Molecular and Cellular Neuroscience 88 (2018) 240–248

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Brain-region specific responses of astrocytes to an *in vitro* injury and neurotrophins

Andrea Beatriz Cragnolini[a], Gonzalo Montenegro[a,1], Wilma J. Friedman[b], Daniel Hugo Mascó[a,*]

[a] IIByT-UNC CONICET, CEBICEM, Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 1611, X5016GCA Córdoba, Argentina

[b] Department of Biological Sciences, Rutgers University, 225 University Avenue, Newark, N.J. 07102, United States

ARTICLE INFO

Keywords:
Astrocytes
BDNF
NGF
Migration
Scratch injury

ABSTRACT

Astrocytes are a heterogeneous population of glial cells that react to brain insults through a process referred to as astrogliosis. Reactive astrocytes are characterized by an increase in proliferation, size, migration to the injured zone and release of a plethora of chemical mediators such as NGF and BDNF. The aim of this study was to determine whether there are brain region-associated responses of astrocytes to an injury and to the neurotrophins NGF and BDNF. We used the scratch injury model to study the closure of a wound inflicted on a monolayer of astrocytes obtained from cortex, hippocampus or striatum. Our results indicate that the response of astrocytes to a mechanical lesion differ according to brain regions. Astrocytes from the striatum proliferate and repopulate the injury site more rapidly than astrocytes from cortex or hippocampus. We found that the scratch injury induced the upregulation of neurotrophin receptor p75NTR and TrkB.t in astrocytes from all brain regions studied. When astrocytes from all regions were treated with NGF, the neurotrophin induced migration of the astrocytes (assessed in Boyden chambers) and induced wound closure but did not affect proliferation. In contrast, BDNF induced wound closure but only in astrocytes from striatum. Our overall findings show the heterogeneity in astrocyte functions based on their brain region of origin, and how this functional diversity may determine their responses to an injury and to neurotrophins.

1. Introduction

Astrocytes comprise a population of glial cells that play critical roles during development as well as in the maintenance of the central nervous system (CNS) functions. These glial cells are often defined as a population that shares common characteristics and functions throughout the CNS, however the existence of morphologically different populations of astrocytes has recently been recognized (Schitine et al., 2015). Different astrocytic morphologies are seen not only in resting astrocytes among brain regions but also in response to a lesion, however, the functional consequences of such diversity have not been fully defined. The diversity of astrocytes is also reflected in the factors they release and how they affect their environment. Thus, it has been reported that astroglial cultures obtained from cortex, cerebellum and spinal cord release unique combinations and amounts of chemokines and cytokines (Fitting et al., 2010). The heterogeneity of astrocytes also impacts their synaptogenic potential, mainly due to distinct gene expression profiles (Buosi et al., 2017). Astrocytes undergo morphological and physiological changes during an animal's life (Schitine et al., 2015). Aging and environmental impoverishment significantly affect astrocytes by reducing their morphological complexity (Diniz et al., 2016). Similar changes are observed after different types of injuries. This phenomenon it is called reactive astrogliosis and it is not a homogeneous response. On the contrary, it is highly regulated by context-specific factors released by neurons, other types of glial cells and astrocytes themselves (Pekny and Pekna, 2014). A CNS injury triggers a finely tuned spectrum of changes ranging from reversible alterations in gene expression and cellular hypertrophy to cell proliferation with compact scar formation and permanent tissue rearrangement (Sofroniew, 2015) and in many cases migration toward the injured site (Zhan et al., 2017).

Reactive astrocytes release a plethora of chemical mediators, including cytokines (Choi et al., 2014), adhesion molecules (Hayakawa et al., 2014), neurotrophins (Patel and Gray, 1993; Rubio, 1997), and extracelular matrix proteins (Wiese et al., 2012) among others. After an injury, neurotrophins can be released by several type of cells, including

* Corresponding author.
E-mail address: dmasco@fcq.unc.edu.ar (D.H. Mascó).
[1] Present address: Instituto Multidisciplinario de Biologia Vegetal (IMBIV), Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 299, 2º piso. 5000 Córdoba

https://doi.org/10.1016/j.mcn.2018.02.007
Received 6 September 2017; Received in revised form 22 January 2018; Accepted 9 February 2018
Available online 11 February 2018
1044-7431/

microglia (Gomes et al., 2013; Tay et al., 2016; VonDran et al., 2014) and astrocytes (Goss et al., 1998; Volosin et al., 2006; Domeniconi et al., 2007; Fulmer et al., 2014) as the main sources. Neurotrophins maintain the survival and differentiation of specific populations of neurons in the central and peripheral nervous system and these actions depend on the interaction with specific Trk receptors and/or p75NTR (Park and Poo, 2012). While the effects of neurotrophins on neurons have been extensively studied, little is known about their functions in astrocytes. Neurotrophin receptors have been detected in astrocytes from several brain areas during development (Hutton et al., 1992; Rudge et al., 1994; Cragnolini et al., 2009, 2012), while in the adult brain they are present in a small population of cells and mainly in injured areas (Cragnolini et al., 2009, 2012; Lu et al., 2013; Schachtrup et al., 2015).

There is evidence showing that astrocytes are a target for neurotrophin actions. NGF can modulate the reactivity of astrocytes by reverting the phenotypic changes associated with astrogliosis after a spinal cord injury (Cirillo et al., 2010; De Luca et al., 2016) and can arrest astrocytes in the cell cycle acting through p75NTR (Cragnolini et al., 2012, 2009). On the other hand, BDNF and the truncated TrkB receptor (TrkB.t) modulate the astrocytic morphology in adult rat neocortex (Ohira et al., 2007).

Astrocytic migration is a crucial process in the developing brain for structural organization as well as during pathological conditions (Cayre et al., 2009; Zhan et al., 2017). The scar formation that occurs after an injury consists of both glial (mainly astrocytic) and fibrotic components. It has been shown that the scar formation has both positive and negative consequences for the surrounding nervous tissue. Astrocytes become dynamic migratory cells when they are performing pathological scar formation (Kawano et al., 2012; Zhan et al., 2017) and this response is a highly regulated process that is not fully understood.

In this study we used an *in vitro* model to compare the closure of a wound inflicted on monolayers of astrocytes obtained from cortex, hippocampus and striatum. We also examined the effects of the neurotrophins NGF and BDNF on the wound closure. Our results indicate that there are region-specific differences in astrocyte responses to an injury and neurotrophins.

2. Methods

2.1. Astrocyte culture

Astrocytes were obtained from P0-P2 Wistar rats maintained under standard 12-h light-dark cycle, 21 °C and *ad libitum* access to food and water. All procedures involving animals were performed in accordance with the international guidelines for the use of laboratory animals, and were approved by the Institutional Committee for Handling and Care of Laboratory Animals. Primary cortical, striatal and hippocampal cultures of astrocytes were performed according to the protocol described by McCarthy and De Vellis (1980). Briefly, cortices, striatum and hippocampi were dissected out from each brain, dissociated by trituration, homogenized and plated on poly-D-lysine-coated 25 cm^2 flasks in growth medium: DMEM (Invitrogen, 10% foetal bovine serum (FBS, Sigma), 50 IU/mL penicillin and 50 IU/mL streptomycin (Invitrogen). Astrocytes were grown to confluence and purified by shaking according to previously published methods (McCarthy and De Vellis, 1980; Cragnolini et al., 2012). The astrocytes were trypsinized and replated onto poly-D-lysine-coated coverslips and placed in 24 multiwell plates, 35 mm plastic Petri dishes or transwell inserts (Corning). The purity of astrocytes was approximately 95%, confirmed by labelling with antibodies against GFAP and devoid of microglia markers staining (anti-CD11) and oligodendrocytes progenitors (anti-NG2). To confirm the specificity and functionality of anti-CD11b and anti-NG2 we tested these antibodies on pure microglial cultures and mixed glial cells cultures, respectively (Supplementary Fig. 1). Mixed glial cultures were obtained from primary cultures without the shaking process used to obtain pure astrocytic cultures.

2.2. Scratch injury

Astrocytes were plated on glass coverslips (50,000 cells per coverslip) and allowed to reach confluence, usually, 7–8 days post-plating.

The day before performing the scratch, the growth medium was aspirated, cells were washed twice with PBS and medium containing low serum was added (DMEM, 1% FBS, 50 UI/mL penicillin and 50 U/mL streptomycin). We used low serum concentration to minimize the effect of growth factors in the medium that could mask the effects of the treatments. At the same time, 1% of FBS allowed the migration induced by the scratch otherwise, in serum free conditions, migration occurs at very low speed even in the presence of neurotrophins.

Twenty four hours later one longitudinal scratch was made in each well using a sterile plastic pipette tip (Etienne-Manneville, 2008), The multiwell plates containing astrocytes were washed once with PBS to remove the debris and incubated with fresh media with low serum and containing the treatments. Immediately following the addition of treatments (time 0), pictures were taken using an inverted microscope in bright field (Nikon Te2000-U). Astrocytes were maintained for 48 h after the scratch and pictures were taken at the end of the period to calculate the area covered by cells post-injury. Ten to twelve pictures were taken along scratch with a 10× magnification objective and superimposing approximately 10% between each contiguous image. Individual images were stitched to reconstruct the scratch at time 0 h and 48 h and the cell-free area was calculated using the FIJI software (Schindelin et al., 2012) to estimate the wound closure.

2.3. SDS-PAGE and western blot

For western blot analysis for neurotrophin receptors expression, confluent astrocyte monolayer grown in 35 mm dishes, were scratched with pipette tips 6 times in one direction followed by 6 times at 90 degree angles to the first set of scratches to increase the area exposed to injury. Twenty four hours later astrocytes were lysed in RIPA buffer, proteins quantified and Western blot was run in standard conditions.

To assess the expression of NGF and BDNF whole cell lysate of astrocytes were used. Cells were lysed in RIPA buffer, proteins quantified and were run in SDS-PAGE, proteins were transferred to a nitrocellulose membrane. The membranes were fixed with 2.5% glutaraldehyde for 30 min, this step was required to detect low molecular weight proteins (Anastasia et al., 2013) and we did not detect NGF or BDNF without the fixation.

2.4. Assessment of astrocyte viability post-scratch injury

To assess cell viability post-scratch injury in astrocyte cultures from cortex, hippocampus and striatum were stained with calcein AM/ethidium (Molecular Probes, Cat # mp03224) according to manufacturer's instructions. Briefly, cells from each brain area were cultured on coverslips and scratched as described above, then astrocytes were washed twice with PBS and a mix of calcein AM/ethidium was added and incubated for 25 min at room temperature. Immediately after coverslips were mounted with FluorSave on glass slides and observed under a fluorescence microscope. Five photos per coverslip were taken along the scratch covering a distance of 650 um from de edge of the scratch. Astrocyte death rate was calculated by determining the percentage of ethidium-positive cells over total cell number. For each experimental group, five fields from at least two coverslips from two separated cultures were imaged were evaluated for each brain region.

2.5. Proliferation assay

To analyze the proliferation rate of astrocytes along the edge of the injury, astrocytes were grown to confluence and scratched as described

above. BdrU (10 μM) was added to the cultures together with the treatments. Forty-eight hours later, cells were fixed with 4% paraformaldehyde and immunostained using a specific antibody against BrdU as described in Cragnolini et al. (2009). Pictures were obtained from fields of equivalent size in three different areas of scratched monolayers.

2.6. Migration assay

The experiments were conducted using P0-P2 Sprague-Dawley rats p75NTR homozygous knockout rats (*p75NTR−/−*) generated by CRISPR technology (Sage Labs/Horizon, USA) or wild type (*p75NTR +/+*). The p75NTR−/− rats were genotyped by PCR to confirm the lack of p75NTR. Cell migration and chemotactic effect of neurotrophins was assayed using transwell chambers containing an 8 μm pore size filter (Corning, cat #3464), following the supplier's directions. Briefly, 15×10^4 cells were plated on the poly-D-lysine-coated upper chamber. 24 h later cells were washed with PBS and medium replaced with medium with low serum, and neurotrophins were added to the lower chamber and maintained for 24 h. At the end of the period, filters were removed from the incubator and the remaining cells on the upper chamber were detached with a cotton swab. The cells on the lower side of the filters were fixed with 4% paraformaldehyde and stained with DAPI. The filters were then placed on glass slides ensuring that the lower surface of the filter was adjacent to the glass slide. One picture per filter was taken with a 4× magnification objective to cover most of the filter surface. The number of cells which migrated to the lower side of the filter were automatically counted with FIJI software (Schindelin et al., 2012).

2.7. Adhesion assay

We performed the adhesion assay as described by Giralt et al. (2016). Briefly, confluent cultured astroglia were trypsinized and suspended in DMEM with 10% FBS. After measurement of cell density with a hemocytometer, 5×10^4 cells per well were plated in 24-well plates coated with poly-D-lysine. Four hours after plating cells were washed with PBS to remove non-adherent cells, lysed and intact cell nuclei were counted with a hemocytometer, a quantification method we have used previously (Friedman, 2000; Volosin et al., 2006). Nuclei of dead or dying cells are irregularly shaped or disintegrated. In contrast, nuclei of healthy cells are phase bright and have clearly defined limiting membranes. Cell counts were performed in triplicate and adhesion rate was defined as the percentage of cells remaining 4 h after plating.

2.8. Chemicals

Recombinant human NGF (hNGF) and hBDNF were purchased from Alomone (NGF, cat # N-245; BDNF, cat # B-250). Culture media was from Invitrogen, poly-D-lysine, ANA-12 and FBS were purchased from Sigma. Antibody against BrdU was from Millipore (Cat# MAB3510 RRID:AB_94897, dilution 1:50), anti-GFAP from Immunostar (Cat# 22522 RRID:AB_572240, dilution 1:8), anti-CD11 (Cat# CBL1512Z RRID:AB_347694, dilution 1:100), anti-NG2 (Cat# sc-33,666, dilution 1:50) and anti-p75NTR for blocking assay (Cat# MAB365 RRID:AB_2152788, dilution 1:500) were from Millipore, anti-p75NTR Rex used for Western blot was kindly provided by Dr. Louis Reichardt (dilution 1:2000) anti-TrkB was from Santa Cruz Biotechnology (Cat# sc-8316 RRID:AB_2155274, dilution 1:1000) anti-BDNF (Cat# sc-546 RRIB:AB_630940, dilution 1:500) and anti-NGF (Cat# sc-548 AB_632011, dilution 1:500).

2.9. Statistics

The results are expressed as mean ± standard deviation (SD), unless otherwise is indicated, of at least 3 independent cell cultures. ANOVA was used to compare groups and was followed by Tuckey *post hoc* comparisons, with $p \leq 0.05$ considered significant.

3. Results

3.1. Scratch injury induces a brain region-dependent astrocytic response

In order to begin to understand the role of neurotrophins and their receptors in the wound closure by astrocytes, we first compared how astrocytes from cortex, striatum and hippocampus responded to the scratch injury inflicted to a cell monolayer. The injury left an empty area of 550–600 μm which was then closed by growing astrocytes. We found that there was a regional heterogeneity in the astrocyte response to the wound. Astrocytes from the three studied areas showed different speed of closure of the injured area (Fig. 1a, b). Thus, striatal astrocytes were the fastest cells to progress toward the injured site, occupying 63 ± 4,3% of the initial empty gap at 48 h post-scratch. In contrast, in cultures from hippocampus and cortex the occupied areas were significantly lower than striatum, 54,5 ± 6,1% and 39,43 ± 5,6%, respectively. Since the cultures were highly pure populations of astrocytes, confirmed by immunostaining of GFAP, we rule out the possibility that other glial cell types can contribute to the closure of the wounded astrocytic cultures obtained from the three brain areas (Supplementary Fig. 1).

3.2. Striatal astrocytes show higher proliferation rates than cortex and hippocampus in response to an injury

The repair process involves both cell proliferation along the edge of the injury and migration of astrocytes toward the injured site. We investigated whether the differences in wound closure were inherent to the astrocytes from the different brain regions, and whether they were due to differences in proliferation or migration. Cultures of astroglial monolayers obtained from cortex, hippocampus or striatum were scratched and incubated with Brdu for 12, 24, 48 and 72 h following injury. At the end of each incubation period, the cultures were subjected to immunostaining and the proportion of the BrdU-positive cells along the scratch and occupying the cell-free gap was calculated as a percentage of the total nuclei in a given microscopic field. In agreement with previous publications (Környei et al., 2000; Yang et al., 2009; Cragnolini et al., 2012) we observed that the injury caused a significant increase in the proportion of proliferating cells along the wound which decreased with the distance to the cell-free gap (Fig. 2a, c). The population in the center of the injury and the invading cells into the gap at 48 h was mainly dominated by proliferating astrocytes obtained from striatum (92%) and cortex (50%), but the proliferating cells in the gap of hippocampal cells was only 17% (Fig. 2c).

We also found that the proportion of proliferating cells over the time along the scratch varied among the astrocytes from different brain areas. Cell growth curve demonstrated that astrocytes obtained from striatum have a higher proliferation rate compared to astrocytes derived from cortex and hippocampus. This difference started to be significant 24 h post-injury and it was sustained over the evaluated period. Astrocytes from cortex showed a slightly higher proliferative rate respect to astrocytes derived from hippocampus that was significant at 72 h post-scratch.

To assess whether astrocytes from different brain region have a different susceptibility to death following the mechanical injury we carried out a survival/death assay staining with calcein-AM and ethidium homodimer-1. Viable cells exhibited green fluorescence generated by the esterase hydrolysis of membrane-permeant dye, calcein-AM and dead cells were marked by red fluorescence ethidium homodimer-1 (Fig. 2d, e). The percentages of dead cell along the edge of the scratch were 2.97 ± 0.22; 2.83 ± 0.53 and 2.76 ± 0.19 for astrocytes from cortex, hippocampus and striatum, respectively. There were no significant differences on cell death between injured astrocytes obtained

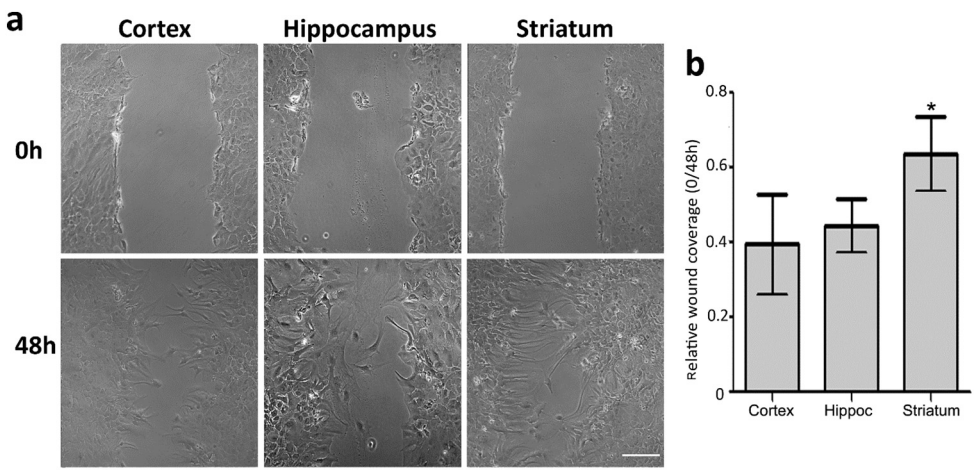

Fig. 1. Different wound occupation in astrocytic cultures from distinct brain areas. a) Representative images of wound healing in monolayers of astrocytes from cortex, hippocampus and striatum, upper row shows images of astrocytes immediately after the injury (time 0), bottom row, 48 h after the injury. b) Quantification of the occupied area 48 h after the injury. Mean ± 95% confidence interval, $n \geq 5$, * $p \leq 0.05$. Scale bar 200 μm.

from the three different areas.

3.3. Neurotrophin receptors p75NTR and TrkB.t are up-regulated in astrocytes from cortex, hippocampus and striatum after a scratch injury

We previously demonstrated that *in vivo* brain injuries, such as *status epilepticus*, or an *in vitro* scratch wound induced the up-regulation of p75NTR expression in astrocytes from hippocampus (Cragnolini et al., 2009, 2012). It has also been shown that TrkB.t1, the main isoform of the TrkB receptor expressed in astrocytes, is up-regulated in these cells after a spinal cord lesion (Matyas et al., 2017). To explore whether the increase in the expression of both receptors, p75NTR and TrkB.t, is a common hallmark of astrocytes from different brain areas after a mechanical injury, we performed a scratch wound on astrocytic

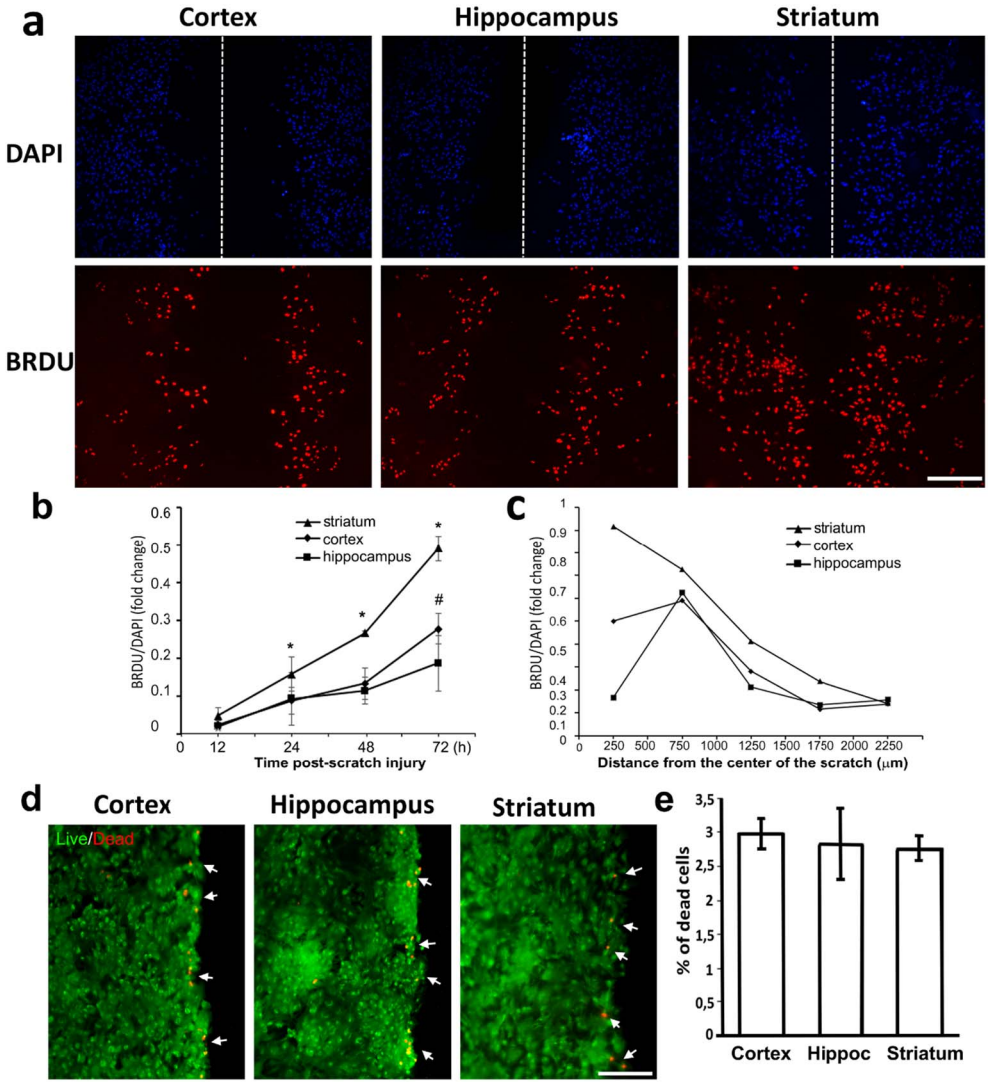

Fig. 2. Scratch wound induces proliferation of astrocytes in a brain region-dependent manner. Astrocytes obtained from cortex, hippocampus or striatum were injured and immediately provided with BrdU (10 μM) for 48 h, then fixed and labeled for BrdU and DAPI. a) Photomicrographs of dual staining for DAPI (top row) and BrdU (bottom row) along the scratch-wounded astrocytes from cortex, hippocampus and striatum. b) Quantification of astrocyte proliferation ratio in wounded regions at 12, 24, 48 and 72 h post-injury. Mean ± SEM, $n = 3$, *$p \leq 0.05$ striatum *vs* hippocampus or cortex, # $p \leq 0.05$ cortex *vs* hippocampus. c) Lateral distribution of proliferating cells in wounded areas; consecutive images were taken along 2250 μm from the center of the scratch (dotted line in Fig. 2a) and the ratio Brdu/DAPI was quantified. d) Representative micrographs showing astrocytic cultures stained with calcein/ethidium immediately after the scratch injury (calcein+ cells, green; ethidium homodimer-1+ cells, red, are indicated with white arrows). e) Quantification of dead cells along the edge of the wound. Mean ± SEM, $n = 2$. Scale bar 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

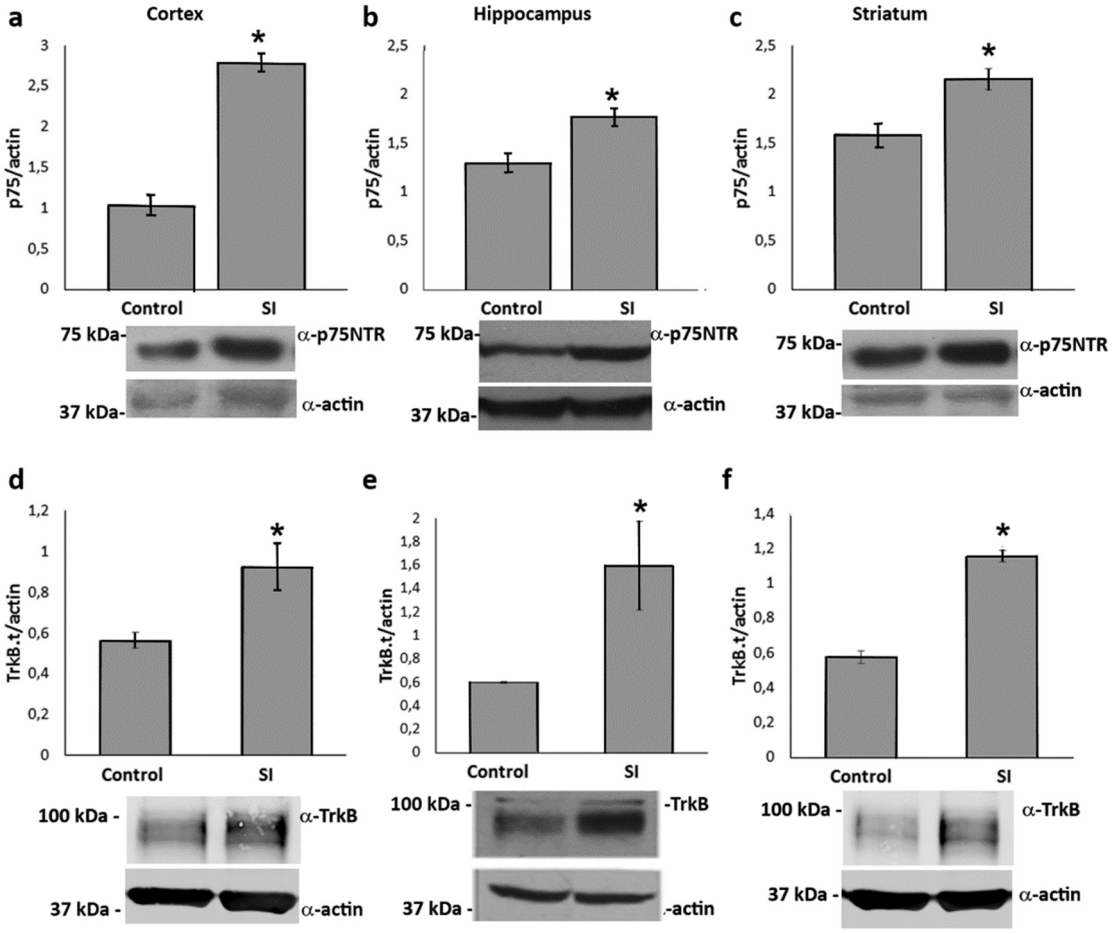

Fig. 3. p75NTR and TrkB.t expression increase after scratch injury in astrocytes from different brain areas. Representative Western blot of protein homogenates from astrocytes obtained from cortex (a, d), hippocampus (b, e) and striatum (c, f) 24 h after the scratch injury or time-matched controls probed with antibodies against p75NTR (a, b, c) or TrkB.t (d, e, f) anti-actin was used as a loading control. Top panels, densitometric quantification of Western blot bands expressed as ratio of p75NTR or TrkB/actin. Mean ± S.E.M., $n = 3$, * $p \leq 0.05$.

monolayers and 24 h later the levels of these receptors were analyzed. Western blot analysis demonstrated that non-injured astrocytes obtained from cortex, hippocampus and striatum express p75NTR and TrkB.T1 receptors (Fig. 3). The *in vitro* injury caused a significant up-regulation in the expression of both receptors, p75NTR and TrkB.t on astrocytes from the three brain areas studied. To detect TrkB on astrocytes by Western blot we used an antibody that recognizes the full length and the truncated isoforms (Cat# sc-8316 RRID:AB_2155274) and we only detected two bands at approximately 90 kDa corresponding to the truncated isoforms on astrocytes from the three brain regions.

3.4. Exogenous neurotrophins induce wound closure of astrocytic monolayers without affecting proliferation

We next examined the effect of NGF and BDNF on wound closure. Astrocytes were scratched and NGF or BDNF (10 ng/mL) were added immediately afterward. Treatment with NGF increased the speed of invading astrocytes obtained from the three brain areas studied (Fig. 4) compared to untreated controls. The effect of NGF was more noticeable in cortical astrocytes (Fig. 4a) which increased the occupied cell-free area 48% more than controls. In astrocytes from hippocampus and striatum the occupied area post-scratch after treatment with NGF were, respectively, 25% and 20% larger than controls. We also assessed the effect of BDNF (10 ng/mL) on wound closure but unlike NGF, only induced an increase in the occupied area post-injury in astrocytes from striatum.

Astrocytes from different brain areas progressed at different rate toward the gap, however all of them had the capability to close the wound. When we observed astrocytes at 72 h porst-sctach (Supplementary Fig. 2) the gaps were completely closed in cell cultures treated with NGF in cortical and hippocampal astrocytes and in the striatum were tightly closed with neurotrophins (Supplementary Fig. 2) that was difficult to distinguish the astrocytes that invaded the scratch from the nearby non-injured cells.

To determine whether the endogenous production of neurotrophins NGF and BDNF varies among astrocytes from different brain regions we analyzed their expression by Western blot in whole cell lysate of uninjured astrocytes. To detect the neurotrophins we used a protocol that includes a fixation of the proteins transferred to the nitrocellulose membranes with glutaraldehyde (Anastasia et al., 2013) otherwise we did not observe any band in our samples (Supplementary Fig. 3). We observed dim bands at approximately 14 kDa corresponding to BDNF as well as a higher molecular weight bands that could correspond to pro-NGF. When we probed our blots with anti NGF we did not detect mature NGF, however we detected a single band between 20 and 25 kDa. We did not observe differences in the levels of expression of NGF or BDNF between astrocytes from cortex and striatum.

The results obtained so far indicate that astrocytes from the cortex and hippocampus responded similarly to the scratch injury as well as to the treatment with neurotrophins. For that reason we decided to continue with the studies comparing only astrocytes of cortex and striatum.

The wound closure mainly depends on cell migration and proliferation. It was therefore important to determine which of these processes was affected by neurotrophins. We analyzed the proliferation of wounded astrocytic monolayers treated with NGF or BDNF by

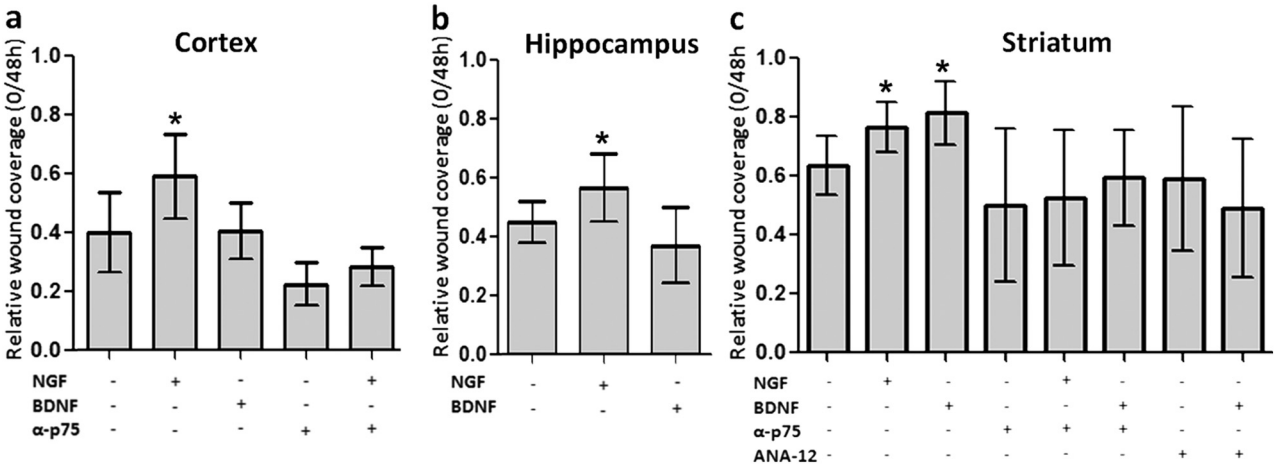

Fig. 4. p75NTR mediates the astrocyte wound closure induced by neurotrophins. Quantification of the occupied area 48 h after the scratch injury on astrocytes obtained from cortex (a), hippocampus (b) and striatum (c). Confluent monolayers of astrocytes were scratched and treated with neurotrophins or vehicle (DMSO) immediately afterward. The blocking antibody against p75NTR (1 μg/mL) (a, c) or the TrkB antagonist, ANA-12 (10 μM) (c) were added to the cultures 10 min before NGF (10 ng/mL) or BDNF(10 ng/mL). Mean ± 95% confidence interval, $n \geq 5$ * $p \leq 0.05$.

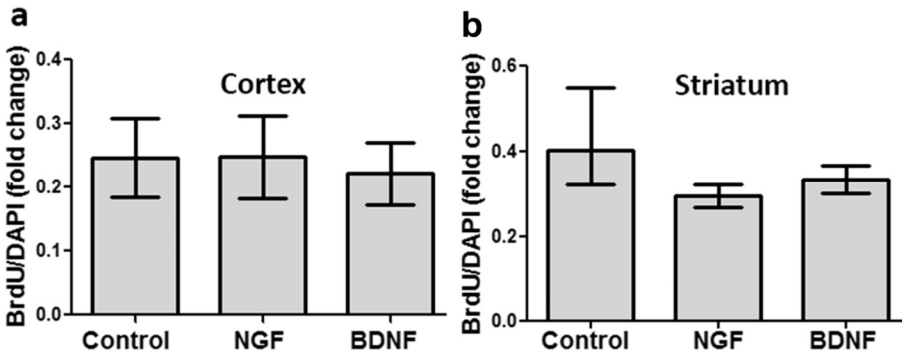

Fig. 5. Neurotrophins did not affect the scratch injury-induced proliferation in astrocytes from cortex and striatum. Astrocytes obtained from cortex (a) or striatum (b) were injured and treated with NGF or BDNF immediately afterward (10 ng/mL) and provided with BrdU (10 μM) for 48 h. Astrocytes were fixed and labeled for BrdU and DAPI and the proliferation rate was quantified as the ratio of BrdU positive cells to DAPI. Mean ± 95% confidence interval, $n = 3$, *$p \leq 0.05$.

quantifying the incorporation of BrdU along the edge of the injured area. We observed that none of the neurotrophins affected the proliferation rate induced by the scratch injury in astrocytes from cortex (Fig. 5a) or striatum (Fig. 5b).

3.5. P75NTR is required for NGF and BDNF-induced astrocytic wound closure

To determine whether the p75NTR receptor mediated the effects of NGF or BDNF on wound closure of astrocytic monolayers we added a function-blocking antibody against p75NTR 10 min before the neurotrophins and the occupied areas were measured at $t = 0$ and 48 h post-treatment. Exposure of the astrocytes to anti-p75NTR (0.5 μg/mL) followed by NGF or BDNF (10 ng/mL) reversed the effects on wound closure of NGF on astrocytes from cortex and striatum, and BDNF on astrocytes from striatum (Fig. 4a,c). Similarly, to assess whether TrkB.t was involved in the effect of BDNF on wound closure of astrocytes from striatum we used the specific TrkB antagonist ANA-12 (10 μM) (Cazorla et al., 2011) administered 10 min before BDNF. The addition of ANA-12 immediately after the scratch abolished the effect of BDNF on wound closure of striatal astrocytes (Fig. 4c).

3.6. The NGF-induced migration is abolished in p75NTR−/− astrocytes

We showed that NGF induced the wound closure of injured astrocytes without affecting the proliferation (Figs. 4 and 5). Although several methods are used to study migration, the Boyden chamber assay is one of the most accepted methods, therefore we utilized this assay to confirm our results obtained with the scratch injury. To ascertain the effect of NGF on migration, a chemotaxis and migration assay using transwell chambers was carried out in non-injured cortical and striatal astrocytes obtained from wild type and p75NTR−/− rats. We used NGF (10 ng/mL) or DMEM with 1%FBS as the control group in the lower chamber. When NGF was added for 24 h the number of astrocytes migrating through the 8 μm pore filter increased 2.5 times in cortical astrocytes and 1.6 times in striatal astrocytes compared to control. The NGF-induced migration was abolished in p75NTR−/− astrocytes obtained from cortex and striatum (Fig. 6).

To rule out the possibility that the decrease in astrocytic migration in $p75^{NTR}$ KO were due to an adhesion deficit we performed an adhesion assay in astrocytes from cortex and striatum. We found that wild type and KO astrocytes obtained from both regions displayed similar adhesion to a poly-D-Lysine-covered substrate (data not shown). These results indicate that the migratory deficit of p75NTR−/− astrocytes are not related to adhesion deficits.

4. Discussion

In this study we used an *in vitro* scratch-wound model to demonstrate that astroglia derived from different regions differ in their responses to a mechanical injury and to the neurotrophins, NGF and BDNF. The scratch injury has been extensively employed to study wound healing and migration, since it recapitulates most of the main astrocytic responses to an *in vivo* injury such as polarization of cell processes, migration and proliferation along the edge of the scratch (Etienne-Manneville, 2008; Cragnolini et al., 2012). In our study, the scratch injury was made on completely confluent astrocytic cultures and maintained for the same time *in vitro*, suggesting that the

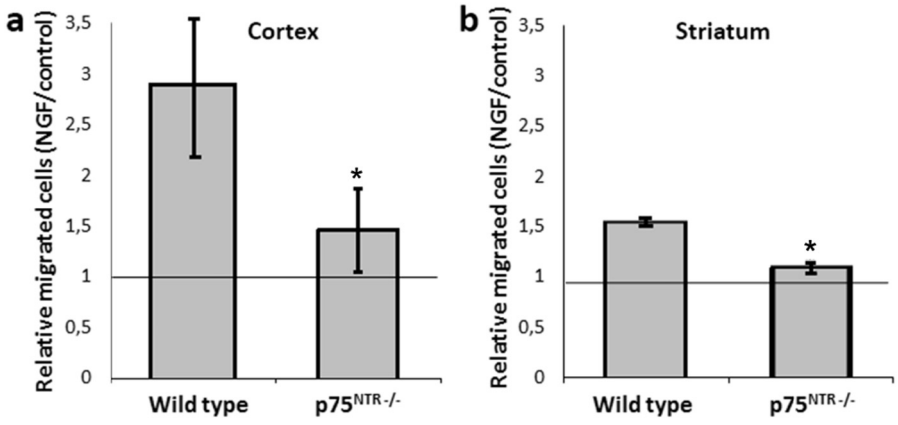

Fig. 6. p75NTR mediates the NGF-induced migration in astrocytes from cortex and striatum. Astrocytes obtained from cortex (a) or striatum (b) were plated on top of a transwell chamber and migration to the bottom side of the filter was quantified 24 h after the addition of NGF (10 ng/mL) to the lower chamber. Results are expressed as the ratio of migrating cells treated with NGF respect to controls. Mean ± SEM, $n = 3$ * $p \leq 0.05$.

differences observed correspond to inherent properties of astrocytes from different regions and not to the age of cultures or confluence levels.

Our results demonstrate that astrocytes obtained from cortex, hippocampus and striatum can respond to the scratch injury by proliferating and invading the empty space that it is left by the lesion. This response was not uniform among the astrocytes derived from different brain regions. Thus, astrocytes obtained from striatum occupied the empty gap post-injury faster than astrocytes from the other brain regions. Astrocytes from cortex and hippocampus were also able to close the injury but it took longer than those from the striatum, indicating that there were temporal differences in the reaction to the injury instead of a fail to respond. In addition, the temporal pattern of proliferation post-scratch injury showed a localized response along the edge of the wound where striatum derived astrocytes proliferated more than those obtained from hippocampus or cortex. Other studies have shown that proliferation of astrocytes varies among brain regions in the healthy adult mouse brain, and the hippocampus was one of the areas with the highest mitotic index (Emsley and Macklis, 2006). The variation may be due to methodological issues such as the type of animals used for the study and the fact that they analyzed the proliferation of astrocytes *in vivo* where the environment is extremely complex and numerous factors may contribute to the response to injury. Together, our results show that the response of astrocytes to a lesion is heterogeneous, and suggest that striatal astrocytes could have a greater capability for wound repair *in vitro*.

At this point is unclear what determines and maintains the brain region differences of astrocytes, but many environmental cues may be involved in determining the astrocytic phenotypes such as the extracellular matrix (Johnson et al., 2015; Dauth et al., 2016) or the availability of trophic factors (Das et al., 2001). We demonstrated that the scratch injury induced an increase in the levels of the neurotrophic receptors p75NTR and TrkB.t of astrocytes from the three brain areas. This observation suggests that the upregulation of p75NTR and TrkB.t, could be a distinctive response of all astrocytes to different types of CNS injuries and make them more sensitive to neurotrophins. This idea is also supported by our previous studies on the expression of p75NTR in injured hippocampal astrocytes *in vitro* (Cragnolini et al., 2012) and animals that underwent *status epilepticus* (Cragnolini et al., 2009). Others have also shown the upregulation of p75NTR in astrocytes after different kind of brain insults (Oderfeld-Nowak et al., 2003; VonDran et al., 2014) as well as TrkB.t1 (Matyas et al., 2017; Wu et al., 2013). Neurotrophins, released by neurons and glial cells, are important signals for migration during development and in the course of CNS repair (Bartkowska et al., 2010; Grade et al., 2013; Oliveira et al., 2013). In this work we did not observe differences in neurotrophin expression among non-injured astrocytes in culture, however it is known that their levels show differential patterns in the rat brain regions during development and adulthood (Das et al., 2001) this could influence on the variability of astrocytic responses in pathological conditions. Further studies would be needed to determine whether the pattern of expression of neurotrophins is affected by an injury in astrocytes from different brain areas.

We demonstrated that neurotrophins added to a confluent glial monolayer has a differential effect on astrocytes from distinct brain areas after a scratch injury. While NGF promoted the repopulation of the wound in astrocytes from all brain areas, BDNF only stimulated the striatal astrocytes. An effect of NGF and BDNF on migration of multipotent astrocytic stem cells has been reported earlier (Douglas-escobar et al., 2012). Although they used higher concentrations of NGF, ranging from 200 to 400 ng/mL to induce the wound closure. This discrepancy may stem from differences in the cell types used in their studies which could have a different sensitivity to NGF. Both neurotrophins, NGF and BDNF, are known to affect proliferation of several cell types (Cragnolini et al., 2009; Cragnolini et al., 2012; Islam et al., 2009; Tu et al., 2017; Waterhouse et al., 2012). Our results demonstrated that the concentration used to induce wound closure did not affect the proliferation induced by the injury.

Using p75KO rats and Boyden chambers to assess the migration of astrocytes, we observed that the absence of p75NTR markedly alters astrocyte migration induced by NGF. This result is in agreement with data obtained using a blocking antibody in the scratch injury assay. NGF binds to both TrkA and p75NTR receptors, however we have previously shown that TrkA is not expressed in astrocytes (Cragnolini et al., 2009), thus we rule out the possibility that this receptor could be involved in NGF effects. Consistent with our results, p75NTR mediates the effect of NGF and BDNF on migration of several cell types, including Schwann cells (Anton et al., 1994), neuroblasts (Grade et al., 2013) and many tumor cells (Bapat et al., 2016; Shonukan et al., 2003). The results obtained so far rule out the possibility that the induction of wound closure is due to an effect on the proliferation of astrocytes suggesting that neurotrophins affect specifically migration instead of proliferation.

On the other hand, BDNF can bind to both neurotrophin receptors, p75 and TrkB.t. We showed that inhibiting either p75NTR with an antibody or TrkB.t with the antagonist ANA-12 caused the effect of BDNF to be blocked in astrocytes from striatum. Since we reversed the effect of BDNF on striatal astrocytes by either blocking TrkB.t or p75NTR we suggest that both receptors are necessary for mediating the BDNF in astrocytes from striatum. In agreement with our results, Matyas et al. (2017), recently observed that TrkB.t1 mediated the scratch injury closure induced by BDNF or serum on astrocytes from spinal cord.

It would be interesting to study if both receptors physically interact and stimulate signaling pathways associated with migration in astrocytes from striatum. In fact, previous studies showed that the interaction of p75NTR and TrkB.t is necessary to induce the formation of filopodia in neurons (Hartmann et al., 2004). It is unknown what makes astrocytes from different brain regions sensitive to distinct

neurotrophins. It is known that p75NTR interacts with other receptors, such as Trk receptors, sortilin and SorCS1, and recruits various cytoplasmic proteins to trigger different signaling pathways (VonDran et al., 2014). It would be interesting to determine whether these co-receptors and interacting proteins have a selective distribution in the CNS that may explain the regional responses of astrocytes to neurotrophins.

The potential consequences of the upregulation of neurotrophic receptors in astrocytes could involve effects on astrocytes itself as well as on the surrounding cells. The upregulation of p75NTR and TrkB.t might increase the migration of astrocytes stimulated by neutrophins facilitating the scar formation, which has recently been demonstrated that supports the axonal growth (Anderson et al., 2016). On the other hand, the activation of TrkB.t by BDNF in astrocytes triggers the release of nitric oxide which has detrimental consequences for neurons (Colombo et al., 2012).

Our overall findings show the heterogeneity in astrocyte functions based on their brain region origin and how this functional diversity may determine their responses to an injury and to neurotrophins. Knowing the mechanisms triggered during wound closure in different brain areas could help to develop new strategies to repair CNS damage. Further studies are clearly needed to determine *in vivo* whether neurotrophins have a brain-region associated effect, and could help to develop new strategies to repair specific CNS areas.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcn.2018.02.007.

Acknowledgment

This study was supported by grants from CONICET (11420110100226), PICT [2011 #1859, 2012 #2100 and 2015 #3199] and SECyT-UNC (113/17). GM was a recipient of a fellowship from Consejo Interuniversitario. We thank to CONICET for a travel grant given to ABC to perform some of the experiments at the Department of Biological Sciences, Rutgers University, Newark, NJ, USA. The authors declare no competing financial interest.

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