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Research Report

Brain stem slice conditioned medium contains endogenous BDNF and GDNF that affect neural crest boundary cap cells in co-culture

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ABSTRACT

Conditioned medium (CM), made by collecting medium after a few days in cell culture and then re-using it to further stimulate other cells, is a known experimental concept since the 1950s. Our group has explored this technique to stimulate the performance of cells in culture in general, and to evaluate stem- and progenitor cell aptitude for auditory nerve repair enhancement in particular. As compared to other mediums, all primary endpoints in our published experimental settings have weighed in favor of conditioned culture medium, where we have shown that conditioned culture medium has a stimulatory effect on cell survival.

In order to explore the reasons for this improved survival we set out to analyze the conditioned culture medium. We utilized ELISA kits to investigate whether brain stem (BS) slice CM contains any significant amounts of brain-derived neurotrophic factor (BDNF) and glial cell derived neurotrophic factor (GDNF).

We further looked for a donor cell with progenitor characteristics that would be receptive to BDNF and GDNF. We chose the well-documented boundary cap (BC) progenitor cells to be tested in our in vitro co-culture setting together with cochlear nucleus (CN) of the BS.

The results show that BS CM contains BDNF and GDNF and that survival of BC cells, as well as BC cell differentiation into neurons, were enhanced when BS CM were used.

Altogether, we conclude that BC cells transplanted into a BDNF and GDNF rich environment could be suitable for treatment of a traumatized or degenerated auditory nerve.

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1. Introduction

1.1. Sensorineural hearing loss and medical bionics

Partial or complete loss of the hearing ability is a serious problem for millions of people around the world. Primary sensorineural deafness is caused by loss of hair cells, which eventually leads to secondary deafness due to consequent degeneration of the spiral ganglion neurons (SGNs) (Zappia and Altschuler, 1989). SGN degeneration is partly due to a reduction in endogenous neurotrophic support (Alam et al., 2007), which has been observed in animal models (Lefebvre et al., 1994) as well as in human cochleae (Nadol et al., 1989). In a limited number of patients, when a regular hearing device is insufficient, there is an option of a neural prosthesis. Implantation of a cochlear implant (CI) is a widely used strategy for these patients (Buchman et al., 1999). The CI electrode directly stimulates on the SGNs. For this implementation to be successful the functional integrity of these receiving neurons is of fundamental importance. An insufficient number or poor condition of SGNs can compromise the performance of the device (Incesulu and Nadol, 1998; Nadol et al., 1989). Due to new stimulation strategies that improve the efficacy of the CI via improved spatial and temporal resolution, preservation of the intact auditory nervous system has come to be of even greater importance (Goldwyn et al., 2010). Also ongoing pathology may lead to continued neural degeneration and cell diameter alterations that further limit strategies to improve performance in a particular patient (Shepherd and Javel, 1997).

Our laboratory has successfully established and used an organotypic tissue slice model of auditory brain stem (BS) to explore the potential use of various donor stem cells (SCs) (Glavaski-Joksimovic et al., 2008, 2009; Novozhilova et al., 2013; Thonabulsombat et al., 2007). This in vitro model contains part of the auditory BS neural circuitry, part of the auditory nerve and the cochlear neurons, all that are often damaged in the major causes of partial or complete hearing loss. The BS slices used are harvested from postnatal rat pups aged between P12 to P14, as at this time in development the rat auditory BS has reached its adult-like tonotopic organization (Friauf, 1992). We have demonstrated by morphological and immunohistological studies that this in vitro model using BS slices is highly reliable, illustrates good survival and keeps its three-dimensional organization for up to five weeks in culture (Thonabulsombat et al., 2007). It has previously been shown that SCs have the ability to migrate on the surface of hippocampal slice cultures (Benninger et al., 2003) and on the auditory BS slice culture (Glavaski-Joksimovic et al., 2009). These results suggest that growth factors, cytokines and/or other unknown substances are released from the BS guiding SCs (Benninger et al., 2003; Glavaski-Joksimovic et al., 2009; Thonabulsombat et al., 2007), which prompts this investigation.

1.2. Neurotrophic factors and the auditory system

Neurotrophins (NTs) were the first neurotrophic molecules characterized. Four essential NTs are expressed in mammals: nerve growth factor (NGF) (Cohen et al., 1954; Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5) (Lewin and Barde, 1996). These constitute a family of proteins with a common gene ancestor (Leibrock et al., 1989) that are instrumental in the regulation of neurons both centrally and peripherally during development. They ensure a suitable match of neuronal density for the target organ (Lewin and Barde, 1996). NTs control cell fate, axon growth and guidance, dendrite structure and pruning, synapse formation and synaptic plasticity (Huang et al., 2001; Kaplan and Miller, 2000; Lewin and Barde, 1996; Poo, 2001). NTs synthesis and secretion are constitutive during development and further modulated by neural depolarization (Blochl and Thoenen, 1996) and autocrine/paracrine regulation by NTs in the adult (Canossa et al., 1997; Kruttgen et al., 1998). In addition to NTs, there are neurotrophic factors of other gene descents. Among these, glial cell derived neurotrophic factor (GDNF) was discovered first. It was initially shown to have trophic effects on developing (Lin et al., 1993) and adult midbrain dopaminergic neurons (Lapchak, 1996), but has also been shown to increase survival and neurite growth of SGNs and thus play a vital role in development of the central auditory pathways and the inner ear (Euteneuer et al., 2013).

1.3. Stem- and progenitor cell based therapies

SCs are shown to be useful in transplantation in degenerative diseases (Gogel et al., 2011). Unlike differentiated cells, these undifferentiated cells can develop highly specialized functions, incorporate into host tissue and improve organ function (Lumelsky et al., 2001; Studer et al., 1998). Due to SCs cells self-renewal and multipotent abilities, application of these cells would make them tentative candidates in a replacement model.

Progenitor cells are multipotent cells that are still able to divide multiple times, like SCs, but have also started their differentiation downstream (Petersen et al., 2004). In adult organisms endogenous progenitor cells serve to replace degenerated cells as well as being recruited for injury recovery. For example, a number of studies have shown promising use of progenitor cells in myocardial neovascularization treatment of ischemic disease (Kawamoto et al., 2001), central nervous system injury treatment (Nandoe Tewarie et al., 2009) as well as liver and acute kidney injury repair (Becherucci et al., 2009; Zhao et al., 2009). Transplantation of neuronal precursor cells have been shown to lead to replacement of damaged neurons in the adult central nervous system (CNS) (Bjorklund and Lindvall, 2000).

Increased attention has been paid to boundary cap (BC) cells as a readily available alternative to SCs for transplantation. BC cells express multipotency markers (e.g. Sox10 and SSEA-1) and can differentiate into both neuronal and non-neuronal cell types, including neurons, glia and smooth muscle-like fibroblasts (Aquino et al., 2006). BC cells are neural crest cell (NCC) derivatives that form clusters at entry and exit points of peripheral nerve roots. These neural progenitors constitute a source of neuronal and glial cells to the peripheral nervous system (PNS) during a second wave of migration of cells from the neural crest. They are not detectable beyond postnatal day 5 in rat, which suggests that they are a late-surviving reservoir of neural precursors (Maro et al., 2004). BC cells can differentiate spontaneously into functionally diverse types of sensory neurons

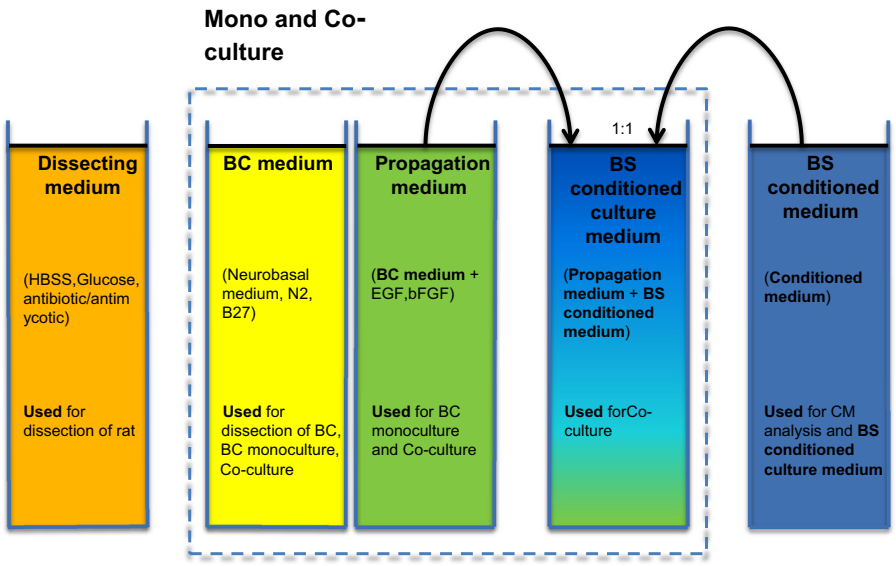

Fig. 1 – Illustration of different mediums that were used in the preparation of cultures and experiments. For reference purposes we here show their names, contents and used. BS conditioned medium production is further illustrated in Fig. 2.

that further support the thesis that they comprise a source of multipotent sensory specified SCs (Hjerling-Leffler et al., 2005).

1.4. *Conditioned medium*

Since researchers started the search for molecules with growth stimulating properties, the knowledge concerning these rare molecules has expanded and the efforts have been organized as field of biological science in its own right. In their hunt for target molecules, the concept of conditioned mediums (CM) were a powerful method to isolate cell specific markers. CM was also found to be useful in cell culture settings where it was used as a supplement to other cell culture mediums.

The CM is, in short, a medium collected after 24–48 h in cell culture. The medium is by then conditioned with the cell secretome of the first culture, containing serum proteins, extracellular matrix proteins, digestive enzymes and low abundant but highly bioactive secreted proteins. Here growth factors, hormones, cytokines, extracellular matrix processing proteases are found, that play key roles in regulation of cell differentiation and cell renewal (Skalnikova et al., 2011). The CM is also depleted of glucose and glutamine, and is for this reason commonly mixed with fresh medium before use. We have previously used CM in co-culture settings (Thonabulsombat et al., 2007) and in the present paper we set out to further examine the neurotrophic factor content in our BS CM.

1.5. *Our study*

In the present study we designed an experiment where BS CM was examined for levels of NTs. Here, after literature searches, we started with analyzing levels of BDNF and GDNF to evaluate any presence of NTs in BS CM at all, and we are happy to report positive findings. Utilizing the BS CM we also co-cultured BC spheres derived from the transgenic green fluorescent protein (GFP) mouse with postnatal rat auditory BS slices and explored its effects on their survival, differentiation and attraction.

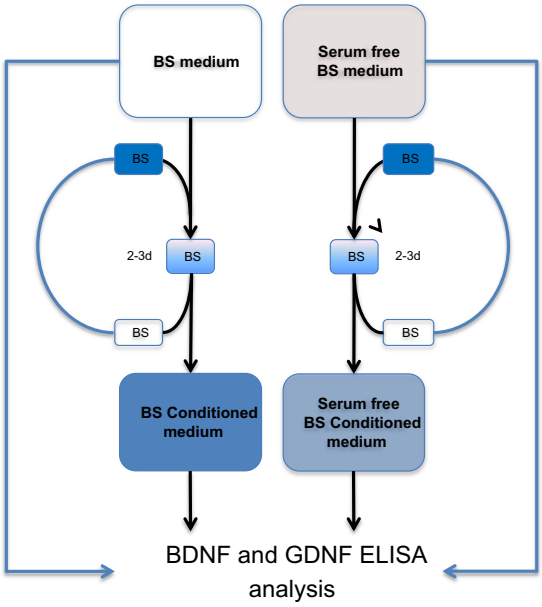

Fig. 2 – Schematic of CM production protocol and points of ELISA evaluation. BDNF and GDNF ELISA analysis of mediums with and without serum. There are two different groups executed according to this protocol, one with one rat BS slice per well, and one with two rat BS slices per well. BS medium contains DMEM, HBSS, FBS, Glucose, Hepes, antibiotics/antimycotic. In the serum free BS medium the serum portion is exchanged for more DMEM.

2. Results

2.1. *BS slice culture*

In BS medium and propagation medium the BS slices survived during the entire culture period of three weeks with intact cytoarchitectonic characteristics. At the same time, in

serum free BS medium, the slices looked destroyed, were lysed and more flattened, precluding any numerical analysis of survival (cf. Figs. 1 and 2).

2.2. BDNF analysis

As compared to undetectable levels in serum free BS medium, BDNF-levels in BS medium were on average 37±18 pg/ml (Figs. 2 and 3A). BDNF levels in BS CM were on average 40 pg/ml in the one slice setting and 62 pg/ml in the two-slice setting (cf. Fig. 3A). The BS CM experiments also showed significant increments in BDNF levels that varied over time. A biphasic release pattern was noticed at days 5 and 15 where two slice BS cultures illustrated statistically significant amounts of BDNF that were threefold to that of one-slice levels (Fig. 3A).

The experimental groups with and without serum supplemented BS medium did not show any statistical difference in BDNF levels between groups (cf. Figs. 2 and 3B). Despite no serum supplementation the serum free BS CM contained significant levels of BDNF at all time points (Fig. 3B).

2.3. GDNF analysis

As compared to undetectable levels in serum free BS medium, GDNF-levels in BS medium were on average 79±32 pg/ml (Figs. 2 and 4A). On average, the level of GDNF in BS CM was 66 pg/ml in the one slice setting and 90 pg/ml in the two-slice setting (cf. Fig. 4A). The BS CM experiments also showed significant increments in GDNF levels that varied over time. A discernable biphasic release pattern was noticed at days 3 and 12 in the two slice setting, though not significant (Fig. 4A).

The experimental two-slice serum free BS medium group showed statistically significant prolonged elevation of GDNF levels between days 8 and 17 (Figs. 2 and 4B).

The experimental groups with and without serum supplemented BS medium did not show any statistical difference in GDNF levels between groups (cf. Figs. 2 and 4B). Despite no serum supplementation the serum free BS CM contained significant levels of GDNF at all time points (Fig. 4B).

2.4. BC cell monoculture

After 24 h in monoculture, following dissection and dissociation of GFP mouse embryonic dorsal root ganglions (DRGs), an adherent cell monolayer was observed (Fig. 5A). In phase microscopy the formation of small clusters as early spheres was observed after five days (Fig. 5B). Within two weeks several free-floating GFP-positive clusters were seen (Fig. 5C and D). Cells in the BC spheres kept propagating for more than six months and appeared to divide indefinitely with

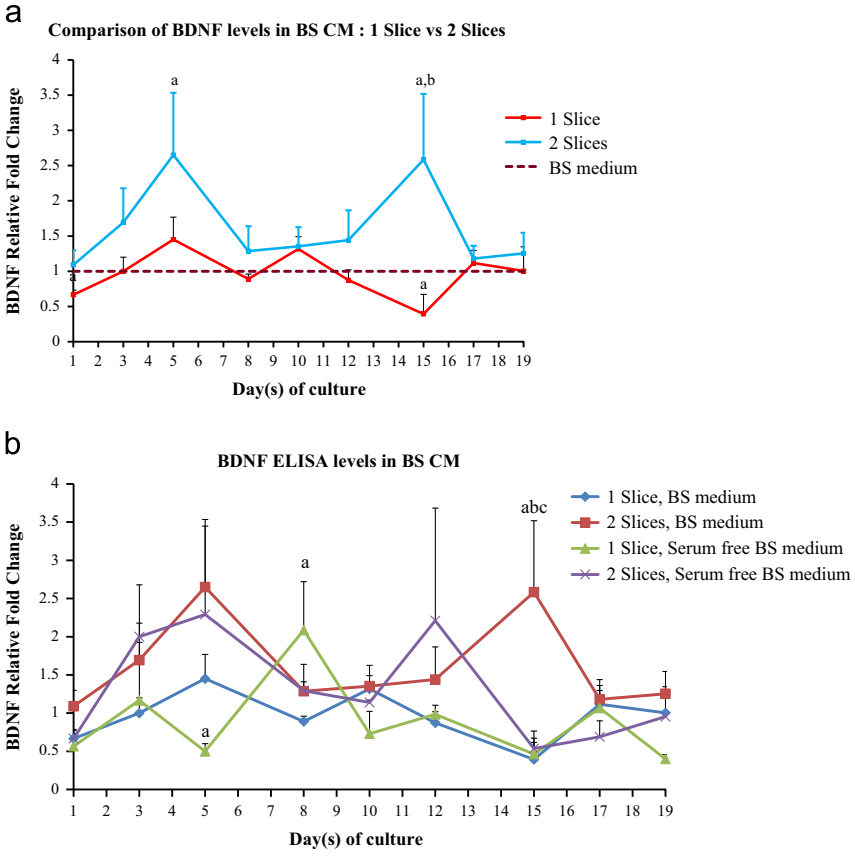

Fig. 3 – (A) Comparison of BDNF levels detected in BS slice CM from cultures of one versus two BS slices. BS cultures were kept for 3 weeks. Graph depicts ELISA analysis in relative fold of BDNF levels in supernatant collected from culture, with BDNF level of BS medium as reference. A biphasic pattern of secretion was seen in the 2 slice group. $^{a}P<0.05$, compared to BS medium; $^{b}P<0.05$, compared to one slice setting. (B) Relative fold change in BDNF levels in CM collected from BS slice cultures in BS medium and serum free BS medium. BDNF level of BS medium is as corresponding reference (=1). $^{a}P<0.05$, compared to 1 slice, BS medium; $^{b}P<0.05$, compared to 2 slices, serum free BS medium, $^{c}P<0.05$, compared to 1 slice, serum free BS medium.

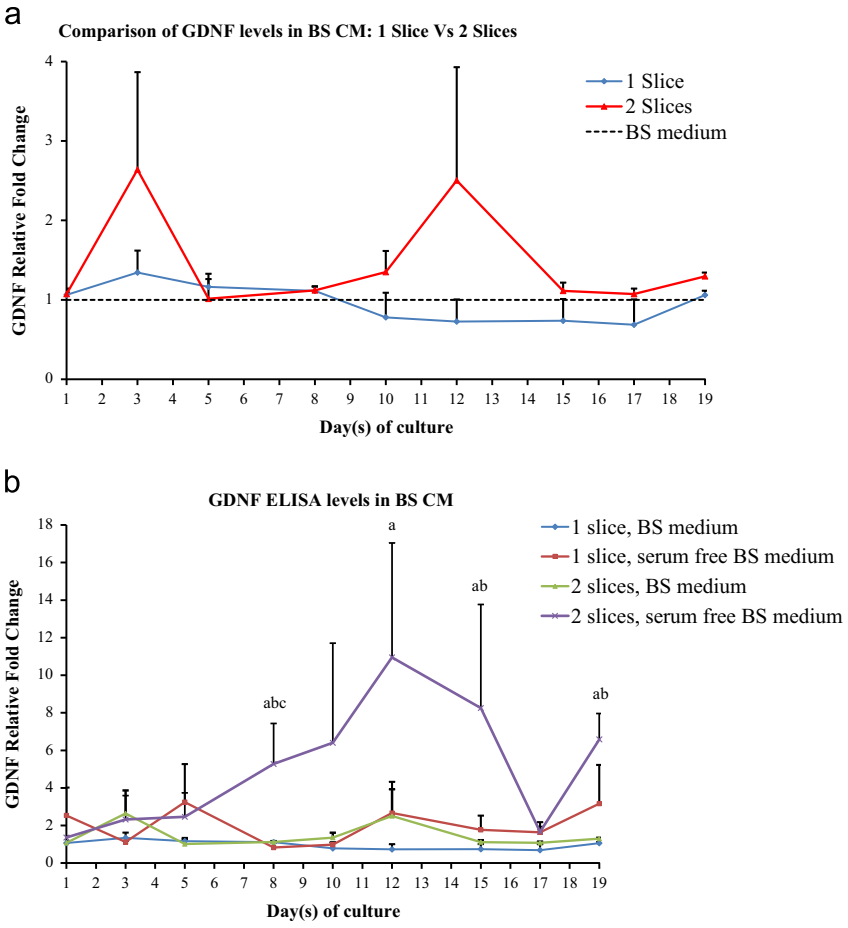

Fig. 4 – (A) Comparison of GDNF levels detected in BS slice CM from cultures of one versus two BS slices. BS cultures were kept for 3 weeks. Graph depicts ELISA analysis in relative fold of GDNF levels in supernatant collected from culture, with GDNF level of BS medium as reference. A biphasic pattern of secretion was seen in the 2 slice group. Levels were not statistically significant. (B) Relative fold change in GDNF levels in CM collected from BS slice cultures in BS medium and serum free BS medium. GDNF level of BS medium is as corresponding reference (=1). $^{a}P<0.05$, compared to 1 slice, BS medium; $^{b}P<0.05$, compared to 2 slices, BS medium; $^{c}P<0.05$, compared to 1 slice, serum free BS medium.

maintained GFP expression (Fig. 5E and F). When BC spheres were isolated and plated on poly-D-lysine and laminin-coated glass slide chambers these attached to the bottom of the well and formed a monolayer.

Plates with attached BC cells were cultured in separate mediums: BC medium, propagation medium and BS conditioned culture medium (cf. Fig. 1). In BC medium, after four days the cells developed peripherin-positive neurite processes (Fig. 6A). Within six days upon culture initiation in BC medium, BC cell survival was seen to be limited with abundant cell death (Table 1). In propagation and BS conditioned culture medium the cell survival was good (Table 1). In propagation medium, the cells illustrated peripherin-negative cells, indicating lack of differentiation towards a neuronal fate (Table 1). The few peripherin-positive cells present were situated in the periphery of the culture and did not show any neurite outgrowth (Fig. 6B). In BS conditioned culture medium, the BC cells illustrated peripherin-positive neurite outgrowths within five days of culture and they maintained their strong peripherin expression during the entire three-weeks period (Fig. 6C; Table 1).

2.5. *BC cell and auditory BS slice co-culture*

In co-culture with the BS slice, BC cells in BC medium and propagation medium did not survive for more than two weeks, whereas in BS conditioned culture medium BC cells were still growing at the three weeks termination point (cf. Fig. 1; Table 2).

Differentiation of BC cells in co-culture using BS conditioned culture medium was strong showing abundant expression of the neuronal marker βIII-tubulin after one week (Fig. 7C and D; Table 2). Co-cultures in BC medium and propagation medium showed substantially less βIII-tubulin marker-expression (data not shown). Directional growth of axons from the differentiated BC cells towards the BS was seen in all three co-culture groups (Fig. 7E and F; Table 2). However, when compared to co-cultures in BC medium, co-cultures in propagation medium and BS conditioned culture medium showed stronger unidirectional growth towards the BS (Table 2). In all experimental groups, BC cells continued to express GFP throughout the entire time in co-culture.

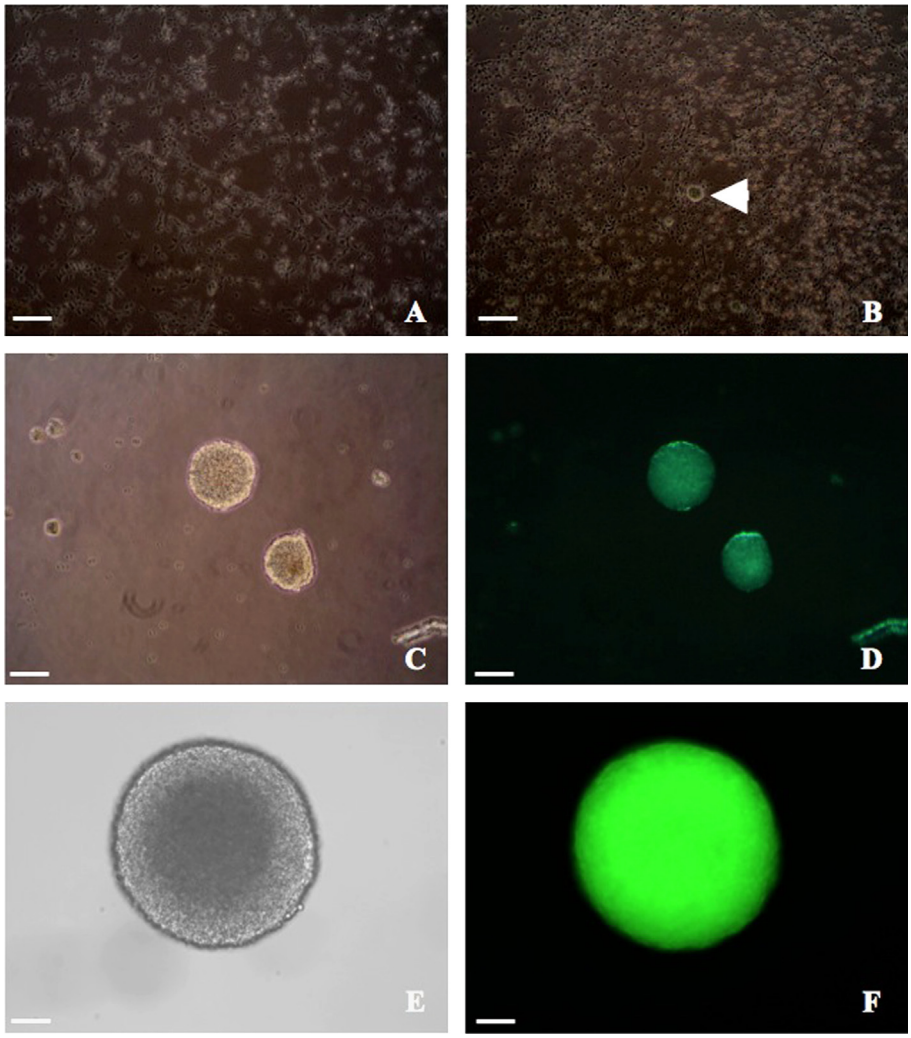

Fig. 5 – (A)–(F) Mouse boundary cap cell culture. DRGs were dissected out and dissociated from E11.5 GFP mice into monoculture. (A) After 24 h in culture a majority of cultured cells had formed an adherent monolayer. (B) Small bright clusters of cells were observed after 5 days of culture (arrow). (C) and (D) After two weeks in culture, BC cells had formed free floating spheres visible in phase contrast and were also expressing GFP marker detectable in fluorescent microscope. (E) and (F) The propagation of BC spheres was possible for more than 6 months and they kept expressing GFP in late passages. Scale bar = 100 μm (A)–(D) and 50 μm (E) and (F).

3. Discussion

3.1. *Stem and progenitor cells*

The use of stem and progenitor cells to replace degenerated SGNs is an attractive approach in hearing restoration. We have previously established a stable in vitro cell culture system to evaluate various implantation candidates including human neural precursor cells, sox cells and olfactory ensheathing cells (Glavaski-Joksimovic et al., 2008, 2009; Thonabulsombat et al., 2007). In the present study we used a neural crest derivative as donor cell. NCC give rise to DRGs and autonomic sympathetic neurons (Hjerling-Leffler et al., 2005). In the DRGs, cells arresting in the boundary caps form a subpopulation of pluripotent cells that prefigure the developing transitional zone between the CNS and PNS. They uphold a key gating mechanism at the boundary cap, arresting neuronal soma migration, confining motorneuron cell bodies to the spinal cord and uphold the integrity of the spinal column (Bron et al., 2007). BC cells are first noticeable at E11.5 until E13 and perform this task until mature motorneuron exit points are formed. After P5, the BC cells are no longer detectable (Golding and Cohen, 1997). The initial general pluripotency of NCC give these cells the capacity to form more than one type of differentiated progeny, depending on microenvironment cues. The majority of neurons and glia in the PNS are generated by the transient NCC migrating from the dorsal neural tube. Further, since BC cells are arresting soma migration during development and, if controlled, this ability could be useful in an in vivo cell implantation paradigm. In such a model it would possibly be preferred to halt implanted cell soma in the periphery of the auditory nervous system, whereas their axons stretch centrally due to attraction from the cochlear nuclei.

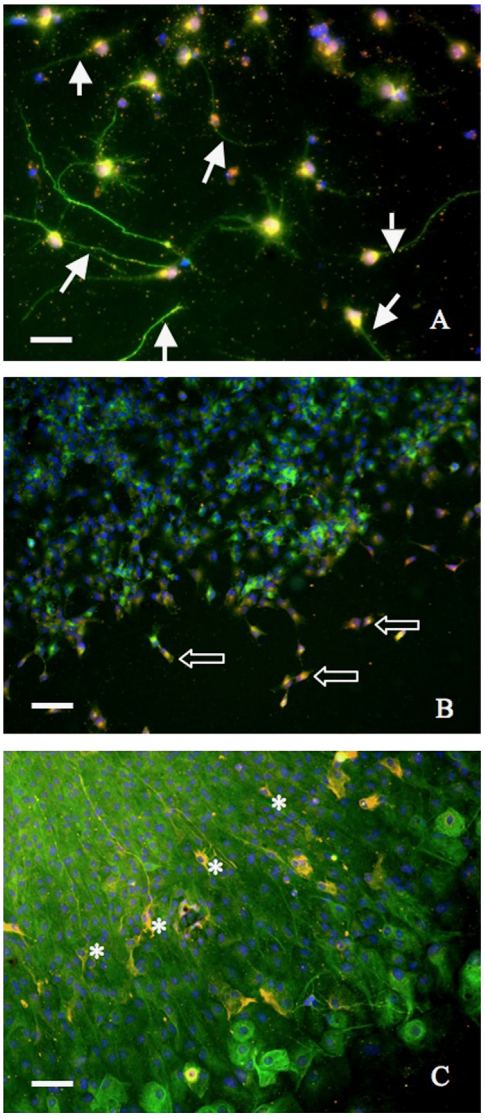

Fig. 6 – (A)–(C) BC cell monoculture from GFP mice. BC cells survive and differentiate to a different extent when cultured in different media. (A) When cultured in BC medium, BC cells show peripherin-positive neuronal morphology with axons stretching from the cell soma (arrows). However, cells survive for less than a week in this medium. (B) BC cells cultured in propagation medium survived for the full three weeks of the experiment period, but exhibited more immature morphology with fewer peripherin-positive cells, and these were situated at the periphery of the transplant (hollow arrows). (C) When cultured in BS conditioned culture medium cells survive well, display neuronal morphology with expression of peripherin throughout the three-week period of culture (asterisks). Immunostaining with peripherin (red), GFP (green), DAPI (blue). Scale bar = 100 μm.

Therefore NCC, and BC cells in particular, are appealing to investigate as implantation candidates to the damaged auditory nervous system.

Since BC-derived sensory neurons are found at E11.5 and until E13 when their proliferation ceases, we isolated embryonic DRGs from E11.5 transgenic GFP mice. After a week in culture small clusters of floating GFP-positive cells were observed. When put to the test, these spheres kept propagating for up to six months in culture without loss of GFP expression. This is in agreement with previous studies and illustrate that at among the E11.5 DRG cells there are BC cells are able to propagate in long-term culture setting with maintained SC characteristics (Hjerling-Leffler et al., 2005; Maro et al., 2004).

When using BS conditioned culture medium and propagation medium our results demonstrated a high survival-rate of BC cells in monoculture. However, the differentiation of the BC cells was more pronounced in BS conditioned culture medium as compared to propagation medium, which only illustrated scarce peripherin-positive cells detected at the periphery of the BC sphere monolayer. BC cells in propagation medium did not develop any neurite outgrowths during the three-week period of culture whereas they did in BS conditioned culture medium. We speculate that the added neurotrophins in the conditioned medium is part of the reason for this effect.

3.2. *Neurotrophins*

As mentioned above, the decision fell on BDNF and GDNF to be the first factors analyzed. BDNF were chosen from the NTs as it is well defined and likely to be expressed in large amounts in the cochlear nucleus at the point of development in interest (Hafidi et al., 1996). NGF were likely to be found here during all adulthood, but at lower levels (Gibbs and Pfaff, 1994). NT-3 have a more pronounced peak of expression during the early postnatal period, and are present at lower levels in the adult (Bernd, 2008; Gibbs and Pfaff, 1994). NT-4/5's effects are mediated via the same receptors as BDNF (Burette et al., 1997), but its properties are less explored.

Exogenous BDNF has in several both in vitro and in vivo studies promoted the survival of SGNs (Miller et al., 2007; Shinohara et al., 2002; Warnecke et al., 2007). In a study where GFP embryonic SCs were transplanted into the rat auditory nerve we have shown that BDNF significantly improved the differentiation rate of the transplanted cells (Palmgren et al., 2012).

GDNF was chosen as it is a neurotrophic factor of a different gene family than NTs, and is shown to significantly promote neurite outgrowth from adult DRG neurons (Sango et al., 2008). GDNF has also been shown to affect the development and survival of a diverse set of both neuronal and non-neuronal cells (Henderson et al., 1994; Pichel et al., 1996; Trupp et al., 1995). Its receptors have been detected in SGNs (Ylikoski et al., 1998) and treatment with GDNF has been reported to protect hair cells and SGNs against various forms of insults (Keithley et al., 1998; Shoji et al., 2000; Ylikoski et al., 1998).

Moreover, GDNF and BDNF genes have shown to interplay in chronic tinnitus (Pg et al., 2012), portraying interdependent role of GDNF along with BDNF not only in neuronal survival and differentiation, but also formation and maintenance of neural circuits and synapse plasticity (Hibi et al., 2009).

Certainly, investigation into the presence of all known neurotrophic factors in our CM is of importance to draw a

Table 1 – Mouse BC cell monocultures were kept in three different mediums. They were checked for cytoarchetectonic integrity as a measure of survival and acquired peripherin expression as a sign of differentiation toward a neuronal fate.

BC monoculture	Survival	Peripherin staining
BC medium	<6d	+
Propagation medium	>3w	–
BS conditioned culture medium	>3w	+

Table 2 – Mouse BC cell-transplants were co-cultured with rat BS slice in different mediums for 3 weeks. All were analyzed visually how well they survived, expressed periferin as a measure of differentiation and to what extent the neuronaly differentiated transplanted cells showed axonal migration towards the BS slice.

Co-culture	Survival	Peripherin staining	Attraction
BC medium ($n=13$)	2w	–	53%±11
Propagation medium ($n=12$)	2w	–	72%±11
BS conditioned culture medium ($n=3$)	>3w	+	66%±13

more complete picture of stimulation/inhibition in our organotypic auditory cell culture model.

3.3. *Serum free medium assay*

It would have been expected that BDNF and GDNF values increased in culture medium when BDNF and GDNF containing serum were added. It was therefore interesting to note that there were almost no differences in the amount of BDNF and GDNF in culture groups with serum supplemented medium as compared to groups with serum free medium. We speculate that the reason(s) for these results is a feedback system in the BS slice with a regulatory mechanism to maintain the respective BDNF and GDNF concentration at a constant level. It was previously shown that BDNF can induce regulated release of similar and other neurotrophins (Kruttgen et al., 1998). Here, we speculate that this, or a similar mechanism, is present in our ELISA analyses, e.g. there is a self-regulatory mechanism in the BS slice causing a feedback-regulated production and/or consumption of neurotrophins in the CM.

Since the amounts of soluble proteins in the CM were very small, a masking effect from proteins in added fetal bovine serum (FBS) could not be ruled out (Dowling and Clynes, 2011). In order to avoid uncertainties regarding the origin of detected BDNF and GDNF, we added a group cultured in a serum-free alternative (serum-free BS CM). However, we conclude that the masking effect from using serum-supplemented medium was weak and did not interfere with our analysis.

The results further illustrate that detected BDNF and GDNF levels are slightly higher in the cultures with more numerous cells.

3.4. *Conditioned medium assay*

We produced BS CM as performed in earlier reports (Thonabulsombat et al., 2007) but in the present paper we analyze the CM BDNF and GDNF content in absolute and relative values using ELISA. The analyses show that there are two distinct time-points where both BDNF and GDNF levels are significantly higher in the two slice settings as compared to the one slice setting. These variations in neurotrophic factor levels were much the same for both BDNF and GDNF and at approximately the same time in culture, but the reason(s) for these time-dependent maxima are uncertain. We can conclude that there is a variable neurotrophic factor production and/or consumption of the slices in culture that shows a biphasic pattern. We speculate that the reason for this is a time-dependent adaptation of the slices to the surrounding milieu. A cell response to the trauma with subsequent upstarts of repair mechanisms could be causing such an effect.

In the one slice setting this response was not noticed at all. We speculate that the total amount of cellular content was relatively small and thereby lacked the ability to produce BDNF and GDNF under these experimental conditions, or that our ELISA analysis was not sensitive enough to detect these changes in levels of neurotrophic factors.

The analysis show that the levels of BDNF in BS conditioned culture medium are in par with that of BS medium. Initially, the BS CM from the two-slice culture contains 62 pg/ml BDNF. After preparation it is further mixed 1:1 with fresh propagation medium for use in cell culture, which results in 31 pg/ml BDNF in the medium used for co-culture. These levels coincide well with the 37 pg/ml of BDNF detected in BS medium. We can therefore speculate that one of the predictors for a successful cell culture would be to use adequate levels of neurotrophins that matches the neurotrophic contents of conditioned medium.

In co-culture, BC cells showing differentiation towards a neuronal fate also showed migration primarily directed towards the BS slice. We hypothesize that the observed targeted migration is triggered by BDNF and/or GDNF produced by the BS, serving as guiding cues for the transplanted cells. This effect does not seem to be diminished by the addition of neurotrophic factors in the levels here supplied by the culture medium. We come to this conclusion since we get similar results from quantifying the attraction between BC cells and BS in all different experimental groups.

In the GDNF analysis we found that when serum-free medium was utilized in a two slice setting control experiment, a biphasic pattern did not occur. Instead the results illustrate a slow but steady climb with a single maximum after twelve days of culture. The reason(s) for this strong and slow elevation of GDNF release are unclear. Further investigation is needed to explain the mechanism(s) for this GDNF increase.

3.5. *Conclusion*

Prominent and attracted growth of neural cells in co-culture using BS CM prompted us to investigate the neurotrophin content of BS CM. The results show a significant content of BDNF and GDNF in the BS CM that seems to have different time maxima and to be self regulated. We further used co-culture techniques to study BC cell survival, differentiation and attraction to the CN in BS slices,

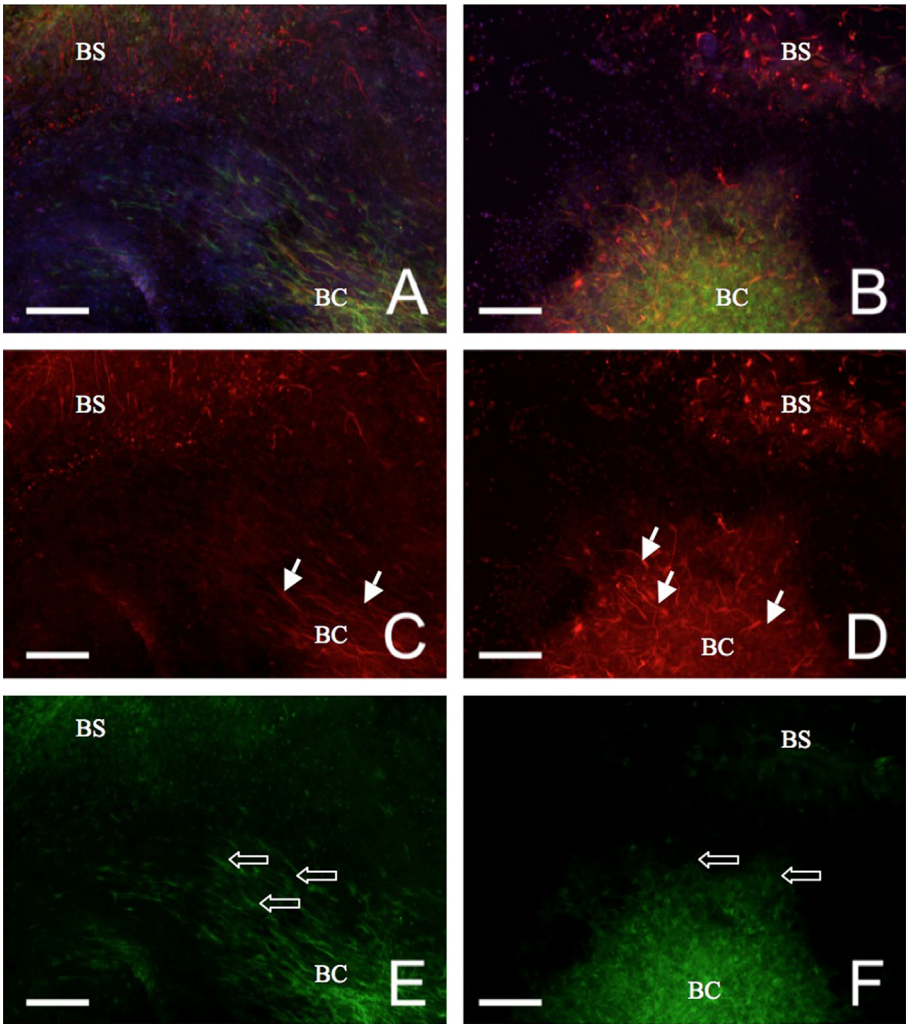

Fig. 7 – (A)–(F) Co-culture of rat BS slice and mouse BC cells in different mediums. In BS conditioned culture medium, GFP-BC cells co-cultured with rat auditory BS slice display neuronal differentiation and axonal attraction towards the BS slice within one week. Here exemplified in two different preparations at seven days of culture. (C) and (D) Neuronal differentiation in the BC ell transplant indicated by expression of βIII-tubulin (arrows). (E) and (F) Attraction of the transplanted BC-cells towards the host BS slice is visible by concentration of GFP-positive cell somas with axonal growth between the transplant and BS slice (hollow arrows). (A) and (B) Merged images. BS slice (BS); BC cell transplant (BC); βIII-tubulin (red); GFP (green); DAPI (blue). Scale bar = 100 μm.

showing that BC cells survive very well in the presence of the BS and a proper mix of growth factors. The BC cells differentiate into neurons and illustrate generous axonal growth towards the BS without any external guidance.

4. Experimental procedures

4.1. *Animals*

Pregnant Sprague-Dawley rats (SD; $n=10$) were obtained from Scanbur (Stockholm, Sweden). Animals were maintained in individual cages under standard conditions with food and water available ad libitum. Postnatal pups (P12-P14, $n=50$) were used for preparing BS slices. GFP-Tau mouse (Charles River Lab, Sulzfeld, Germany) embryos were used as donors for BC cell preparation (E11.5). All animal-related procedures were conducted in accordance with local ethical guidelines and approved animal care protocol (N329/07, N3/11, N4/11).

In obtaining BS slices, pups were deeply anesthetized intramuscularly with 4 mg per 100 g body weight ketamine (Pfizer AB) and 1 mg per 100 g body weight xylazine (Bayer AG). Following decapitation the skulls were opened longitudinally along the midline and excised brains were placed in ice cold dissecting medium (Hank's balanced salt solution (HBSS), supplemented with 20% glucose and 1% antibiotic/antimycotic). 250 μm thick transverse sections of the brain were obtained using tissue-chopping device (McIlwain).

Slices encompassing the proximal part of the cochlear nerve and the CN were chosen with anatomical landmarks (Herlenius et al., 2012; Thonabulsombat et al., 2007) and the size of each BS slice and amount of CN were approximated to

be equal for all slices. The slices were propagated as interface cultures (Stoppini et al., 1991) on sterile polyester membranes (24 mm diameter inserts with 0.4 μm pore size, Corning), coated with 5 μg/ml poly-D-lysine and 10 μg/l laminin. Slices placed on the insert were maintained in BS medium (DMEM supplemented with 30% HBSS, 10% FBS, 6.5 g/l glucose, 25 mM Hepes and 1% antibiotic/antimycotic) (Illustation 2). Cultured BS slices with abundant gliosis and visible neuronal loss were discarded. BS slices were thoroughly washed with BS medium to remove cell debris the next day after transplantation. Further on the medium was changed every other day.

4.2. *Cultures*

CM was prepared from rat BS slice monocultures that were cultured for a three-week period. Supernatant from cultures was collected every two to three days. Supernatant was then filtered through a 0.2 μm cell culture filter, pooled into one solution and mixed with equal amounts of fresh propagation medium, resulting in BS conditioned culture medium (Figs. 1 and 2).

For neurotrophic factor assay of BS CM, BS slices were prepared and CM collected as above ($n=18$). Since the BS medium is made with FBS, which contains BDNF and GDNF, we prepared a serum-free medium group where the serum part of the BS medium was substituted with more DMEM (Fig. 2).

Two groups with different amounts of BS slices were prepared, one with one slice and another with two slices per well.

Control monocultures of BS slices in BS medium and serum free BS medium were prepared for evaluation of overall survival.

BC cells were isolated as previously described (Hjerling-Leffler et al., 2005). Briefly, GFP-Tau mouse E11.5 DRGs were dissected out with sharpened tungsten forceps in BC medium (Neurobasal medium supplemented with N2 and B27) (cf. Fig. 1). Following dissociation using 1 mg/ml Collagenase/Dispase (Roche) the cells were plated in propagation medium (BC medium with 20 ng/ml epidermal growth factor (EGF) and 20 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems) added) (cf. Fig. 1). From the DRG cell culture BC sphere formations could be observed within two weeks of culture. BC spheres expressing GFP were selected after 2–3 passages and then cultured as monolayer or co-cultured with auditory BS slices.

To evaluate BC survival and differentiation response to different culture mediums, BC spheres were plated on glass slide chamber (Gibco) coated with 50 μg/ml poly-D-lysine and 20 mg/ml laminin and maintained as monolayers for three weeks in three different mediums: BC medium, propagation medium and BS conditioned culture medium (Fig. 1).

To observe BC cells survival and attraction in co-culture with rat auditory BS slice, a BC sphere was placed near the area of the CN in the BS slice under the dissecting microscope at 1 mm distance ($n=28$). Immediately after transplantation BS medium was replaced by the designated medium for each particular experiment. Medium was changed every other day. Cultures were maintained for three weeks.

4.3. *ELISA assay*

From collected CM, BDNF and GDNF levels were assayed with ELISA kit reagents (Cat. No. KA0330; Abnova Corporation, Taiwan). Standard solutions were diluted by addition of dilution buffer and 100 μl aliquots of standards and culture medium samples were added in duplicates, respectively, in individual pre coated plate wells. The plates were sealed with a removable cover and the contents were incubated for 90 min at 37 °C temperature. The plates were emptied and blotted on a paper towel. 100 μl of biotinylated anti-rat antibody working solution was added to each well and the contents were incubated for 60 min at 37 °C temperature. The plates were emptied and the wells were washed with 0.3 ml of 0.01 M PBS solution buffer for 3 times; 100 μl of ABC working solution was added to each well and the contents were incubated for 30 min at 37 °C temperature. The plates were emptied and the wells were washed with 0.3 ml of 0.01 M PBS buffer solution for 5 times; 90 μl of TMB color developing agent was added to each well and the contents were incubated in dark for about 20–25 min at 37 °C temperature. The reaction was stopped by addition of 100 μl of TMB stop solution to each well. The optical density of each well was read at 450 nm.

4.4. *Immunocytochemistry and immunohistochemistry*

Cell morphology was studied after fixation with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4), followed by immunocytochemistry (ICC) and immunohistochemistry (IHC). Monocultures on glass slide chambers were fixed for 20 min, while co-cultures with BS were kept for 2 h, all at room temperature. Cells were then permeabilized overnight with 5% Triton X-100 in PBS. Cultures were washed again three times in PBS for 5 min followed by incubation in blocking solution for 2 h. The blocking solution contained PBS, Triton X-100 (0.4% for mono- and 1% for co-culture), 5% normal goat serum (NGS; Jackson Immuno Research), 5% bovine serum albumin (BSA; Sigma). Membrane inserts were cut out for further immunostaining and mounted between a glass slide and a coverslip. Along with TUJ1, peripherin was used as a marker of neuronal development as it is expressed concomitantly with axonal growth following differentiation of sensory neurons (Escurat et al., 1990). To enhance the endogenous BC cells GFP epifluorescence an anti-GFP antibody (GFP-FITC, Abcam) was used. Primary antibodies were diluted in the blocking solution: goat anti-GFP-FITC (Abcam) 1:200; rabbit anti-neuronal class III β-Tubulin (Tuj1; Covance) 1:500 and mouse anti-peripherin (Chemicon) 1:200. Incubation with primary antibodies was done at 4 °C for 48 h. The primary antibody-antigen complexes were visualized with secondary antibodies Cy-3 and Cy-5 (Jackson Immunoresearch). The secondary antibodies were applied for 2 h at 1:250 dilution for monocultures and for 3 h at 1:200 ratio in case of co-cultures. After rinsing with washing buffer three times for 10 min, cells nuclei were counterstained using 2 mg/ml DAPI (4′,6-diamindino-2-phenylindole dihydrochloride; Molecular Probes) nuclear stain. All samples were mounted using Vecta-Shield mounting medium (Vector Laboratories) and allowed to dry by storing overnight at 4 °C. The stained cultures were observed on Zeiss Axioplan 2 microscope equipped with a spot digital camera. The images were obtained using AxioVision software. Image editing was done using Adobe Photoshop software.

4.5. *Quantification*

Following fixation and IHC all BC-BS co-cultures were subjected to quantitative analysis of directional growth of axons ($n=28$). The BC transplant area was divided into quadrants with the center of the BC cell transplant after fixation as origo. The total number of Tuj1-positive neurons with fiber growth in the absolute vicinity of the transplant was counted at 100× magnification. Care was taken to blind the group affiliation and orientation of transplants on the slide. The cells found in the quadrant encompassing the hiatus between the transplanted cells and the BS slice were accounted for as attracted. The data are presented as a percentage of neurons in the attracted quadrant, out of the total number of neurons in the transplant area. This is presented separately for each experimental group (Table 2).

Mean BDNF and GDNF concentrations were analyzed by two-way ANOVA, followed by Tukey's HSD test. Differences of $P \leq 0.05$ were considered to be statistically significant. To analyze trends we looked at relative fold of change in BDNF levels as compared between groups with BS medium as baseline.

Conflict of interest

Authors have declared no conflict of interest.

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