Brain injury activates microglia that induce neural stem cell proliferation *ex vivo* and promote differentiation of neurosphere-derived cells into neurons and oligodendrocytes

Tomas Deierborg, Laurent Roybon, Ana R. Inacio, Jelena Pesic and Patrik Brundin

Neuronal Survival Unit, Wallenberg Neuroscience Center, Department of Experimental Medical Science, Lund University, Lund, Sweden.

Corresponding author:

Tomas Deierborg

Neuronal Survival Unit, BMC A10, Wallenberg Neuroscience Center,

221 84 Lund, Sweden

Phone: + 46-46-2229827, Fax: + 46-46-2220531

E-mail: tomas.deierborg@med.lu.se

Abbreviations

BrdU: 5-Bromodeoxyuridine; ANOVA: analysis of variance; bFGF: basic fibroblast growth factor; CNPase: 2', 3'-cyclic nucleotide 3'-phosphodiesterase (oligodendrocyte specific marker); DAPI: 4',6-diamidino-2-phenylindole; EGF: epidermal growth factor; GFAP: Glial fibrillary acidic protein (astrocyte specific marker); Iba1: ionized calcium binding adaptor molecule 1 (macrophages/microglia specific marker); IFN: interferon; IL: interleukin; LPS: lipopolysaccharide; MCAO: middle cerebral artery occlusion; MHC: major histocompatibility complex; NSPCs: neural stem/progenitor cells; OX42/CD11b: single-pass type-1 α chain 1136 aa glycoprotein (microglia/macrophage-specific marker; clone of antibody that detects cell surface antigen-cluster of differentiation 11b, anti-complement receptor 3); PBS: phosphate-buffered saline; QA: quinolinic acid; SVZ: subventricular zone.

Abstract

Brain damage, such as ischemic stroke, enhances proliferation of neural stem/progenitor cells (NSPCs) in the subventricular zone (SVZ). To date, no reliable *in vitro* systems, which can be used to unravel the potential mechanisms underlying this lesion-induced effect, have been established. Here, we developed an *ex vivo* method to investigate how the proliferation of NSPCs changes over time after experimental stroke or excitotoxic striatal lesion in the adult rat brain by studying the effects of microglial cells derived from an injured brain on NSPCs. We isolated NSPCs from the SVZ of brains with lesions and analyzed their growth and differentiation when cultured as neurospheres. We found that NSPCs isolated from the brains 1-2 weeks following injury consistently generated more and larger neurospheres than those harvested from naïve brains. We attributed these effects to the presence of microglial cells in NSPC cultures that originated from injured brains. We suggest that the effects are due to released factors because we observed increased proliferation of NSPCs isolated from non-injured brains when they were exposed to conditioned medium from cultures containing microglial cells derived from injured brains. Furthermore, we found that NSPCs derived from injured brains were more likely to differentiate into neurons and oligodendrocytes than astrocytes. Our *ex vivo* system reliably mimics what is observed *in vivo* following brain injury. It constitutes a powerful tool that could be used to identify factors that promote NSPC proliferation and differentiation in response to injury-induced activation of microglial cells, by using tools such as proteomics and gene array technology.

Keywords: Ischemia, Neurogenesis, SVZ, Neural Progenitor, Stroke, Microglia

In the adult human brain, newborn neurons arise in the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) lining the lateral ventricles (Eriksson et al., 1998, Curtis et al., 2007).

Brain damage, such as experimental stroke (Arvidsson et al., 2002) or intrastriatal injection of the excitotoxin quinolinic acid (QA) (Tattersfield et al., 2004) enhances the proliferation of neural stem/progenitor cells (NSPCs) that are located in the subventricular zone (SVZ) or the ependymal cell layer (Carlen et al., 2009). Understanding the cellular mechanisms that regulate the proliferation and differentiation of NSPCs following brain injury is important, as these cells have been suggested to contribute to endogenous brain repair and can constitute a potential source of donor tissue for cell transplantation therapies (Deierborg et al., 2008, Burns et al., 2009).

In the hippocampal subgranular zone (SGZ), microglial cells have been reported to impede lesion-induced neurogenesis (Monje et al., 2003). However, recent data suggest that microglia have diverse functions in relation to neurogenesis. For example, microglia activated by endotoxins inhibit neurogenesis *in vivo*. Conversely, when activated by cytokines IFN-g/IL-4 they enhance neuronal differentiation (Butovsky et al., 2006). Moreover, microglia activated by lipopolysacharide (LPS) for 24 h (acute activation) release pro-inflammatory cytokines, in contrast to chronically activated microglia that adopt an anti-inflammatory phenotype (Cacci et al., 2008).

In light of the above findings, it is valuable to define how different durations of activation of microglia, in clinically relevant models of brain injury, affect the proliferation and the fate of NSPCs. Therefore, we developed an *ex*

vivo system to study the effect of activated microglia on NSPCs. We stimulated microglia by inducing ischemic or excitotoxic lesions in the brain of rats, and examined their effect on NSPCs. We found that microglia harvested during the first 1-2 weeks following brain injury exert profound effects, mediated via soluble factor(s), on the proliferation of NSPCs grown *in vitro.* The effect resembles the injury-induced enhacement of neurogenesis previously observed *in vivo* (Thored et al., 2008). These results suggest that the *ex vivo* model we have developed is highly relevant to the *in vivo* situation and can be used to further clarify mechanisms underlying the effects of activated microglia on NSPCs in the injured brain.

Experimental procedures

Animals

We used male Sprague Dawley rats (B&K Stockholm, Sweden), 10 - 12 weeks old of age at the start of the experiment. All experiments were approved by the Malmö-Lund Ethical Committee. Animals had free access to food and water and were housed under a 12h light/12h dark cycle.

Experimental Model of Stroke

We induced experimental stroke, focal brain ischemia, by transient middle cerebral artery occlusion (tMCAO) using an established protocol (Memezawa et al., 1992). Briefly, the rats were anesthetized with halothane (1.5%) and an intraluminal filament was inserted into the internal carotid artery and placed at the origin of the MCA. An occlusion time of 2 h was used to induce both striatal and cortical damage. We assessed neurological symptoms after a 2 h recovery period. The neurological symptoms were assessed using two behavioral tests: contralateral rotation/circling behavior and contralateral fore-/hindpaw impairment (lack of paw withdrawal when hanging over the edge of a table). A combination of these two tests can predict stroke-injury affecting both the striatum and the cortex (Rickhag et al., 2008).

Excitotoxic lesion to the striatum by injection of quinolinic acid (QA)

We lesioned the striatum by an intracerebral injection of QA, using an established protocol (Nakao and Brundin, 1997). Briefly, we initiated anesthesia by intraperitoneal injection of Fentanyl (50 µg/ml, Pharamlink, Sweden) / Domitor (1 mg/ml Medetomidine, Orion Pharma, Finland);

proportions: 2 ml Fentanyl + 0,1 ml Domitor; dose: 6,3 ml/kg rat and placed the rat in a stereotaxic frame. We induced a large striatal lesion by an injection of 120 nmoles of QA dissolved in 1 µL of 0.1 M phosphate buffered saline (PBS) into the right striatum (rostral 1.2 mm/lateral 2.8 mm/ventral 4.5 mm to bregma). The anesthesia was discontinued by a subcutaneous administration of Antisedan (1 ml/kg) and Temgesic (1.7 ml/kg) was used as an analgesic.

Neurosphere generation

We generated neurospheres using a standard protocol as previously described (Svendsen et al., 1998, Roybon et al., 2009). Briefly, the SVZ was isolated from the ipsilateral side 7 days after a MCAO or QA lesion, except for the study designed to study temporal features of microglial activation (fig. 3) where the animals were sacrificed 1, 2, 3 and 4 weeks following QA lesion. We dissected out the ipsilateral SVZ from coronal sections starting at 1 mm caudal to the optic cross and ending 5 mm rostrally. The tissue was treated with trypsin/deoxyribonuclease (0.1/0.05%) and was mechanically dissociated to generate a single-cell suspension. The cells were plated at a density of 200,000 cells/ml in uncoated T25 flasks in 7 ml of proliferation medium containing DMEM:HAMS-F12 (3:1), B27 (2%; Invitrogen-Gibco, Stockholm, Sweden), Heparin (5 µg/mL), penicillin:streptomycin (50 µg/ml. Sigma, Stockholm, Sweden), epidermal growth factor (EGF) (20 ng/mL, R&D Systems, Stockholm, Sweden) and basic fibroblast growth factor (bFGF) (20 ng/mL, R&D Systems, Stockholm, Sweden). The medium was supplemented with mitogens by the addition of 3 ml of proliferation medium at 2 and 5 days

in vitro. Secondary neurospheres (P_1) and later passages were generated by mechanical dissociation of primary neurospheres (P_0). Individual flasks represent individual animals.

Neurosphere Differentiation

After 7 days *in vitro,* the neurospheres were plated on Poly-L-Lysine/Laminin (15/0.01 mg/ml, Sigma, Stockholm, Sweden) coated glass coverslips. We induced the differentiation by withdrawal of EGF and bFGF. The differentiation medium was changed after 3 days. At day 5, we fixed the cultures using 4% paraformaldehyde in PBS.

Immunohistochemistry

For immunohistochemical analysis, animals were deeply anesthetized with pentobarbital and transcardially perfused with saline for 5 min, followed by 4% paraformaldehyde in PBS for 10 min.

Brains were post-fixed in the same fixative overnight at 4°C, cryopreserved in 30% sucrose in PBS and subsequently, 30 µm thick coronal sections were cut using a microtome (Zeiss) and were preserved in an antifreeze solution. For immunohistochemistry, we pre-incubated the brain sections/cultures for 1 h with a blocking solution (5% goat serum, 0.25% triton X-100), followed by an overnight incubation of the appropriate primary antibody at room temperature. The following primary antibodies were used: mouse anti-β-III-tubulin (1:1000), mouse anti-CNPase (1:300, monoclonal, Sigma, Stockholm, Sweden), rabbit anti-GFAP (1:1000, polyclonal, DAKO, Stockholm, Sweden) OX-42/CD11b (1:200, Serotec, Oxford, UK) and Iba1 (1:500, rabbit, Wako Chemicals,

Osaka, Japan). The corresponding secondary antibodies were incubated for 1 h (Alexa-488 goat anti-mouse and Alexa-595 goat anti-rabbit; 1:200; Molecular Probes, Stockholm, Sweden) followed by incubation with DAPI (1:1000, Sigma, Stockholm, Sweden) for 15 min and rinsing before mounting on glass slides with PVA-DABco. We performed the analysis with a fluorescence microscope (Leica BX60) using FITC/CY3 filters at 40X magnification.

Neurosphere quantification and differentiation analysis

Using a 4X objective, we counted all the neurospheres (>40 μm in diameter) in the visual field in 5 different areas of the T25 culture flask (each corner and the center). The number of neurospheres in individual flasks was estimated by taking the sum of all neurospheres counted in the 5 fields. We estimated the mean diameter of neurospheres by randomly measuring 10 neurospheres in each culture flask using a 20X objective and an eyepiece grid scale. The number of neurons (β-III-tubulin), astrocytes (GFAP) and oligodendrocytes (CNPase) was assessed for each experimental condition (individual animals) by selecting 5 neurospheres of similar size (≈100 μm in diameter) (Roybon et al., 2005), and analyzing only the cells that migrated out from the neurospheres and attached to the Poly-L-Lysine/Laminin-coated surface. The numbers of neurons, astrocytes and oligodendrocytes were counted as a percentage of approximately 150 DAPI positive cells per field (monitored in 5 fields for each sphere). Only cells with a clear immunoreactivity/morphology were included in the analysis.

Conditioned medium and flasks with microglial cells

Cultures of SVZ-derived NSPCs from a lesioned brain gave rise to a monolayer of microglial cells, possibly originating from the SVZ or the adjacent injured striatal tissue. We studied the effect of soluble factors (secreted from microglial cells) on neurosphere generation by using conditioned medium (centrifuged at 450 g, for 7 minutes, at 4ºC) from the different experimental groups (Ctrl, MCAO and QA) obtained 7 days after neurosphere generation. Dissociated cells from neurospheres (P_4) derived from naïve brains were plated at a density of 200 000 cells/ml. We evaluated the number and the size of the neurospheres after seven days in culture. The total number of cells was counted following mechanical dissociation of all the neurospheres from each culture flask in a Bürker chamber. To determine whether the microglia were proliferating, microglial cultures were incubated (at 5-7 days *in vitro*) with the thymidine analogue 5-Bromodeoxyuridine, BrdU, (50 mM, Chemicon) for 2 days and the number of proliferating cells was counted.

Statistics

For statistical comparison of the number and the size of neurospheres from control, MCAO and QA lesions, the data was log transformed and analysed using ANOVA followed by Fischer's post-hoc test. The non-parametic Mann-Whitney U-test was applied for the time and differentiation studies followed with Bonferroni correction to compensate for multiple comparisons between experimental groups. We used ANOVA with Scheffe's *post hoc* test to analyze groups treated with conditioned medium. Experiments were done in

duplicates or more. Data are expressed as mean±SEM and $P<0.05$ was considered as significantly different.

Results

Distinct microgliosis in the striatum of rats at one and four weeks following brain injury

To develop an *ex vivo* system to study the effect of microglial cells on NSPCs, we activated microglial cells by inducing experimental stroke (tMCAO) and excitotoxic brain injury in rats. We first examined striatal microgliosis following brain injury using immunohistochemistry for the microglial marker CD11b at 1 and 4 weeks post-injury. At both time points, the microglial hyperplasia appeared identical at the cellular level with hypertrophic microglial cells that exhibited characteristic bushy cellular ramifications (fig. 1 A2, B2, C2 and D2). At 1 week post-injury, the microgliosis was evenly distributed in the injured brain parenchyma (fig. A1, C1), whereas, after 4 weeks, the microglial distribution had changed to a more dense cell accumulation at the border of the injury (fig. 1 A1, D1), resembling the glial scar microgliosis (Arranz et al., 2010) known to hamper neuronal plasticity (Rolls et al., 2009).

Increased number and size of neurospheres derived from brain lesions

We next examined whether brain injury induces dynamic changes in the proliferation of NSPCs located in the SVZ. We assessed the effects of excitotoxic lesion and ischemic stroke on the proliferation of NSPCs grown as neurospheres *in vitro*. We isolated the SVZ 7 days after injury from rats with ischemic brain injury (n=5), with a striatal QA-lesion (n=5) and naïve animals (n=5) and compared the number and size of neurospheres generated over a

period of 7 days in culture. Strikingly, we observed that both types of brain injury increased the number and the size of neurospheres. Neurospheres derived from ischemic and QA lesions were both more numerous (by 69% and 102%, respectively; fig. 2a) and larger in diameter (by 23%, and 40%, respectively; fig. 2b) than neurospheres derived from naïve animals. These results confirm that different brain injuries increase proliferation of NSPCs derived from the SVZ and imply that NSPCs grown as neurospheres are a valid model that can be used to study neurogenesis following brain injury. Moreover, these *in vitro* data are consistent with previous reports showing that brain lesions promote proliferation of NSPCs in the SVZ *in vivo* (Arvidsson et al., 2002, Tattersfield et al., 2004, Zhang et al., 2004a).

Time-dependent increase in neurosphere proliferation

We next examined the temporal effect of a brain lesion on NSPC proliferation. We assessed the number and the size of neurospheres generated from SVZ isolated 1, 2, 3 and 4 weeks following brain injury, e.g. after a striatal QA-lesion (n=5 animals for all time points). Following a 7-day culture period, we found a 4-fold increase in the number of neurospheres generated from NSPCs isolated 1 and 2 weeks following a QA-lesion (fig. 3, c). Notably, the neurospheres harvested a week after lesion were 30% larger compared with control (fig. 3b, c). However, the number and the size of neurospheres isolated 3 and 4 weeks following a QA-lesion were comparable to control neurospheres (fig. 3a, b, c). These data are in agreement with earlier observations showing that maximal cell proliferation in the SVZ occurs 7 days after brain injury (Zhang et al., 2004a).

Cultures of neurospheres derived from lesioned brains generate a monolayer of microglial cells

We next investigated whether the proliferation of NSPCs was influenced by the presence of microglial cells in our cultures. Immunocytochemistry for the specific microglial markers Iba1 and CD11b consistently revealed the presence of a microglial cell layer, on the bottom of the culture flask, in neurosphere cultures derived from ischemic or QA-lesioned brains (fig. 4a, c-d). These cells actively proliferated, as shown by the incorporation of BrdU, which was added to the medium (fig. 4d). The microglia were most likely derived from the medial striatum adjacent to the SVZ (Duan et al., 1998). Microglial cells were rarely found in cultures derived from sham-lesioned brains (fig. 4b). Similarly, we rarely detected microglial cells when neurosphere cultures were prepared from SVZ tissue isolated from 3 and 4 weeks following brain injuries (data not shown). The results strongly suggest an intimate link between the appearance of a monolayer of microglial cell in cultures derived from injured brains and the increase in neurosphere proliferation. In analogy with our results, a recent study described that microglia proliferate and accumulate in the rat SVZ during the first 2 weeks following experimental stroke (Thored et al., 2008).

Microglial cells, derived from SVZ of injured brains, secrete soluble factors with a robust proliferative effect

To confirm whether the microglia present in our cultures increases neurosphere formation and proliferation we used complementary paradigms;

microglia-conditioned medium and the co-culture of microglia and neurospheres (fig. 5a).

We first plated single cell suspensions of dissociated neurospheres derived from NSPCs generated from the SVZ of naïve brains, grown in the presence of EGF and bFGF, according to conventional protocols. We then exposed these single cell cultures to conditioned medium obtained from cultures containing microglial cells harvested from ischemic (n=4) and QA-lesioned brains (n=4). As a control (n=4), we used conditioned medium from cultures generated from naïve brains, in which only a few or no microglial cells were present. We found a substantial increase in the proliferation of “naïve neurospheres” (generated from naïve NSPCs) when grown for 7 days in conditioned medium derived from microglia originating from ischemic (9-fold) and QA-lesioned (11-fold) brains (fig. 5b). The increase in proliferation was evident not only in the number (fig. 5b1) and the size (fig. 5b2) of neurospheres, but also when we counted the total number of cells in the neurospheres (fig. 5b3). These data suggest that microglial cells release soluble factors that stimulate the proliferation of NSPCs.

Next, we asked whether the factors secreted by the microglial cells could efficiently induce the formation and growth of neurospheres from naïve NSPCs in the absence of exogenously added growth factors. We grew naïve neurospheres in the presence of microglial cells derived from brains that had sustained lesions. In this set of experiments, we first studied the proliferative effect induced by the microglial cells using basic medium, without the addition of EGF and bFGF. In absence of EGF and bFGF, we observed a 5-fold increase in the number of neurospheres compared to cultures grown without

microglia, suggesting that microglial cells produce mitogenic factors that partially compensate for the lack of EGF and bFGF. Next, we grew the naïve NSPCs in new fresh proliferation medium containing EGF and bFGF in the presence of microglia and observed an additional 58% increase in the proliferation of the neurospheres (fig. 5c3). These results indicate that microglia from injured brains secrete mitogenic factors that exert a proliferative effect beyond the effect of conventional proliferation medium (containing EGF and bFGF). Thus, it is likely that, in this context, microglia from injured brains secrete trophic factors other than EGF and bFGF.

Minocycline inhibition of activated microglial cells decreases the proliferation of SVZ-derived NSPCs

To elucidate whether microglial activation *per se* is necessary for their ability to increase the proliferation of NSPCs, we treated cultured microglial cells obtained from injured brain with minocycline to limit the microglial activity (200 nM for 24 h) prior to co-culturing them with neurospheres. Pre-treatment with minocycline dramatically reduced the number of neurospheres even after only 2 days in culture (39.5% reduction; n=8, p=0.01) compared to neurospheres grown in the presence of untreated microglia. In the presence of minocycline treated microglia, the size (sphere diameter) of the neurospheres decreased by 20.7% after 6 days in culture (n=8, p=0.02). These results demonstrate that inhibiting the activation of microglia exposed to injury prevents them from exerting a positive effect on NSPC proliferation. These results are in line with a recent *in vivo* study describing how

minocycline treatment suppresses the microglial response in stroke-injured rats and leads to a reduction in cell proliferation in the SVZ (Kim et al., 2009).

Brain lesion stimulates differentiation of NSPCs into neuronal and oligodendrocytic lineages

We next studied the phenotype of the cells present in the neurospheres. After cultivating free-floating neurospheres in the presence (from lesined brain) or absence of microglia (from naïve brain) for 1 week, we induced the cells to differentiate over 5 days. We found that the NSPCs from naïve brain neurospheres gave rise to all three neural cell types: β-III-tubulin-expressing neurons (3.4%), CNPase-expressing oligodendrocytes (22.4%) and GFAP-expressing astrocytes (11.2%) (fig. 6a-i). The remaining 60% had not yet matured and expressed the neural progenitor marker nestin (data not shown). SVZ-derived neurospheres from rat brains with ischemic stroke injury (n=5) differentiated to a higher extent into neurons and oligodendrocytes, with a 3.7- and 1.8-fold increase, respectively, compared to naïve control cultures (n=5), (fig. 6j and k). Equally, striatal QA-lesion (n=5) increased the differentiation of SVZ-derived neurospheres into neurons and oligodendrocytes (3.0- and 1.6-fold increase, respectively, fig. 6j and k). Interestingly, we observed some cells that co-expressed β-III-tubulin and GFAP (fig. 6g, h, i), in analogy to what has been observed in the SVZ of human fetuses (Draberova et al., 2008). These data suggest that brain injury could increase the cellular differentiation of NSPCs into neurons and oligodendrocytes. Possibly, inflammatory molecules and microglia could have an active role in this differentiation (Taupin, 2008).

Discussion

We have developed an *ex vivo* model to study activated microglia-induced proliferation of NSPCs following brain injury *in vivo*. We found that microglia activated due to brain injury influenced NSPC proliferation. Specifically, we demonstrated an increase in cell proliferation in neurospheres generated from NSPCs isolated from the ipsilateral SVZ tissue following both ischemic stroke and excitotoxin-induced lesion. Enhanced neurogenesis has been suggested to occur in stroke patients (Jin et al., 2006, Macas et al., 2006). The notion that enhanced proliferation of NSPCs following stroke promotes functional recovery (Nygren et al., 2006, Thored et al., 2006) makes these findings potentially clinically relevant. The ambiguous response of microglia (pro-/anti- inflammatory) to injury depends on the types of stimulus (Hanisch and Kettenmann, 2007) and receptor activation (Ribes et al., 2009), The effects of microglia often differ between *in vivo* and *in vitro* models (Zietlow et al., 1999, Eskes et al., 2003). It is, therefore, important to create *in vitro* models that closely mimic the activation pattern of microglia *in vivo* and can replicate their effects on other cells *in vivo.* We believe that our *ex vivo* assay is a valuable tool which can help to clarify how activated microglia affect the proliferation and the differentiation of NSPCs.

Brain injury increases cell proliferation of SVZ-derived NSPCs

Microglia are activated following ischemic brain injury (Morioka et al., 1993) and excitotoxic brain lesions (Topper et al., 1993). We observed an increase in neurosphere proliferation using tissue harvested 1-2 weeks

following ischemic and excitotoxic brain lesions. This increase in neurosphere proliferation was induced by injury-activated microglia. These early time points following brain injury correspond to the period when microglia accumulate in the SVZ following experimental stroke (Thored et al., 2008). Moreover, the number of MHC-II positive microglia peaks in the injured striatum within the first 2 weeks (Thored et al., 2009) and they are suggested to regulate neurogenesis (Butovsky et al., 2006, Ziv et al., 2006).

Experimental stroke and intrastriatial injection of the excitotoxin QA are known to increase the proliferation of NSPCs in the SVZ, with a maximal effect one week after the injury (Tattersfield et al., 2004, Zhang et al., 2004b, Deierborg et al., 2009). We confirmed this increase in the proliferation of neurospheres by dissecting out the SVZ and dissociating the cells one week following either ischemic stroke or excitotoxic striatal lesion. Earlier studies suggest that cell proliferation in the SVZ is sustained up to two weeks after excitotoxin-induced brain injury (Deierborg et al., 2009) and thereafter decreases to baseline levels (Zhang et al., 2004b). In this respect, our *ex vivo* model robustly mimics the dynamic changes in cell proliferation observed *in vivo*. Moreover, the enhanced (3- to 4-fold) neuronal differentiation of NSPCs that we observed among cells derived from neurospheres harvested from injured brains is consistent with previous observations showing increased neurogenesis *in vivo* following these brain lesions (Arvidsson et al., 2002, Tattersfield et al., 2004, Zhang et al., 2004b). Interestingly, this stroke-induced neurogenesis in the SVZ is concomitant with trophic microglial cells expressing insulin-like growth factor-1 in the vicinity of the SVZ (Thored et al., 2006). In addition, these neurospheres generated higher numbers of

oligodendrocytes in line with previous studies reporting an increased density of oligodendrocytes following focal ischemia in the rat (Gregersen et al., 2001).

Lesion-induced microglial activation promotes NSPC proliferation

Early literature often suggested that microglia might negatively influence plasticity (including neurogenesis) following brain injury (Monje et al., 2003, Liu et al., 2006). Recent reports convey a more complex role of microglia, stressing that they sometimes promote brain repair following injury (see Introduction). Thus, the classical view of microglia, as a uniform cell type that exacerbates neuronal damage and inhibits neurogenesis, is under question (Schwartz et al., 2006). When we treated microglia derived from injured adult brain with minocycline, we observed reduced proliferation of NSPCs derived from the SVZ, indicating that, in our model, activated microglia actually enhance stem/progenitor cell proliferation. In a similar way, inhibition of microglial activation with minocyline *in vivo* following stroke in rats reduces the cell proliferation in the neurogenic SVZ (Kim et al., 2009), in stark contrast to the effect of minocycline on hippocampal neurogenesis. Possibly, activated microglia in the SVZ/striatum secrete other factors than those present in the dentate gyrus. Indeed, microglia in the SVZ show a greater response to injury, in terms of activation and proliferation, compared to microglia in non-neurogenic regions (Goings et al., 2006).

Microglia activated by brain injury secrete factors promoting NSPC proliferation

We are currently investigating which microglia-derived molecules stimulate the proliferation of NSPCs and their mechanism(s) of action. Earlier work suggests that microglia produce multiple pro- and anti-inflammatory cytokines, as well as growth factors, with diverse cellular effects (Hanisch, 2002). For example, microglia can produce trophic factors and exert a positive effect on: neurogenesis *in vivo* (Butovsky et al., 2006); oligodendrocytic differentiation (Nicholas et al., 2001) and astrogliogenesis (Zhu et al., 2008). Several mitogens are also known to positively regulate the proliferation of NSPCs in the SVZ, e.g., erythropoietin (Wang et al., 2004), vascular endothelial growth factor (VEGF) (Jin et al., 2002) and endocannabinoids (Carrier et al., 2004, Aguado et al., 2005). We found that microglia-conditioned medium had additive effects on cell proliferation in the neurospheres in the presence of high concentrations of EGF/bFGF, suggesting that the unknown factor acts via a different pathway.

The complexity of microglial activation and its contribution to NSPC proliferation

Hypoxia, which clearly occurs in experimental stroke, activates microglia (Abraham et al., 2001). In our study, the pro-neurogenic effect of microglia activated by excitotoxic brain damage was equally strong to that seen after ischemic hypoxia. Therefore, the microglial activation we observed might instead be a direct response to cell death where factors, such as nucleotides, released from dying cells could trigger activation (Nimmerjahn et al., 2005, Haynes et al., 2006).

One week after striatal lesion, we observed microglia with a bushy and hypertrophic morphology. They remained at 4 weeks after the lesion, when we found the microglia to no longer be pro-neurogenic. Thus, the morphology of individual microglia does not necessarily correlate with their pathophysiological state (Perry et al., 2007). Interestingly, between one and four weeks post-injury the anatomical distribution of activated microglia changed. Thus, initially, they were present in the whole striatum and at 4 weeks they concentrated around a scar-like region medially, close to the SVZ. The cells present initially are likely to represent supportive microglia reported to occur shortly after a brain injury (Neumann et al., 2006, Lalancette-Hebert et al., 2007), whereas those at 4 weeks may have a more phagocytic role (Rolls et al., 2009). Studies on the effects of activated microglia from a transgenic model of neurodegenerative disease further exemplify the complexity of their roles. For instance, culture medium conditioned by activated microglia harvested from a Presenilin 1 transgenic Alzheimer's mouse model impairs NSPC proliferation (Choi et al., 2008). In conclusion, the nature of the molecules secreted by activated microglia and their effects on NSPCs depend strictly on the type of activating stimulus.

Conclusion

We have generated an *ex vivo* model to study the response of NSPCs to microglial cells obtained from injured brains. Our data imply that microglial cells present in acutely damaged SVZ/striatum release factors that initially promote proliferation. We also show that NSPCs derived from an injured brain differentiate to a higher extent into neurons and oligodendrocytes. These

results suggest that a microglial changes might underlie the increased cell proliferation observed in the SVZ and the striatum following stroke. Our results also support the notion that anti-inflammatory treatment may not necessarily be beneficial for NSPC proliferation following injury to the striatum, but rather might inhibit the proliferative effect of activated microglia in this brain region.

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Disclosures: None

Figure legends

Figure 1. Microgliosis in the striatum at 1 and 4 weeks following brain injury induced by excitotoxic lesions (A, B) or ischemic stroke (C, D). The microglial/macrophage marker CD11b demonstrates general and homogenously distributed microglial activation in the injured striatum adjacent to the lateral ventricle (left) at 1 week following a striatal lesion (A, C). Four weeks after brain injury, the microglial cells show a scar-like accumulation of cells making up a microglial barrier that delineates the striatal injury (B, D). Note the similarity in the morphology of the activated bushy-shaped microglia at 1 and 4 weeks post-injury. A1 and A2 have the same magnification as B1, C1, D1 and B2, C2 D2, respectively.

Figure 2. Neurospheres cultured from brains lesioned by an ischemic (MCAO) or intrastriatal excitotoxic injury with QA (QA) increase the proliferation of neurospheres. The number of neurospheres (A) and the diameter of the neurospheres (B) are increased following a brain lesion. The injury was induced 7 days before the dissection and dissociation of SVZ cells. Evaluation was done after seven days of culturing. Mean±SEM (n=5), * denotes $P<0.05$.

Figure 3. The proliferation of neurospheres cultured from lesioned brains is increased only if they are isolated and cultured during the first two weeks following the lesion. A) The number of neurospheres is increased if the SVZ is dissected out within 2 weeks following a QA lesion. B) The size of

neurospheres, measured as the diameter, was increased only if the SVZ was cultured 7 days after a QA lesion. The values are expressed as a percentage of the corresponding controls (neurospheres cultured from naïve rats and cultured at the same time). C-F) Representative pictures showing the difference in the number and the size of neurospheres taken out 1 and 4 weeks following a brain lesion and cultured for one week. Mean±SEM (n=5). M-W U-test, * denotes $P<0.05$, ** denotes $P<0.01$.

Figure 4. Proliferating microglial cells attached to the bottom of a culture flask in neurosphere cultures from a lesioned brain. Phase contrast image of the cell layer found on the bottom of all cultures derived from the SVZ from a QA-lesioned brain (A) and from a naïve brain (B). The cells could be identified as microglia/macrophages by the use of immunocytochemistry for the specific markers CD11b (C, green; DAPI, blue) and Iba1 (D: Iba1, green). The microglia were in a proliferative state as shown by their incorporation of BrdU (D, Iba1, green; BrdU, red). Scale bar: A, B, 400 µm; C, 50 µm; D, 200 µm.

Figure 5. Conditioned medium from flasks containing microglial cells, harvested from a lesioned brain, have a proliferative effect on neurospheres. A) Experimental outline. To obtain microglial cultures, SVZ and medial striatum were dissected out and dissociated from naïve animals (Ctrl), and from ischemic and QA-lesioned brains 7 days after the lesion was induced. After 7 days of culturing, a confluent layer of microglial cells could be found on the bottom of the culture flask and all neurospheres were discarded from the culture medium. Dissociated neurospheres from naïve brains were seeded in

respective medium or flasks. Following 7 days of culturing, neurospheres in culture flasks containing conditioned medium from lesioned brains increased in number (B1), size (B2) and total number of cells/ml (B3) compared to neurospheres maintained in control medium, *i.e.*, culture from naïve brains and without the presence of microglial cells. C, Culturing of dissociated naïve neurospheres with new proliferation medium in new flasks (Ctrl) or 7 days old flasks from QA-lesioned brain with microglial cells on the bottom further increased the neurosphere proliferation (cells/ml, C3). Mean±SEM, (n=4). ANOVA and Scheffe's post hoc, B1-3. Student's t-test, C3 was applied. * denotes $P<0.05$; ** denotes $P<0.01$; ** denotes $P<0.001$.

Figure 6. Differentiation towards neurons and oligodendrocytes was enhanced after a brain lesion. Neurospheres from naïve control brains could be differentiated into all neural lineages; neurons (ß-III-tubulin: A, G), astrocytes (GFAP: B, E and H) and oligodendrocytes (CNPase: D). Neurospheres obtained from ischemic or QA lesioned brains and cultured for 7 days and subsequently differentiated for 5 days have a markedly increased differentiation into neurons (ß-III-tubulin, J). The differentiation into oligodendrocytes (CNPase) was also increased (K), whereas the astrocytic differentiation was unaffected (L). Mean±SEM (n=5). M-W U-test with Bonferroni correction, * denotes $P<0.05$.

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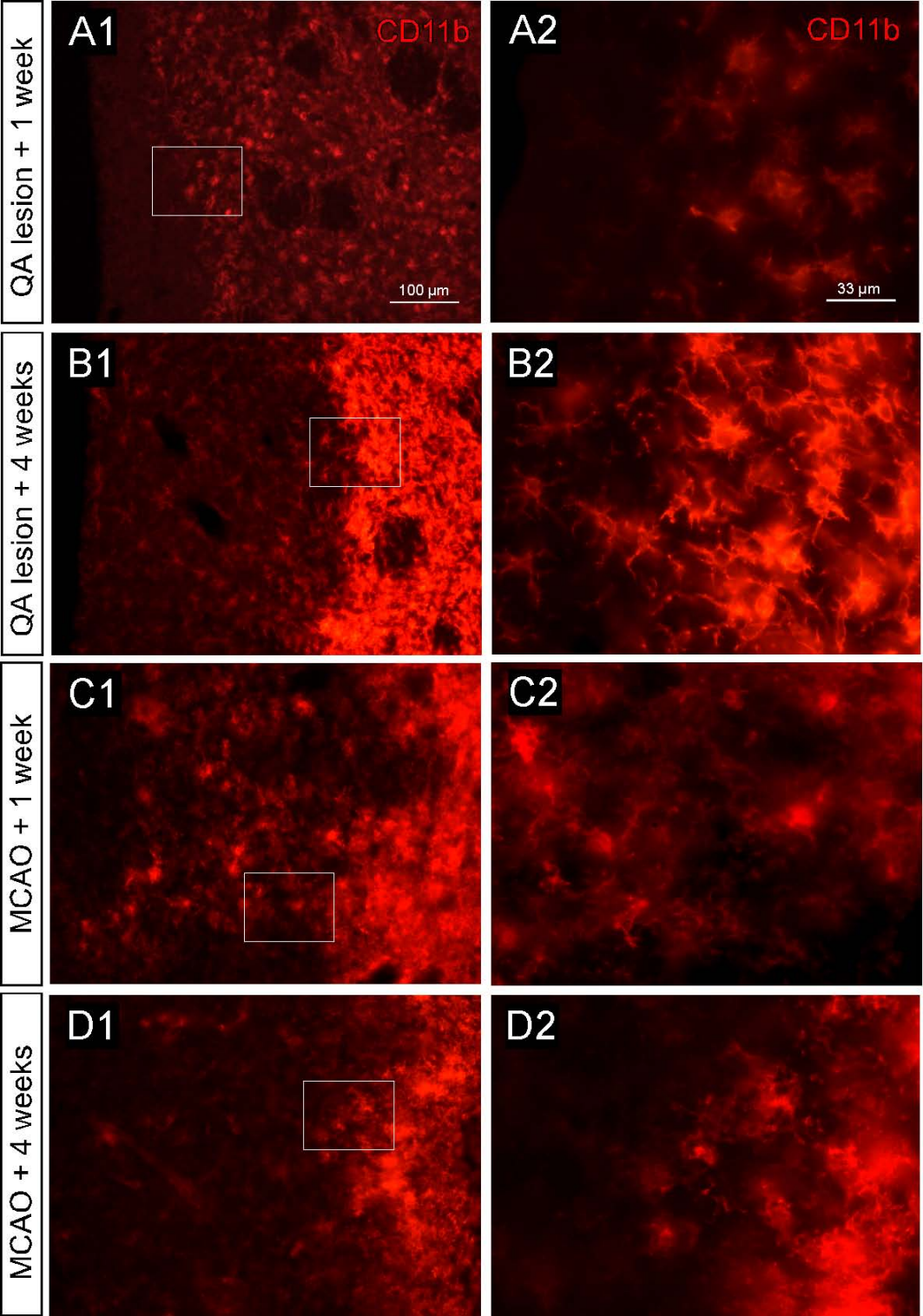
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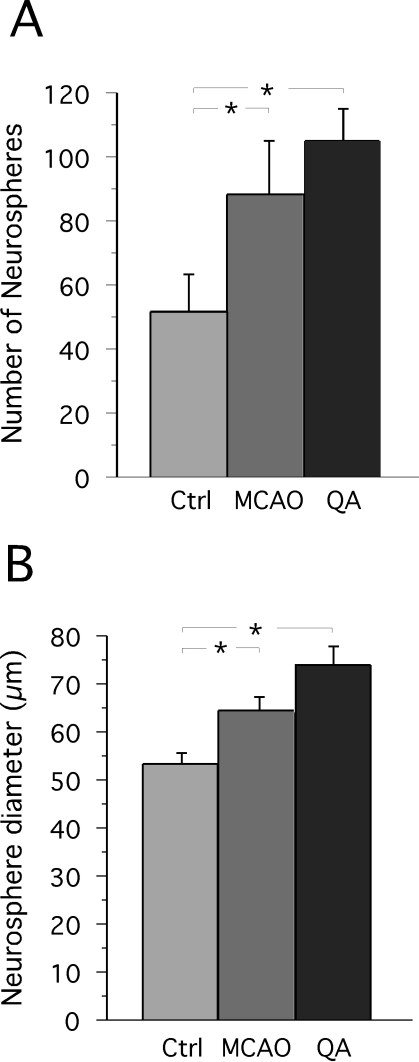
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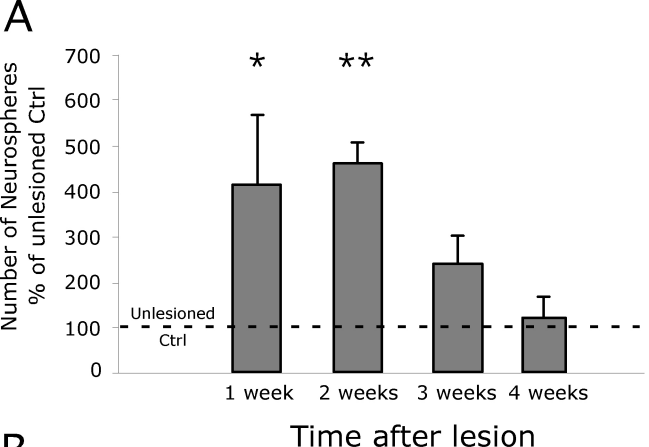
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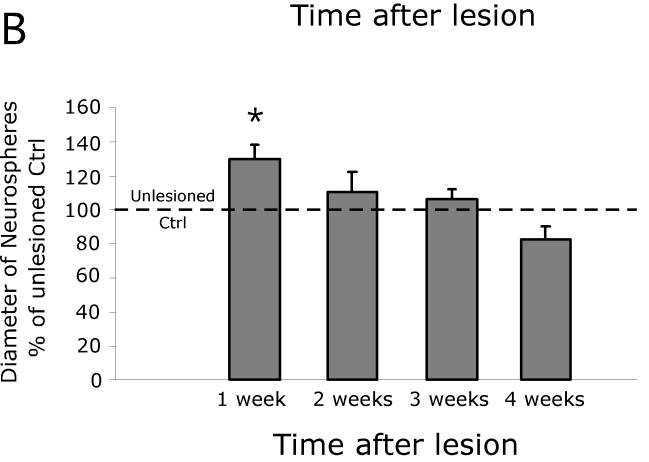
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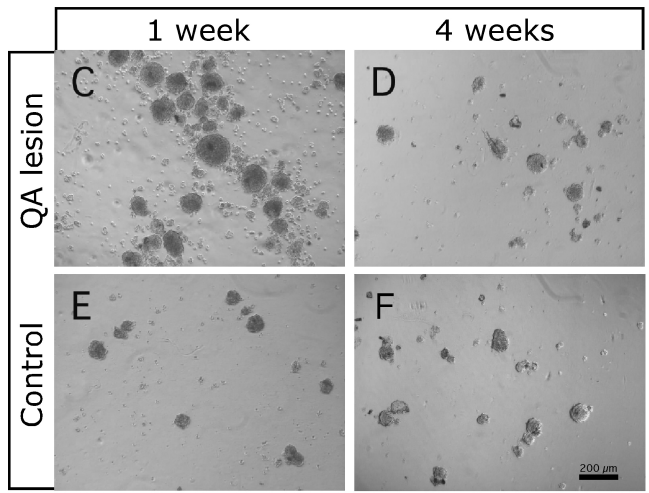
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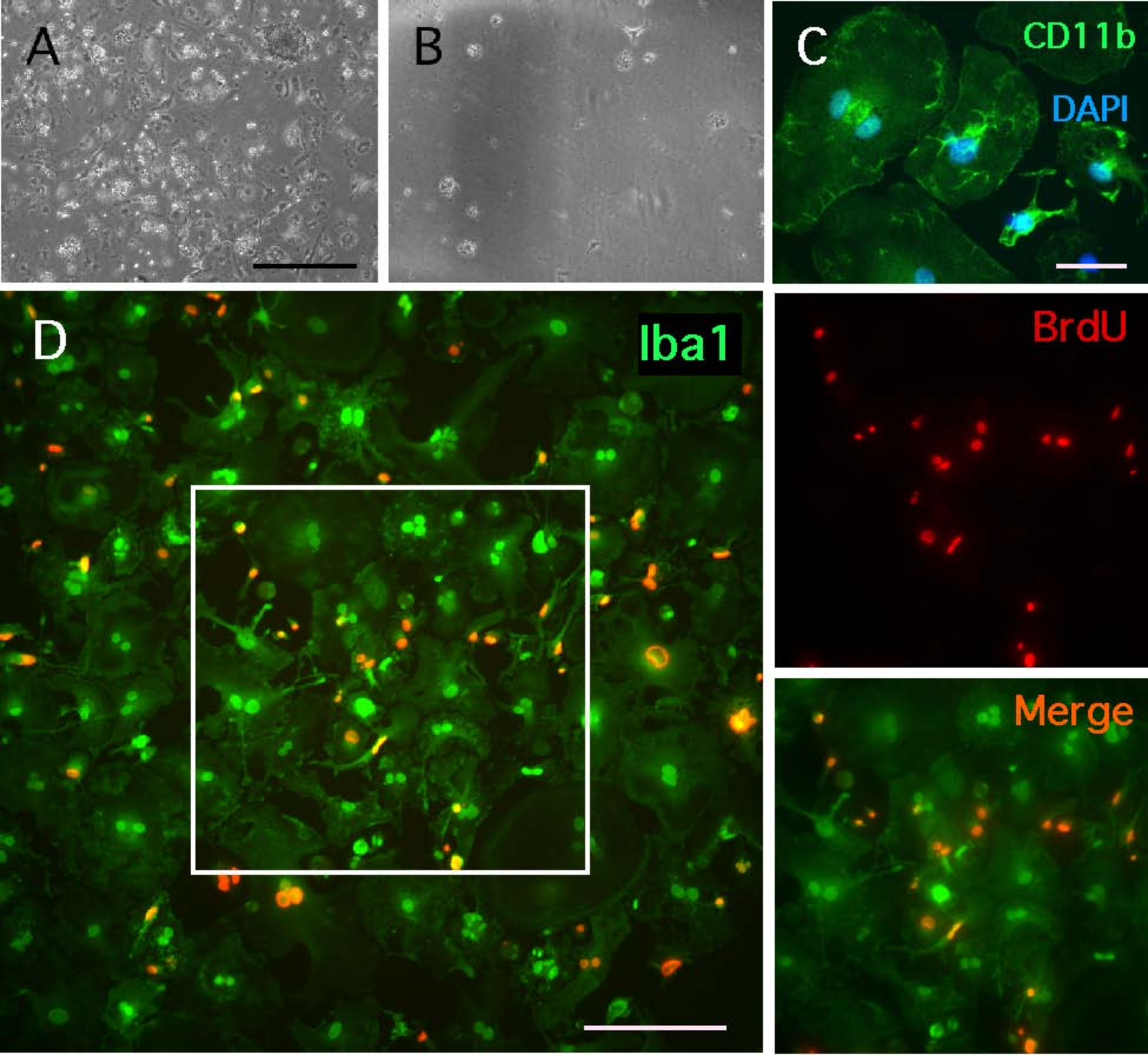
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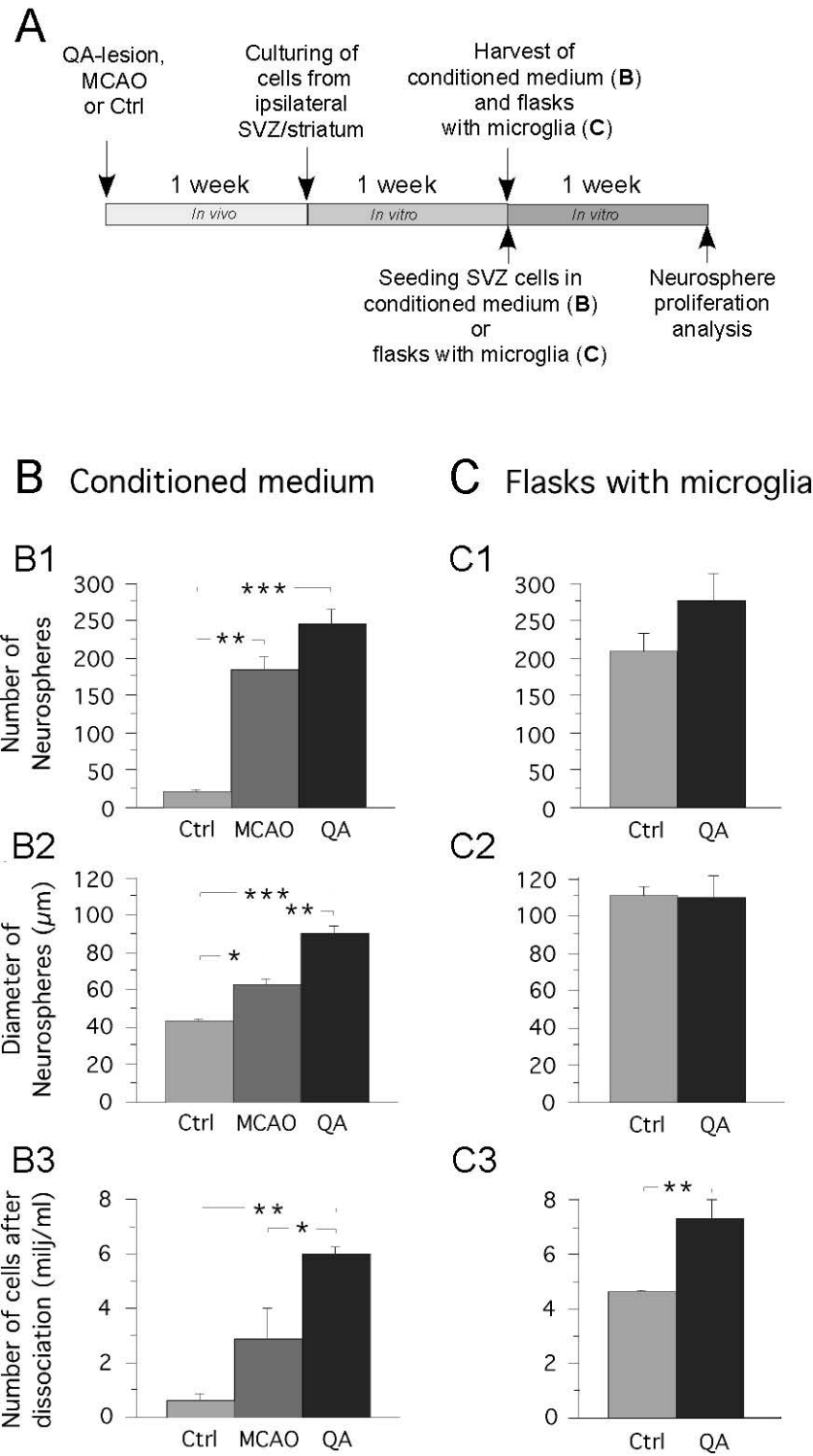
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Culturing of cells from ipsilateral SVZ/striatum
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In vitro
In vitro
Seeding SVZ cells in conditioned medium (B) or flasks with microglia (C)
Neurosphere proliferation analysis
B Conditioned medium
C Flasks with microglia
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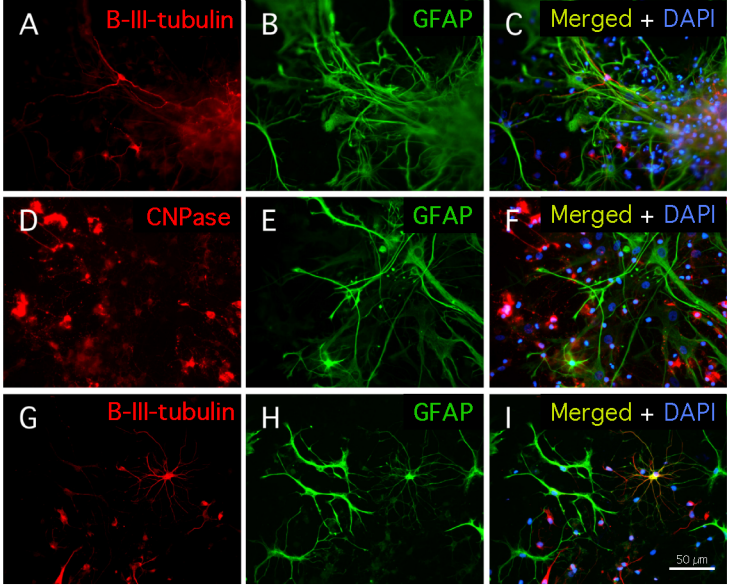
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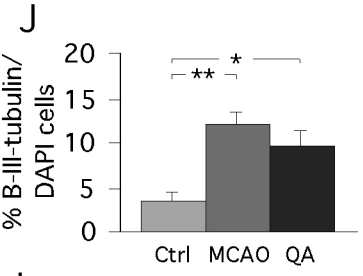
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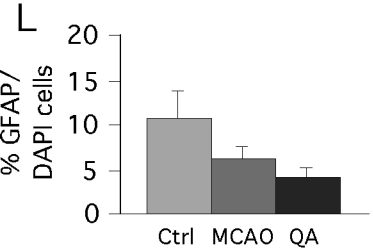
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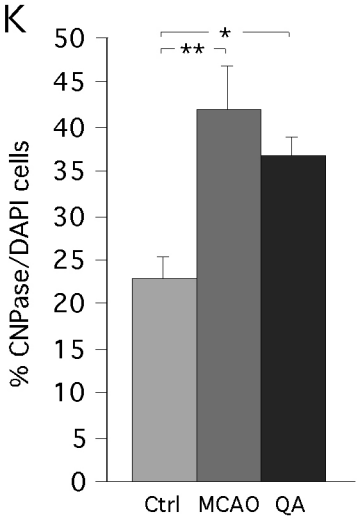
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