neurospheres. The spheres increased in size and their appearance in phase contrast microscopy was similar to that of neurospheres isolated from the CNS [25]. The size of chromospheres reached up to 100 µm after two weeks days in culture (Fig. 1 A-C).

The composition of fourteen day-old chromospheres was then analyzed with single cell RT-PCR followed by subsequent nested-PCR for higher sensitivity. Out of 51 cells analyzed from dissociated chromospheres 12 were $nestin^+/PNMT^-$ and 7 $nestin^-/PNMT^+$; 32 cells were $nestin^-/PNMT^-$. Cells expressing both nestin and PNMT were not detected. On the electron microscopical level, 14 day old chromospheres were composed of differentiated chromaffin cells with characteristic large dense cored vesicles and progenitor-like small cells with large nuclei and little cytoplasm (Fig. 1 D, E).

Evidence of self-renewal in sphere cultures is based on the capability of single cells derived from primary spheres to form secondary

spheres [26]. Cells, enzymatically and mechanically dissociated from primary chromospheres, were cultured as one cell per well and the formation of secondary chromospheres was traced. Cells from primary chromospheres were able to proliferate and to form clonal secondary chromospheres from single cells. From 1150 wells analyzed with one single cell each from 6 independent experiments, 170 wells contained secondary chromospheres after 2 weeks of culture (14.79 ± 1.47%; mean ± s.e.m.; n=6; Fig. 1 F).

To clarify if the proliferating cells are indeed progenitor cells, chromospheres were double stained for BrdU incorporation, indicative of DNA replication and cell division, and the intermediate filament protein vimentin. The BrdU signal could be detected by 6 hr administration, and the signal was stable after 24 hr treatment. The specificity of the anti-vimentin antibody to bovine cells was confirmed by western blotting. Chromospheres showed higher vimentin expression than chromaffin cells not only at the mRNA (Fig. 2A), but also the protein level (Fig. 2B). Double immuno-staining for vimentin and BrdU (incubation for 2 days), revealed $BrdU^{+}$/$vimentin^{-}$ and $BrdU^{+}$/$vimentin^{+}$ cells (Fig. 2C).

Chromosphere Characterization

Analyses of mRNA expression by RT-PCR revealed the expression of several progenitor markers including nestin, Sox1, Sox9, musashi1, vimentin and NGFR (neural growth factor receptor) in chromospheres which were either undetectable or at low levels in primary chromaffin cells. Primary chromaffin cells expressed the chromaffin cell markers TH and PNMT. Neither smooth muscle nor endothelium markers, MYH11 (smooth muscle myosin heavy chain 11) and PECAM1 (platelet/endothelial cell adhesion molecule), respectively, were detected in chromospheres but only in the primary isolated chromaffin cells (Fig. 2A). As revealed by quantitative real-time PCR, culture in low-attachment environment significantly influenced PNMT expression ($p<0.0001$) and nestin expression ($p<0.05$, One-way ANOVA). The expression of PNMT (the enzyme catalyzing the final step in epinephrine formation) decreased from 0.687 (relative copy number to GAPDH) to 0.007 after 13 days in culture in a time-dependent manner during chromosphere formation in low-attachment conditions ($p<0.01$, Dunnett's multiple comparison test, Fig. 3A). In parallel, nestin expression increased over time of culture with a 6 times increase of nestin expression after 4-days in culture (0.237) as compared to the freshly isolated chromaffin cells (0.039, $p<0.01$, Dunnett's multiple comparison test, Fig. 3B).

Cell Differentiation

Singularized cells dissociated from chromospheres were seeded onto poly-D-lysine coated plates or coverslips. Differentiation was induced by incubating the cells for 14 days with Dex, BMP4, or NGF, respectively, in serum free medium containing bFGF, EGF and LIF. Cells cultured in the presence of bFGF, EGF and LIF (basal conditions) did not express PNMT mRNA. Expression of PNMT, however, was significantly induced by treatment with Dex (Fig. 3F). Neither BMP4 nor NGF induced significant up-regulation of B3T mRNA expression (Fig. 3G). In addition to PNMT, the enzyme catalyzing the conversion of norepinephrine to epinephrine, the upstream enzymes including TH, DDC, and DBH were analyzed after induction of differentiation (Fig. 3 C-F). Although not significant, DDC was slightly up-regulated by dexamethasone treatment (unpaired *t*-test to basal treatment: $p=0.14$, n=6) while BMP4 treatment lead to a decreased DBH expression compared to basal condition (unpaired *t*-test: $p<0.05$, n=6); no influence was found on TH mRNA expression.

Immunofluorescent staining revealed CgA and B3T staining in the cells under basal conditions (Fig. 4). The intensity of staining for CgA was increased in cells treated with Dex, predominantly in the peri-nuclear area. Treatment with NGF induced the development of neurite-like extensions with strong immunofluorescent staining for B3T. After treatment with BMP4, B3T positive processes appeared in CgA-positive cells, resembling neurite outgrowth. Cells showing this neurite-like outgrowth were never observed after Dex-

treatment and scarcely observed at basal conditions. The processes in BMP4-treated cells showed strong immunostaining for B3T; a punctuate immunostaining for CgA appeared on these processes in addition to the peri-nuclear region. No fluorescence was observed in negative control where the antisera were replaced by BSA contained PBS.

Electrophysiology

To validate functional maturation of neurons derived from chromospheres, we performed whole-cell voltage-clamp recordings to measure voltage-gated sodium and potassium currents and current-clamp recordings to search for the capacity to generate action potentials after differentiation for 4-14 days. The resting membrane potential of differentiated chromosphere cells was -51±24 mV (n=21), the input resistance was 1643±991 MΩ (n=21), and the cell membrane capacity was 5.9±3.8 pF (n=10). Differentiated chromosphere cells expressed a sustained outward current of a few hundred pA up to 2 nA (Fig. 5B). These currents showed a voltage dependence and kinetics characteristic for delayed rectifier potassium channels (Fig. 5C). In 52% of cells (11 out of 21 cells), we could identify TTX-sensitive inward currents of up to 1 nA with voltage-dependence and kinetics typical for voltage-activated sodium channels (Fig. 5B,D). Current clamp recordings revealed that 24% of differentiated chromosphere cells (5 out of 21 cells) generated mature short-duration action potentials (mean±s.e.m. duration: 2.9±1.7 ms; n=5) with typical afterhyperpolarizations (Fig. 5E).

DISCUSSION

Although adrenomedullary chromaffin cells are one of the most intensively studied neural crest derivates, it is unclear whether sympathoadrenal progenitors exist within the adrenal medulla. Due to the close relation of chromaffin cells to sympathetic neurons, however, the isolation, propagation, and differentiation of sympathoadrenal progenitors from the adult adrenal medulla hold great promise for the treatment of neurodegenerative diseases. A major advance in stem cell research was achieved with the discovery that an undifferentiated multipotent population of neural cells [27, 28] can be isolated from differentiated neural tissue and can be grown in suspension as neurospheres. Analogous to primary neural cells, we have now established in this study a strategy to isolate chromaffin progenitors from bovine adrenal medulla (Fig. 6) and provide evidence that proliferation and differentiation competent chromaffin progenitor cells can be isolated from adult adrenal medulla. The morphological and functional similarities of bovine adrenal to the human adrenal [3, 29, 30] make these cells an ideal model to establish methods for the future isolation and transplantation of human chromaffin progenitors.

Unlike the closely related sympathetic neurons, adrenal medulla is able to proliferate throughout life and it has been hypothesized that progenitors continue to exist within the adult adrenal medulla [31]. To investigate whether chromaffin progenitors could be obtained from the adult adrenal medulla, we adopted the method used to enrich neuronal stem cells in neurospheres from adult differentiated tissue. Primary chromaffin cells were isolated through differentiatial plating thereby removing most non-chromaffin cells, *e.g.* adrenocortical cells and endothelial cells. Subsequent culture in low-attachment environment provided selection conditions for chromospheres formation. Through the formation of chromospheres, this selective environment not only resulted in a dramatic reduction of differentiated PNMT expressing chromaffin cells, but also in the removal of traces of endothelium and smooth muscle cells that where not completely eliminated by the differential plating. Isolation of free-floating progenitor/stem cell clusters was in addition to the culture of neurospheres also applied for the isolation of others progenitor/stem cells from different tissues [24, 32-37]. In the present study, the proliferation of chromosphere cells was indicated by their ability to incorporate BrdU. Furthermore, clonal growth of secondary chromospheres from single cells dissociated from primary chromospheres provides direct evidence for the self-renewing capability of the cells.

Are the progenitor cells self-renewing? To answer this question, chromospheres were immunostained for vimentin one week after BrdU-incorporation. Intermediate filament proteins such as vimentin or nestin, have been shown to characterize stem cells [38]. Self-renewal of vimentin-positive progenitor cells within chromospheres is indicated by the incorporation of BrdU into their nucleus. Therefore, a proliferation competent progenitor population does exist within chromospheres. In addition, vimentin$^+$/BrdU$^-$ cells and vimentin$^-$/BrdU$^+$ cells exist in chromospheres; those might be quiescent progenitor populations and proliferating non-progenitor cells or cells that differentiated between BrdU labelling and staining for vimentin, respectively.

In addition to the ability to self-renew, "stemness" of chromosphere cells was indicated by the expression of several neural precursor markers. In contrast to primary chromaffin cells, chromosphere cells expressed progenitor markers including genetic markers for neural stem cells (nestin, vimentin, NGFR and musashi1) [24, 39-41]. They also expressed Sox9, a member of the SoxE subgroup that plays an important role in the migration and differentiation of neural crest derivatives during embryonic development [42, 43]. In addition, Sox9 has recently been shown to be a common marker for multiple tissue-specific progenitors [44] and neural stem cells in the brain [45]. Sox1 is a transcription factor from the SoxB1 subgroup that maintains a stem cell-like state and is widely used marker of neural stem cells [46].

The decrease of PNMT expression is due to the fact that the majority of cells in the primary culture from adrenal medulla - differentiated chromaffin cells - did not survive in the non-adherent culture. Furthermore, PNMT expression depends on glucocorticoids [47] and therefore was down-regulated in the glucocorticoid-free culture conditions. Undifferentiated, nestin expressing progenitors, however, keep proliferating and form three dimensional cell clusters as suspense spheres. Similar to our data, sphere-like PNMT-negative structures, which might also contain progenitor cells, have been observed in primary cultures of human fetal chromaffin cells [14].

The composition of chromospheres was then investigated with single cell RT-PCR followed by subsequent nested PCR for higher sensitivity. Single cell RT-PCR has been widely used to study gene expression profiles in stem cells [48, 49]. This method allowed detection of even the low levels of PNMT expression that persisted in glucocorticoid-free conditions. Low-attachment environment promoted nestin$^+$ cells and reduced PNMT$^+$ cells resulting in 24% nestin$^+$ progenitor cells and 14% differentiated chromaffin cells after two weeks in culture. No cells were detected expressing both, nestin and PNMT, indicating two different populations with the characteristics of differentiated or progenitor cells, respectively.

Self-renewal and multipotency are hallmarks of progenitor/stem cells. To analyse the differentiation potential of chromosphere cells, dissociated cells were seeded on poly-D-lysine coated slides and differentiation was induced by treatment with specific factors in serum-free medium supplemented with bFGF, EGF and LIF. Serum-free medium supplemented with these growth factors provides a well-characterized, defined culture condition, used to maintain and expand progenitor cells [50-52]. In these basal conditions, cells from chromospheres sustained their undifferentiated status, indicated by very low levels of PNMT mRNA and increased nestin expression. It is well known that PNMT expression is induced by adrenocortical glucocorticoids. In addition, glucocorticoids including the synthetic glucocorticoid dexamethasone, induce a chromaffin phenotype such as the up-regulation of CgA and TH *in vivo* and *in vitro* [53, 54]; indeed, Dex treatment induced PNMT and CgA in differentiating cells isolated from chromospheres. Dex slightly, although not significantly, also increased the expression of upstream catecholaminergic enzymes TH, DDC and DBH. In addition, on the morphological level, Dex-treated cells showed an endocrine phenotype, with a large, CgA-positive cell body and no neuron-like extensions. These data indicate the potential of

chromosphere cells to differentiate into epinephrine-producing chromaffin cells.

NGF treatment induced a neuron-like differentiation of chromosphere-derived cells with B3T positive neurite outgrowth suggesting neuronal differentiation of the isolated progenitor cells [55, 56]. A similar induction of neuronal differentiation was observed after treatment of the cells with BMP4. BMP4 induced the expression of B3T mRNA and at the immunocytochemical level, B3T-positive neurite outgrowth. The effect of BMP4 on neural differentiation is controversial and seems to depend on the developmental stage of the stem cells. On the one hand, BMP4 inhibits the neural differentiation of embryonic stem cells [57-59]. On the other hand, a promoting role for BMP4 in the differentiation of neural crest derived neurons is well established [60, 61]. Under the influence of dorsal aorta-derived BMPs, neural crest cells develop into sympathoadrenal progenitor cells next to the primary sympathetic ganglia and BMP4 is likely to induce TH in sympathoadrenal progenitors [62]. Overexpression of BMP4 in multipotent neural crest-derived precursors initiated noradrenergic properties and mRNA expression for synaptotagmin I and neurexin I, which are proteins involved in neurotransmitter secretion [63]. Since neurexin is expressed only in neurons and not in neuroendocrine cells of the adrenal medulla, BMP4 differentially induces a neuronal development of sympathoadrenal progenitors versus a chromaffin development. Therefore, our data indicate the existence of BMP4-sensitive progenitor cells in the adult adrenal medulla and their potential to develop neuron-like properties under the influence of BMP4. BMP4 decreased, although not significantly, DBH expression in differentiating chromosphere cells. This might indicate the potential of BMP4 to direct these differentiating cells into dopamine producing neurons.

It has been suggested that chromaffin cells are able to transdifferentiate into neuron-like cells under the influence of NGF as first shown by Unsicker et al. [10]. In the present study, most differentiated chromaffin cells were removed from the culture in the low-attachment environment that led to an enrichment of nestin$^+$ or vimentin$^+$ progenitor cells. Therefore, the subsequent differentiation into neuron-like cells most likely is due to the differentiation of these progenitor cells and not due to transdifferentiation of chromaffin cells. The functional maturation of neurons derived from chromospheres was validated by whole-cell voltage-clamp recordings. The NGF-induced differentiated chromosphere cells acquired functional all major properties of mature nerve cells such as expression of sodium currents and generation of action potentials similar to primary neurons in culture [64-66] or differentiated neural stem cells from various origins [67-70].

Regarding the development of chromaffin cells and sympathetic neurons, two hypotheses are discussed (reviewed in [1]). The widely accepted hypothesis suggests the existence of common TH-positive progenitors of chromaffin cells and sympathetic neurons located in the primary sympathetic ganglia, co-expressing neuronal markers and chromaffin-specific marker. The second hypothesis proposes that chromaffin and sympathetic cells develop independently, based on the fact that distinct precursors of chromaffin cells and sympathetic neurons with different marker expression have been found during embryonic development before the formation of discrete adrenal anlagen [71, 72]. Chromosphere cells isolated from adult adrenal medulla in the present study reveal progenitor properties together with bipotential differentiation ability, possibly indicating the existence of common progenitor cells for sympathetic and chromaffin cells, a subpopulation of which may persist in the adult adrenal medulla.

Thus, this study provides evidence that neural crest-derived progenitor cell cells can be efficiently generated from adrenal medulla. When prevented from adherence, these cells form spheres, have the ability to self-renew, and are able to differentiate into chromaffin cells and neurons. The existence and isolation of chromaffin progenitor cells from adult adrenal medulla might harbour the potential for the use of these cells in the treatment of

neurodegenerative diseases, e.g. Parkinson's Disease.

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Figure 1. Chromospheres culture. After differential plating, adrenomedullary chromaffin cells were cultured for two weeks in low-attachment plates. (A-C): Dissociated cells (A) started to form clusters after 3 days (B). After 13 days in culture chromospheres reached diameters of 100 µm (C). (D-E): Electron micrographs of chromosphere cells after 14 days culturing. Chromospheres were of heterogeneous composition with differentiated chromaffin cells with typical large dense cored vesicles (D) and small cells with large nuclei surrounded by little cytoplasm (E). (F) Chromosphere cells at clonal density formed secondary spheres. From 1150 wells analyzed with one single cell each from 6 independent experiments, 170 wells contained secondary chromospheres after 2 weeks of culture (14.79 ± 1.47%; mean ± s.e.m.; n=6).

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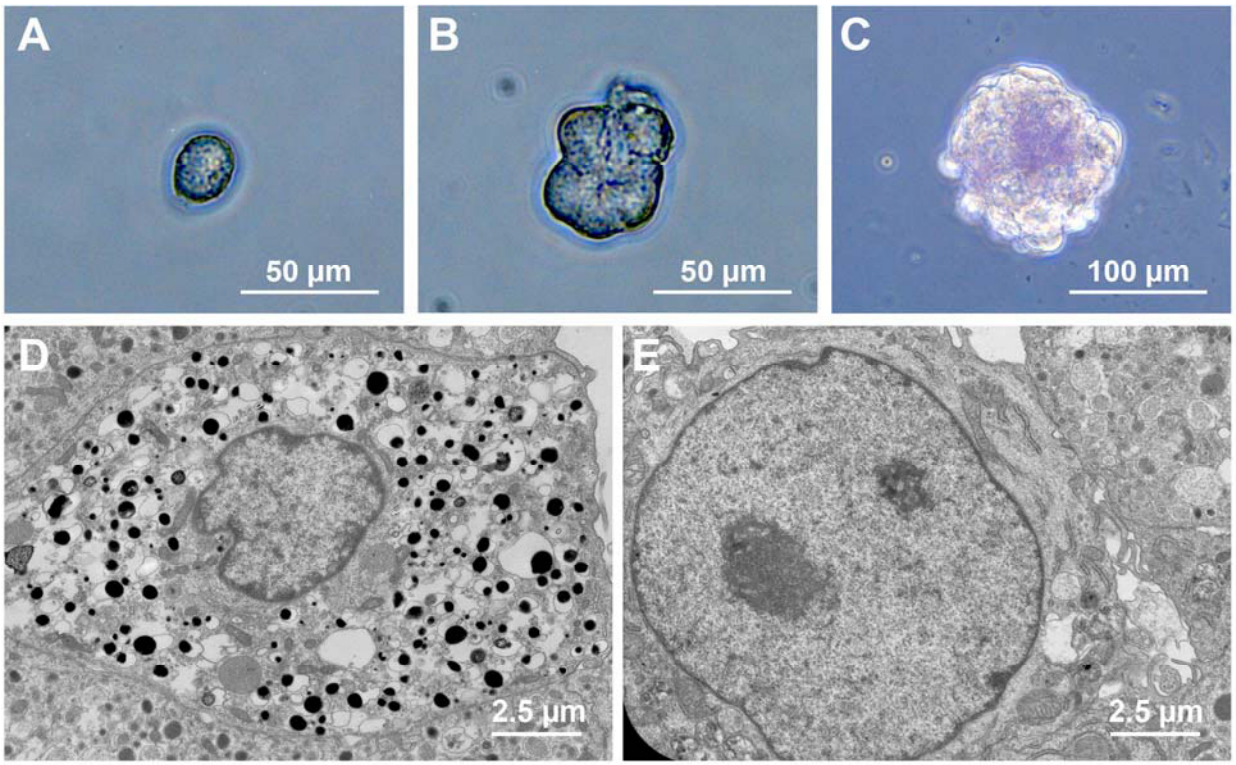

F

Analyzed Single Cells	Colony Formation
1150	170

Figure 1. Ehrhart-Bornstein

Figure 2. Characterization of chromosphere cells. (A): Chromospheres expressed several neural and neural crest progenitor markers including nestin, Sox1, Sox9, musashi1, vimentin and NGFR, which were not expressed or expressed at lower levels (musashi1) in chromaffin cells. TH expression in chromospheres was higher than in chromaffin cells. PNMT was undetectable in chromospheres. MYH11 and PECAM1 showed higher expression in the primary chromaffin cell preparation. (B): The higher expression of vimentin in chromospheres could also be detected at protein level by western blot analysis. (C): Vimentin immunoreactive cells (green) and BrdU-positive cells (red) in chromospheres. Arrow heads indicate vimentin immunoreactive cells that are BrdU-positive (enlarged in the insert).

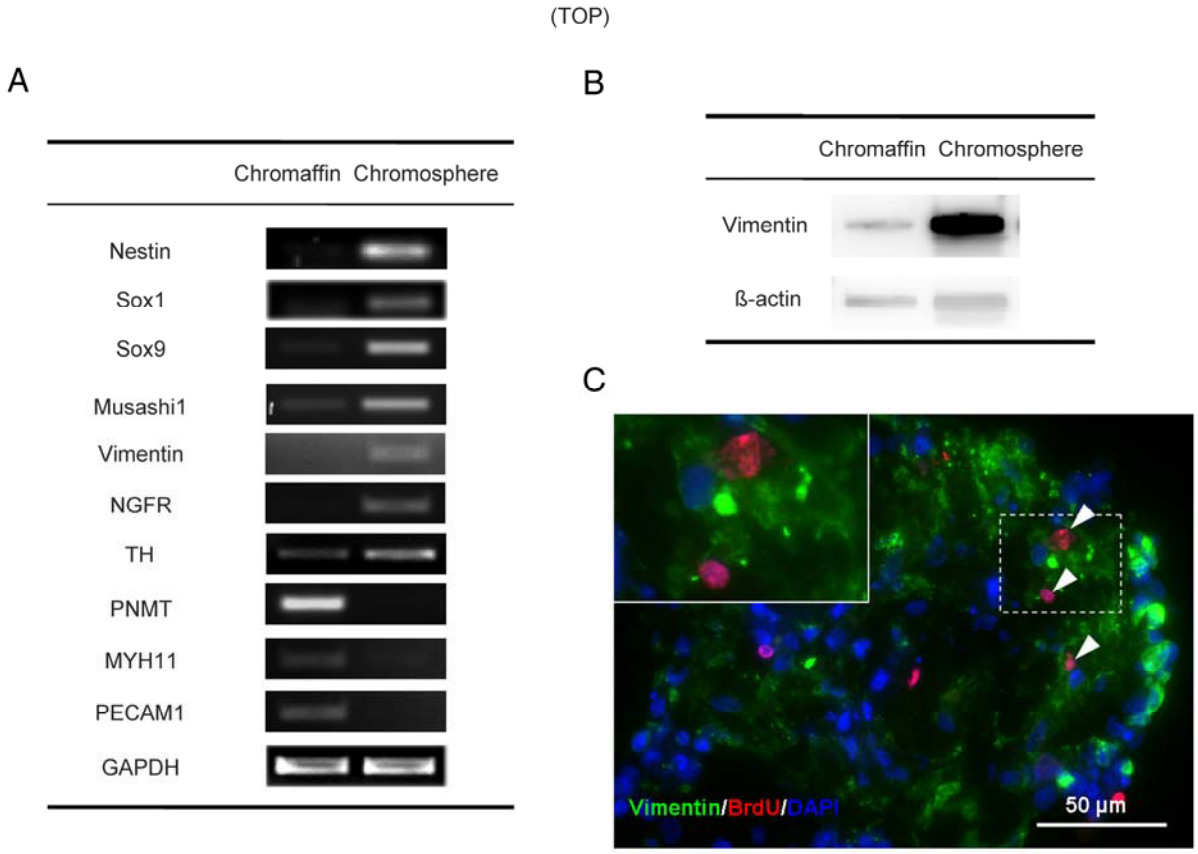

Figure 2. Ehrhart-Bornstein

Figure 3. mRNA expression in chromospheres and differentiated cells. (A-B): Nestin and PNMT expression during chromosphere formation. Culture in low-attachment environment significantly influenced PNMT expression ($p<0.0001$) and nestin expression ($p<0.05$, One-way ANOVA). After 4 days in culture, PNMT expression was significantly reduced and nestin expression was significantly increased as compared to day-0 chromaffin cells (**: $p<0.01$, *: $p<0.05$, Dunnett's Multiple Comparison Test). (C-G): mRNA expressions of catecholamine synthesis enzymes TH, DDC, DBH, and PNMT and B3T in chromosphere cells after induction of differentiation. (F): Dex induced a significant upregulation of PNMT expression (One-way ANOVA, $p=0.018$; *: $p<0.05$ compared to basal treatment, Dunnett's Multiple Comparison Test). (G): B3T mRNA expression was slightly increased after induction of differentiation with NGF or BMP4 (One-way ANOVA, $p=0.648$). Gene expression was normalized to GAPDH expression.

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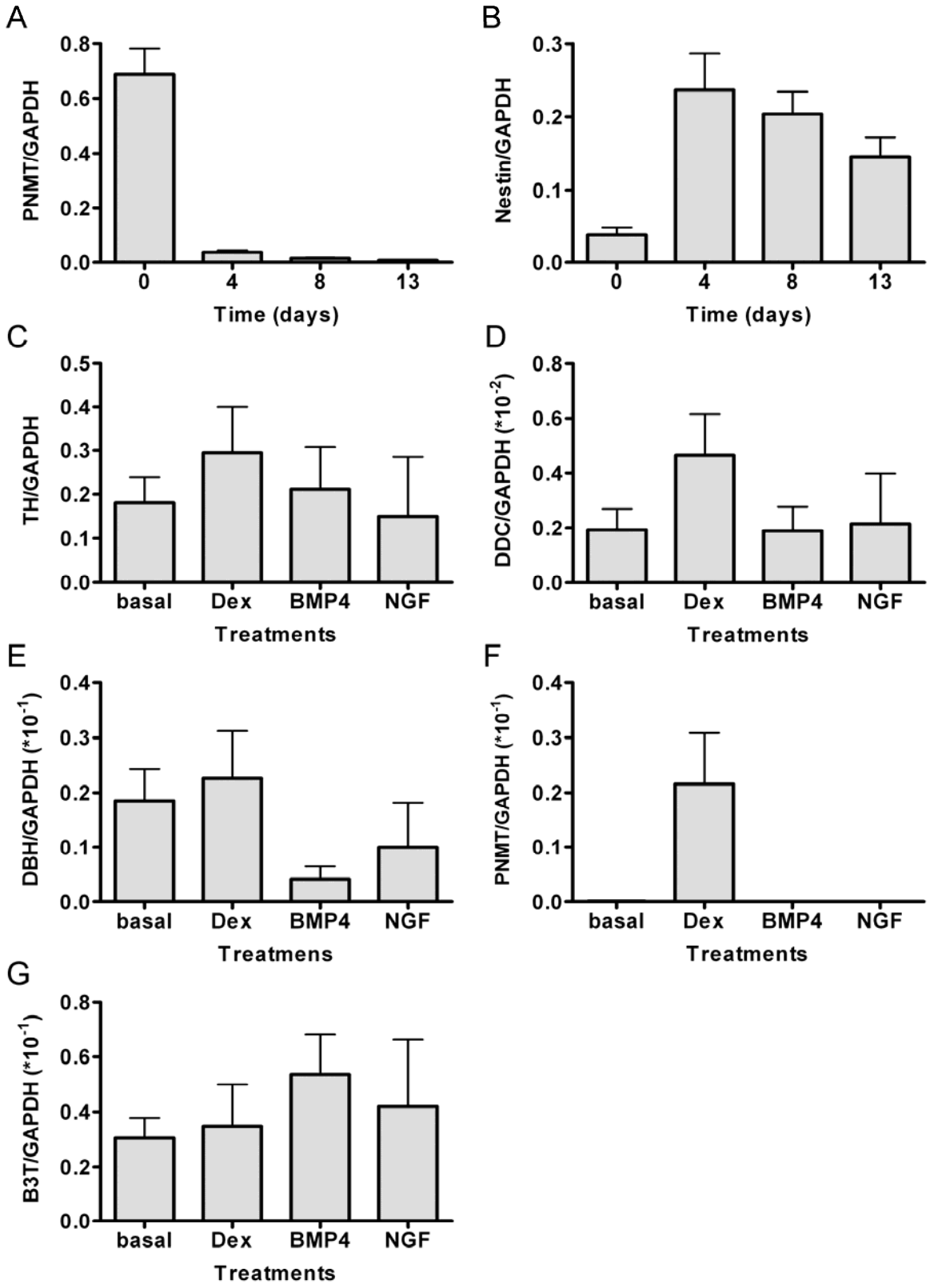

Figure 3. Ehrhart-Bornstein

Figure 4. Immunofluorescent staining of differentiated cells. Dex treatment induced strong perinuclear CgA expression (green). Both NGF and BMP4 treatment induced neurite-like B3T-positive extensions (red). The extensions induced by BMP4 also stained for CgA. Nuclei were counter-stained with DAPI (blue). No signal was detected in the negative control (Neg.) where the specific primary antibodies were replaced by BSA. Scale bar: 20 μm.

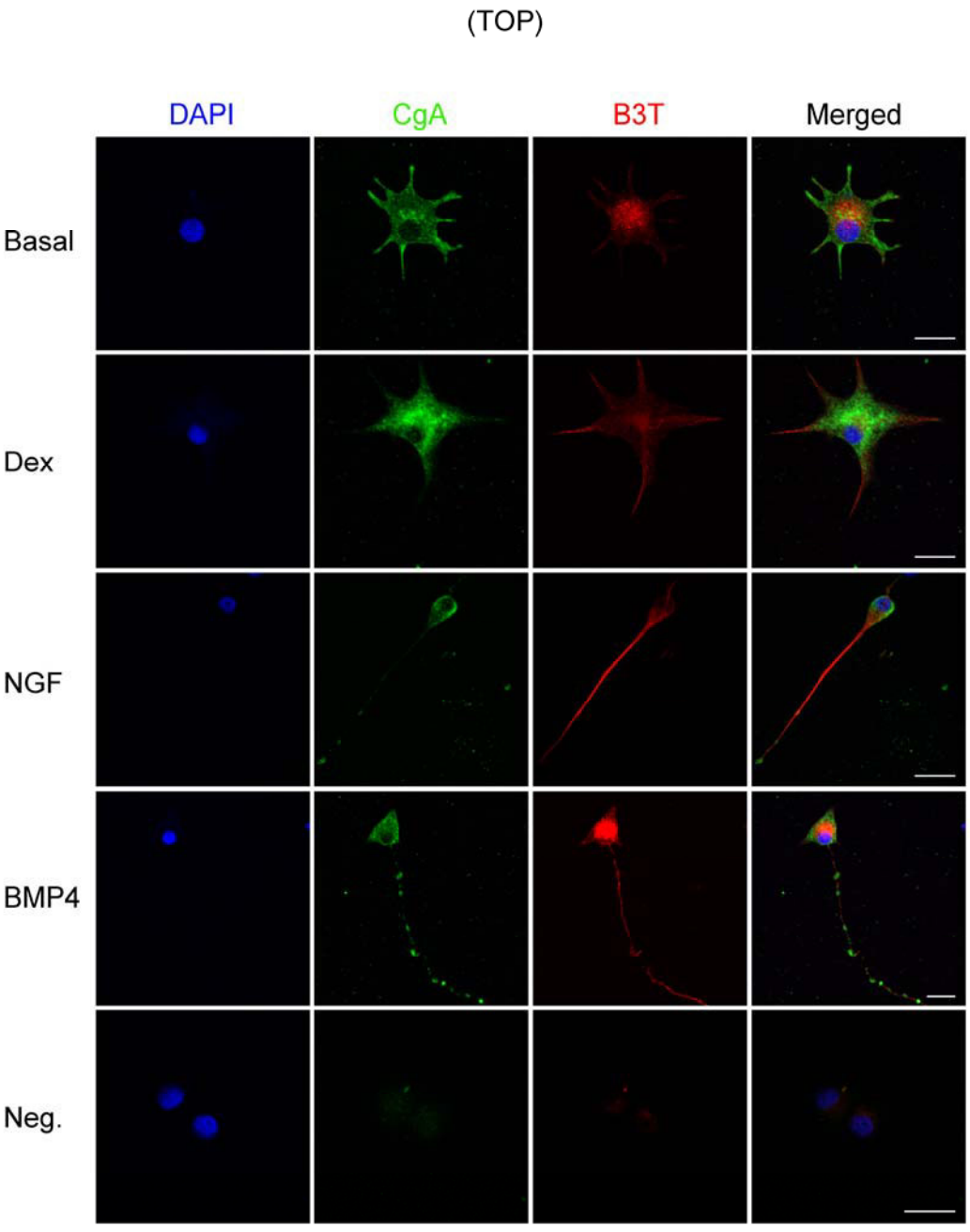

Figure 4. Ehrhart-Bornstein

Figure 5. Electrophyisological recordings on chromosphere-derived neurons. (A): Microphotograph showing a chromosphere-derived neuron with the patch pipette during the recording. (B): Representative traces from voltage-clamp recordings of a chromosphere-derived neuron displaying inward and sustained outward currents. The cells held at –120 mV and depolarized in 5 mV steps between –80 and +40 mV. (C): Current-voltage (*I*-*V*) relationship of the normalized outward currents in steady state at the end of the voltage steps. (D): Peak current-voltage (*I*-*V*) relationship for fast inward currents displayed the characteristic *I*-*V* relationship of voltage-gated sodium channels. The line represents a fit to the following equation: $I(V)/I\mathrm{max} = g(V - V\mathrm{rev})/1\exp[(V - V0.5)/k\mathrm{V}]$, where *I* (*I*max) is the (maximum) membrane current; *g*, the maximum conductance; *V*, the applied voltage; *V*rev, the reversal potential for Na; *V*0.5, the potential of half-maximal activation and *k*V, a slope factor. Data are mean values of three independent experiments. S.E.M. were ~3% and 12% for outward and inward currents, respectively, and error bars are thus omitted in the figure for clarity. (E): Current-clamp recordings in response to depolarizing current pulses of increasing amplitude showing the firing of repetitive action potentials.

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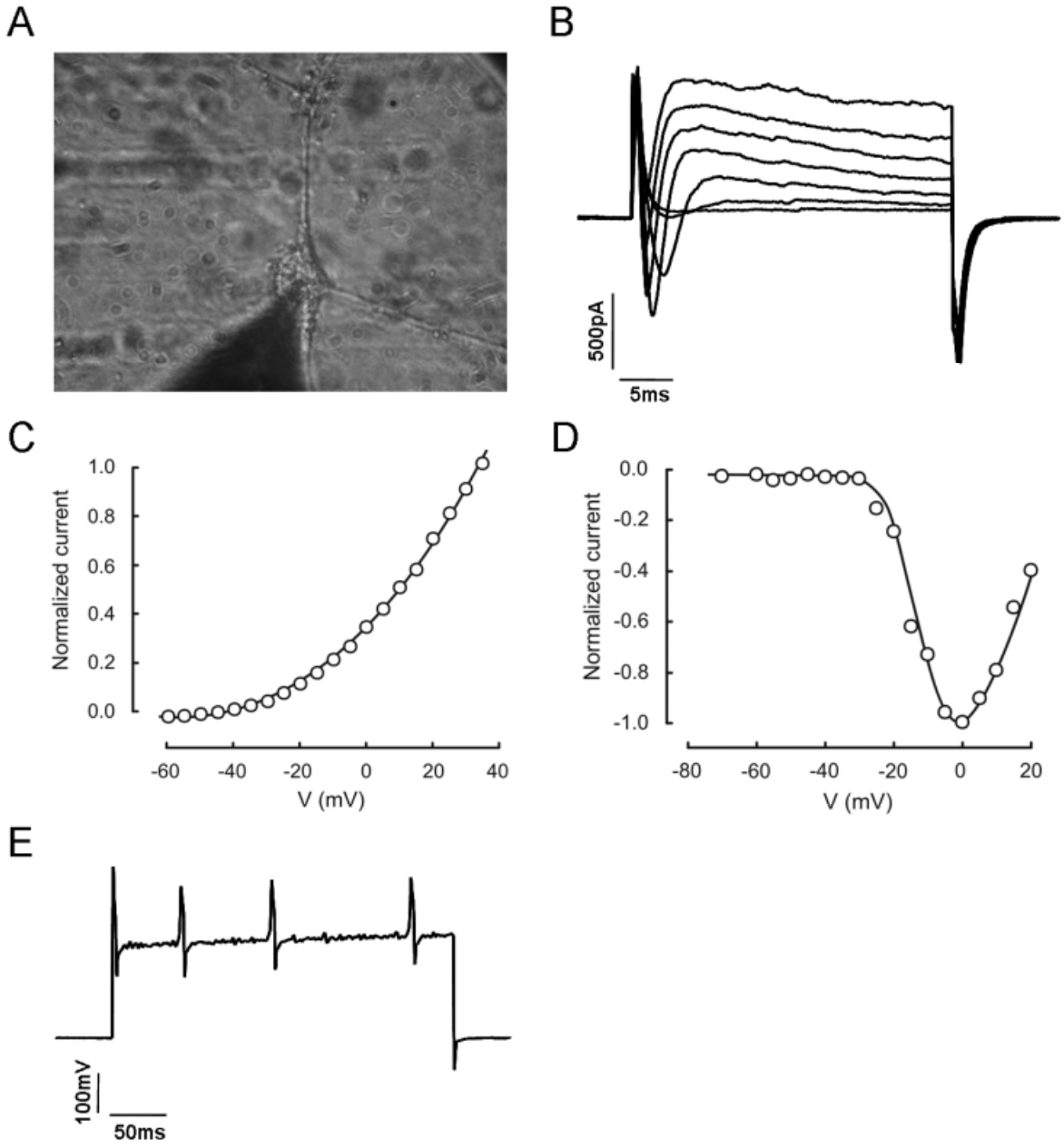

Figure 5. Ehrhart-Bornstein

Figure 6. Chromaffin progenitor isolation and characterization. In low-attachment environment chromospheres formed from isolated chromaffin cells. They expressed the progenitor markers nestin, Sox1, Sox9, musashi1, vimentin and NGFR, not expressed by chromaffin cells. Chromosphere cells showed differentiation ability: Dex induced differentiation into PNMT and CgA expressing chromaffin cells. NGF or BMP4 induced $PNMT^-$ neuron-like cells with $B3T^+$ neurite-like extensions.

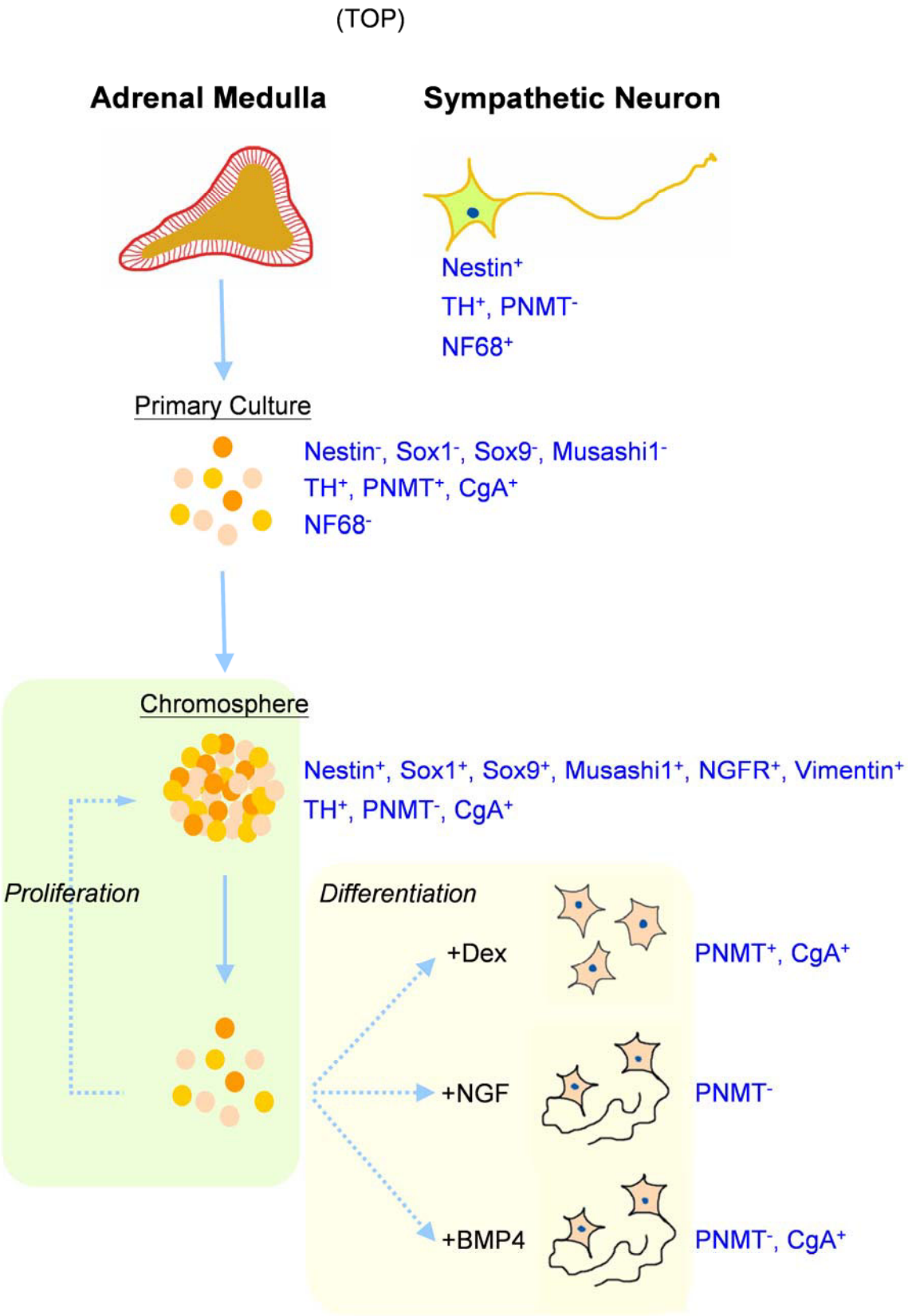

Figure 6. Ehrhart-Bornstein

Table 1

Nestin-n2	AGAAACAAGGCCTCCAGAGCCAAAT GTAGCTCCAGCTTAGGGTCCAGAAA	65	192
PNMT-n1	CGCCTACCTCCGCAACAACTA GCTGTACACGCTCCAGTCGAAA	64	283
PNMT-n2	CTGCTTGGCTCAGACCTTCG AGGCGCTGAGCAGCTGGTAT	65	95
MYH11	GTGACAGATTTCACCAGATCCA AAGATCTCAAACCCAGCAATGT	58	233
PECAM1	CAGAGTATGAGGTGTGGGTGAA GTATGGTACGGTCTTGCTCCTC	60	246
B3T	GTGGACCTCGAGCCTGGAAC TGCAGGCAGTCGCAGTTCTC	62	197
DBH	CTCCGCCTGGAAGTTCACTA GAGCTGAGAGGCGAAGATGT	63	252
DDC	GCTGCTCCTTTGACAACCTC TCCCTTGACTCCGTACATCC	60	377