STEM CELLS (TISSUE-SPECIFIC STEM CELLS)

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TITLE PAGE

Running head: Functional identification of oligodendrocytes

Title: Thrombin activates PAR-1 receptors in neural stem cell-derived cultures: a gateway to identify oligodendrocyte differentiation

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ABSTRACT (249 words; limit: 250 words)

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system. Current immunosuppressive treatments fail preventing long-term motor and cognitive decline in patients. Excitingly, glial cell transplantation arises as a promising complementary strategy to challenge oligodendrocytes loss occurring in MS. A potential source of new oligodendrocytes is the subventricular zone (SVZ) pool of multipotent neural stem cells. However, this approach has been handicapped by the lack of functional methods

for identification and pharmacological analysis of differentiating oligodendrocytes, prior transplantation.

In this work, we questioned whether SVZ-derived oligodendrocytes could be functionally discriminated on the basis of intracellular calcium levels ($[Ca^{2+}]_i$) variations following KCl, histamine and thrombin stimulations. For this, P1-3 mice SVZ cultures were treated with triiodo-L-thyronine (T3) promoting oligodendrocytic differentiation. We have previously shown that SVZ-derived neurons and immature cells can be discriminated due to their selective $[Ca^{2+}]_i$ rise upon KCl and histamine stimulations, respectively. Herein, we demonstrate that O4-oligodendrocytes, do not respond to these stimuli but display a robust $[Ca^{2+}]_i$ rise following thrombin stimulation, whereas other cell types are thrombin-insensitive. Thrombin-induced calcium increase in oligodendrocytes is mediated by protease-activated receptor-1 (PAR-1) activation and downstream signaling through $G_{q/11}$ and phospholipase C (PLC), resulting in calcium recruitment from intracellular compartments.

This method allows the analysis of functional properties of oligodendrocytes in living SVZ cultures, which is of major interest for the development of effective grafting strategies in the demyelinated brain. Additionally, it opens new perspectives for the search of new pro-oligodendrogenic factors to be used prior grafting.

TEXT (5458 words; limit: 5000 words, excluding abstract, figures, legends, references)

INTRODUCTION

Central nervous system demyelinating diseases, namely multiple sclerosis (MS), are usually slow-progressing debilitating diseases, resulting in moderate to severe permanent neurological deficits. Briefly, an auto-immune attack on myelin-forming oligodendrocytes

causes axonal demyelination, and consequently neuronal communication becomes deficient, leading to brain function impairments. In early phases of MS, endogenous oligodendrocyte precursor cells (OPCs) spontaneously remyelinate newly nude axons in damaged area. However, as the disease progresses, they gradually lose this capability. Furthermore, conventional immunomodulatory therapies are insufficient to prevent devastating progression of the disease, with common relapsing events occurring. (see review [1]).

Recent advances in neural stem cell (NSC) biology raised considerable prospects for the development of MS therapies, based in glial cell replenishment. Multipotency of NSCs has been well-characterized, unveiling a renewable source of new oligodendrocytes for use in regenerative medicine concerning myelin pathologies [2,3]. Indeed, subventricular zone (SVZ) stem cells originate oligodendrocyte progenitors that differentiate into mature and functional myelinating oligodendrocytes [3-7]. Nevertheless, remaining hurdles still limit the grafting success, including a lack of functional pre-transplantation studies on reparative cells that identify key pharmacological targets, in order to drive their differentiation in myelinating oligodendrocytes, as well as their survival and integration in the diseased brain. Currently, characterization of oligodendrocytes or their progenitors is mainly performed by immunocytochemical studies which are far limitative to support an engrafting approach.

In the present study, we describe a functional method to study oligodendrocytes derived from SVZ cultures. Thus, monitoring the intracellular calcium variations ($[Ca^{2+}]_i$) of single cells, upon a defined sequence of stimulations allows the identification of oligodendrocytes among other cells in the SVZ culture and perform their pharmacological characterization. Our group has previously developed a calcium imaging protocol that rapidly discriminates neurons and immature cells in SVZ cultures [8]. Indeed, KCl-induced depolarization and consequent $[Ca^{2+}]_i$ rise is typical of excitable neuronal cell lineage, whereas histamine triggers a response in immature cells. This method has unveil the pro-neurogenic effects of NPY and

TNF in mice SVZ cultures [9, 10]. Here, we hypothesized whether thrombin can be similarly used as a specific stimulus for SVZ-derived oligodendrocytes. Accordingly, Wang *et al.* 2004 [11] show that rat OLN-93 oligodendrocyte cell line displays an increase of $[Ca^{2+}]_i$ following thrombin stimulation, an effect mediated by protease activated receptor-1 (PAR-1) activation.

The present work describes the development of a reliable single cell calcium imaging (SCCI) protocol to identify new SVZ-derived oligodendrocytes, useful to perform pharmacological studies and cell fate identification in SVZ neural stem cell cultures. Our results show that oligodendrocytes in SVZ cultures can, indeed, be distinguished according to the uniqueness of their response to thrombin.

MATERIALS AND METHODS

All experiments were performed in accordance with NIH and European Union (86/609/EEC) guidelines for the care and use of laboratory animals.

SVZ cultures

Briefly, brains from P1-3 C57Bl/6 mice were removed following decapitation and placed in Hanks' balanced salt solution (HBSS; Gibco, Rockville, MD, USA) plus 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco). Meninges were gently removed and the brains sectioned in 450 µm coronal slices from where fragments of SVZ were dissected out. After digestion of these fragments with 0.025% trypsin and 0.265 mM EDTA (Gibco) in HBSS (10 min, 37 ºC), and subsequent mechanical dissociation with a P1000 pipette, the cell suspension was diluted in serum-free culture medium (SFM) composed of Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium with GlutaMAX-I (Gibco), supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% B27 supplement (Gibco), 10 ng/ml epidermal growth

factor (EGF; Gibco) and 10 ng/ml basic fibroblast growth factor (FGF-2; Gibco). Individualized cells were then plated on uncoated Petri dishes at a density of 3000 cells/cm^2 and incubated during 6-8 days, in a 95% air-5% CO_2 humidified atmosphere, at 37 ºC. At this time, generated primary neurospheres were collected and deposited on 0.1 mg/ml poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) coated coverslips placed within cell culture plates, and covered with SFM devoid of growth factors. For the experiments correlating SCCI with phenotypic immunodetection, SVZ neurospheres were seeded onto poly-D-lysine coated microgrid coverslips (Eppendorf CELLocate, Hamburg, Germany).

The neurospheres were allowed to develop during 7-10 days, in the absence or presence of 30 nM 3,3',5-triiodo-L-thyronine (T3; Fluka, Sigma-Aldrich), or 10 ng/ml ciliary neurotrophic factor (CNTF; Promokine, Heidelberg, Germany), or 20 ng/ml stem cell factor (SCF; Chemicon, Millipore, Billerica, MA, USA). Following the treatment period, SCCI and immunostaining procedures were performed.

Hippocampal neuronal culture

Primary cultures of hippocampal neurons were prepared from the hippocampi of E18-19 C57Bl/6 mice embryos, after treatment with 0.06% trypsin (Sigma-Aldrich) in Ca^{2+}/Mg^{2+}-free HBSS (121 mM NaCl, 25.5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.8 mM KCl, 14.3 mM glucose, 10 mM HEPES and 0.001% phenol red, pH 7.4), for 10 min at 37 ºC. The hippocampi were then washed in Ca^{2+}/Mg^{2+}-free HBSS supplemented with 1.5 mg/ml of trypsin inhibitor (Sigma-Aldrich) in order to stop trypsin activity. After removing the excess of trypsin and trypsin inhibitor, cells were mechanically dissociated with a P1000, in fresh Ca^{2+}/Mg^{2+}-free HBSS. The cells suspension was centrifuged and the pellet of cells resuspended in serum-free Neurobasal medium (Gibco), supplemented with 2% B27, 25 µM glutamate (Sigma-Aldrich), 200 µM glutamine (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells

were plated on 0.1 mg/ml poly-D-lysine coated coverslips placed within cell culture plates (370 000 cells/cm^2) and maintained in the same medium. The cells were kept in 95% air-5% CO_2 humified atmosphere, for 7-8 days before SCCI/immunostaining analysis. Half of the medium was changed every four days for Neurobasal medium supplemented with 2% B27, 200 μM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, without glutamate.

Cortical astrocytic culture

Astrocytes were cultured from P6-7 C57Bl/6 mice. Brains were removed following decapitation and freed of meninges in HBSS solution plus 100 U/ml penicillin, 100 g/ml streptomycin. The entire cerebral cortex was harvested and digested in 0.025% trypsin and 0.265 mM EDTA, then, mechanically dissociated with a P1000 pipette. Cell suspension was centrifuged and the pellet was resuspended in DMEM/Ham's F-12 medium with GlutaMAX-I supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 0.37 g/l $NaHCO_3$, 10% fetal bovine serum (FBS; Gibco) and 25 mM HEPES. Cells were seeded onto 0.1 mg/ml poly-D-lysine coated coverslips placed within cell culture plates at a density of 25 000 cells/cm^2. The next day, the medium was changed to remove non-adhering dead cells and the culture was then allowed to develop in a 95% air-5% CO_2 humidified atmosphere at 37 ºC, during 7 days, before experiments.

SCCI analysis

To functionally discriminate and characterize oligodendrocytic differentiation in SVZ mixed cultures, we analyzed variations of $[Ca^{2+}]_i$ upon stimulation with high potassium (50 mM KCl) Krebs solution, 100 μM histamine (Sigma-Aldrich) and 0.1 U/ml thrombin (Sigma-Aldrich), as depicted in Figure 1 A.

Following the 7-10 days differentiation period, SVZ cultures were loaded for 40 min, at 37 ºC, with 5 μM Fura-2 AM (Molecular Probes, Eugene, OR, USA) in Krebs solution (132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 2.5 mM $CaCl_2$, 6 mM glucose, 10 mM HEPES, pH 7.4) plus 0.1% fatty acid free bovine serum albumin (BSA; Sigma) and 0.02% pluronic acid F-127 (Molecular Probes). Afterwards, cells were washed for 10 minutes in Krebs to remove the probe that remained outside the cells, and subsequently mounted on RC-25 chamber (Warner Instruments, Hamden, CT, USA, www.warneronline.com) in a PH3 platform (Warner Instruments) on the stage of an inverted fluorescence microscope Axiovert 200 (Carl Zeiss, Göttingen, Germany, www.zeiss.com). Cells were continuously perfused with Krebs and stimulated at defined periods of time by 50 mM KCl (isosmotic substitution with NaCl), 100 μM histamine and 0.1 U/ml thrombin. Solutions were applied by a fast-pressurized system (95% air-5% CO_2 atmosphere) (AutoMate Scientific, Berkeley, CA, USA, www.autom8.com). $[Ca^{2+}]_i$ was assessed by quantifying the ratio of fluorescence emitted at 510 nm following alternate excitation (750 msec) at 340 nm and 380 nm, using a Lambda DG4 apparatus (Sutter Instruments Company, Novato, CA, USA, www.sutter.com), and a 510 nm bandpass filter (Carl Zeiss). Fluorescence acquisition was given by a 40x objective and a Coll SNAP digital camera (Roper Scientific, Tucson, AZ, USA, www.roperscientific.com). Approximately 100 cells/field were analyzed and the acquired values were processed using MetaFluor software (Universal Imaging Corporation, Marlow, UK).

KCl, histamine and thrombin peaks given by the normalized ratios of fluorescence at 340/380, at the proper time periods, were used to calculate the ratios histamine/KCl (Hist/KCl) and thrombin/histamine (Throm/Hist) for the quantification of the percentage of astrocytes plus oligodendrocytes, or oligodendrocytes only, respectively, in SVZ cultures. Additionally, the same SCCI experimental protocol (Figure 1 A) was carried out on hippocampal neurons and cortical astrocytes, in which oligodendrocytes are absent.

To identify the receptor of thrombin that mediates thrombin-induced Ca^{2+} rise in SVZ-derived oligodendrocytes and further explore the activated transduction pathway, we designed other protocols of stimulation along Krebs perfusion of T3-treated SVZ cultures, represented in Figure 1 A-E. Among the three protease-activated receptors (PARs) that bind thrombin, PAR-1 is the receptor most probably involved in the cascade event, according to the literature [12, 13, 14]. Therefore, after KCl and histamine perfusions we stimulated the cells with 100 μM TFLLR-NH_2 (Tocris Cookson, Bristol, UK), a PAR-1 activating peptide, instead of thrombin (Figure 1 A). Furthermore, we analyzed the variations of $[Ca^{2+}]_i$ upon stimulation with thrombin concomitantly with 30 μM SCH79797 (Tocris Cookson), a potent and selective non-peptide PAR-1 antagonist, in SVZ cells pre-incubated for 60 minutes with 30 μM SCH79797, for full inhibition of thrombin binding to PAR-1 [15] (Figure 1 B).

To assess whether the elevation of $[Ca^{2+}]_i$ induced by PAR-1 results from Ca^{2+} release from intracellular stores or extracellular Ca^{2+} influx, we replaced the regular Krebs solution by a Krebs solution containing low calcium concentration (0 Ca^{2+} Krebs: 132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 38 μM $CaCl_2$, 6 mM glucose, 10 mM HEPES, 50 μM EGTA, pH 7.4) 2 minutes before applying TFLLR-NH_2, prepared in 0 Ca Krebs as well. At the end of TFLLR-NH_2 pulse the cells were perfused with normal Krebs till the end of the protocol (Figure 1 C). To further explore the signaling mechanism triggered by PAR-1 activation, particularly the coupling to heterotrimeric G proteins, a set of SVZ cells was pre-incubated with 200 ng/ml pertussis toxin (PTX; Sigma-Aldrich), which inhibits $G_{i/o}$ proteins, or 10 μM Y-27632 (Calbiochem, Beeston, UK) which inhibits the $G_{12/13}$ effectors Rho/ROCK, 24 hours before SCCI experiment, where cells were stimulated with TFLLR-NH_2 (Figure 1 D). To investigate whether $[Ca^{2+}]_i$ rise resulted from phospholipase C (PLC) activation cells were first stimulated with TFLLR-NH_2, KCl and histamine, then, incubated for 10 minutes with 5 μM

U73122 (Sigma-Aldrich), a PLC inhibitor, before a last pulse of TFLLR-NH_2 plus U73122 (Figure 1 E).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich) diluted in phosphate-buffered saline (PBS) for 30 minutes at room temperature, washed in PBS, and then incubated for 1 hour in PBS plus 3% BSA and 0.25% Triton X-100 (Sigma), to block non-specific binding sites and permeabilize the cells. Thereafter, cells were incubated overnight at 4º C with the following primary antibodies, all prepared in PBS plus 0.3% BSA and 0.1% Triton X-100: rabbit polyclonal anti-NG2 (1:500; Chemicon, Millipore), mouse monoclonal anti-MAP-2 (1:200; Sigma-Aldrich), rabbit monoclonal anti-GFAP (1:100; Sigma-Aldrich), mouse monoclonal anti-Nestin (1:200; Chemicon, Millipore) rabbit polyclonal anti-doublecortin (1:200, Cell Signaling Technology, Danvers, MA, USA) and goat polyclonal PAR-1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). In the case of mouse monoclonal anti-O4 (1:100, Chemicon, Millipore), the immunobinding was performed in living cells, for 30 minutes at 37 ºC, before 4% PFA for 10 minutes. Then, coverslips were washed in PBS, and subsequently incubated for 1 hour at room temperature with the proper secondary antibodies diluted in PBS: donkey anti-rabbit Alexa Fluor 488 (1:200, Molecular Probes), rabbit anti-mouse Alexa Fluor 488 (1:200, Invitrogen, Carlsbad, CA, USA), rabbit anti-goat Alexa Fluor 594 (1:200, Invitrogen) and goat anti-mouse Alexa Fluor 594 (1:200, Molecular Probes). Following PBS rinses, the cells were incubated for 5 minutes at room temperature, with Hoechst 33342 (2 μg/ml, Molecular Probes) in PBS, for nuclear staining. After a final PBS rinse, the coverslips were mounted in Dako fluorescent medium (Dako, Glostrup, Denmark). Fluorescent images were taken in an Axioskop microscope (Carl Zeiss).

Data Analysis

In SCCI experiments, fluorescence measurements were performed in cells located at the outside border of the seeded neurospheres. These cells migrated from the periphery of the sphere forming a pseudo-monolayer where the analysis is done. Each experimental condition was assessed at least in three different wells (about 100 cells per coverslip). The experiments were replicated at least in three different cultures except where otherwise specified. Data are expressed as means ± SEM. Statistical significance was determined by using the unpaired two-tailed Student's t test, with $*p<0.05$, $**p<0.01$, $***p<0.001$.

RESULTS

Thrombin triggers the increase in $[Ca^{2+}]_i$ in SVZ-derived oligodendrocytes

To functionally evaluate oligodendroglia differentiation in postnatal mice SVZ cultures, we took advantage of cell-specific responses to different calcium-coupled stimuli. We have previously demonstrated that neurons and immature cells in SVZ cultures can be rapidly distinguished on the basis of their unique responses to KCl or histamine, respectively [8]. Accordingly, the membrane depolarization evoked by KCl on neurons results in calcium entry via voltage-sensitive Ca^{2+} channels, whereas histamine activates H1R receptor expressed in immature cells inducing the mobilization of calcium from the intracellular stores into the cytoplasm. It was also shown that neither KCl nor histamine trigger a significant $[Ca^{2+}]_i$ rise in astrocytes. Herein, we characterize the intracellular calcium profile of oligodendrocytes upon perfusion with KCl and histamine. Furthermore, we used thrombin as a putative $[Ca^{2+}]_i$ rise inducer stimulus for SVZ-derived oligodendrocytes [11].

For this purpose, we used mice SVZ neurospheres, grown over 6-7 days in proliferative conditions, and subsequently seeded on poly-D-lysine coverslips, remaining in the absence of

growth factors for 7-10 days. During this differentiation period, cells emerge continuously from the spheres and differentiate in neurons, astrocytes, and also oligodendrocytes albeit in a minor extent, forming a heterogeneous pseudo-monolayer of diverse cells in various stages of maturation. As our interest is focused on studying the oligodendroglia lineage resulting from SVZ stem/progenitor cells, spheres were treated with 30 nM T3 during the differentiation period, in order to obtain a SVZ culture highly enriched in oligodendrocytes among the progeny. Indeed, there was a robust increase in the proportion of cells of the oligodendroglia lineage upon 7-10 days of T3 treatment, as demonstrated by immunocytochemistry for the oligodendrocyte markers O4 and NG2 (Figure 2). We used T3-treated SVZ cultures to perform SCCI analysis when we intended to focus on the oligodendrocyte population subset.

To correlate profiles of $[Ca^{2+}]_i$ oscillations with the respective cell phenotype, we performed SCCI analysis in SVZ neurospheres seeded and differentiated on microgrid coverslips, allowing single cell identification, and subsequent immunostaining for the different cell type markers. Cells were pre-loaded with Fura-2AM calcium probe and then continuously perfused with Krebs solution, and stimulated subsequently with 2 minutes pulses of 50 mM KCl, 100 μM histamine and 0.1 U/ml thrombin, in accordance with Figure 1 A. The calcium oscillations within delimited single cells were given by the normalized ratios of Fura-2AM fluorescence at 340/380, monitored along the 20 minutes experiment. By this method, we demonstrated that O4 oligodendrocytes do not respond to KCl nor histamine, underlining the specificity of the two stimuli in the identification of neurons or immature cells, respectively. Excitingly, thrombin clearly induces an increase in the intracellular calcium content of SVZ-derived O4 oligodendrocytes (Figure 3 A). Moreover, increase of $[Ca^{2+}]_i$ upon thrombin stimulation was not seen in MAP-2 neurons (Figure 3 B), nor in DCX neuroblasts (data not shown), Nestin immature cells (Figure 3 C) or GFAP astrocytes (Figure 3 D), revealing the specificity of thrombin stimulus for oligodendrocytes. In agreement with the previous work,

cells from the neuronal lineage presented a calcium rise upon KCl stimulation, and none (MAP-2 neurons) or a weak (DCX neuroblasts) response to histamine, whereas immature cells expressing Nestin responded solely to histamine pulse. The profiles of fluorescence depicted are representative (at least, 8 cells per phenotype were analyzed).

SVZ-derived oligodendrocytes can be identified on the basis of the selective response to thrombin

Normalized data was used to calculate the ratio of responses to thrombin and histamine (Throm/Hist) (Figura 3 E). The high Throm/Hist values for O4-positive oligodendrocytes (1.43 ± 0.04; 14 cells analyzed) were due to their marked response to thrombin and no observed response to histamine, whereas the low values of Throm/Hist for Nestin-positive cells (0.84 ± 0.01; 8 cells analyzed) reflect their null response to thrombin but high response to histamine. Other cell types had a Throm/Hist ratio of approximately 1 as they do not respond to any of these two stimuli (11 MAP-2 neurons [1.03 ± 0.01], 10 DCX neuroblasts [1.02 ± 0.01] and 8 GFAP astrocytes [1.00 ± 0.01] were analyzed). According to these findings, SVZ-derived oligodendrocytes can be functionally discriminated among other cells in SVZ cultures. We concluded that, in our cultures, cells that present a Throm/Hist ratio above 1.3 are oligodendrocytes.

Thrombin-induced $[Ca^{2+}]_i$ increase in SVZ-derived oligodendrocytes is mediated by PAR-1 activation

To further explore the mechanism of $[Ca^{2+}]_i$ increase in oligodendrocytes derived from SVZ cultures, following thrombin perfusion, we firstly investigated which receptor of thrombin was triggering the cytosolic Ca^{2+} rise in these cells. Thrombin receptors are included in the protease-activated receptors (PARs) family, characterized by a particularly interesting process

of activation, which consists on the recognition and cleavage of the receptor by the enzyme with consequent exposure of a new N-terminus that acts as a tethered ligand subsequently activating the receptor. The PARs family is composed of four receptors, PAR-1, PAR-2, PAR-3 and PAR-4. Among these, PAR-1, PAR-3 and PAR-4 are activated upon cleavage by the serine protease thrombin. Many of the effects of thrombin are mediated by PAR-1, being the most extensively studied subtype and the prototype of the family [12, 16]. PAR-4 is a low affinity receptor requiring higher concentrations of thrombin for activation in comparison with the other thrombin receptors [13]. PAR-3 does not signal by itself having a possible co-factor role in the cleavage and activation of PAR-4 [14]. Based on this knowledge we focused on PAR-1 expression and function in SVZ cultures. Our hypothesis was also supported by the finding that PAR-1 is the only thrombin receptor functionally expressed in the oligodendrocyte cell line OLN-93, where it was associated with an increase of $[Ca^{2+}]_i$ evoked by thrombin [11]. Besides, the same authors showed that primary cultured rat oligodendrocytes express high levels of PAR-1 but almost undetectable levels of PAR-3 and no PAR-4.

Therefore, we run a perfusion protocol consisting on the subsequent application of KCl, histamine and TFLLR-NH_2, a PAR-1 activating peptide (Figure 1 A), in T3-treated SVZ cultures. Although we have already an enriched culture in oligodendrocytes, we focused on the subset of cells responding with Hist/KCl ratio between 0.9 and 1.1, regarding that cells with Hist/KCl above 1.1 are immature cells and below 0.9 are neuronal cells, based on Agasse *et al.* 2008 [8]. By this data selection, we are assuring that we work on a population where the majority of cells are oligodendrocytes. As shown in Figure 4 A (on top), cells were highly responsive to TFLLR-NH_2, showing a similar calcium profile to the one obtained upon thrombin stimulation (see Figure 6 B). Additionally we performed another set of experiments where we used SCH79797, a potent and selective PAR-1 antagonist. After a 60 minutes pre-

incubation of T3-treated SVZ cultures with SCH79797, we run the perfusion protocol as depicted in Figure 1 B, and verified that the thrombin-induced rise of $[Ca^{2+}]_i$ was abolished (Figure 4, on bottom). We measured the peak of response due to thrombin, or TFLLR-NH_2, or thrombin plus SCH79797. A similar magnitude of response to TFLLR-NH_2 (1.42 ± 0.01; 854 cells analyzed) as compared to thrombin (1.41 ± 0.01; 431 cells analyzed) was observed, as well as a complete inhibition of thrombin peak by SCH79797 (1.06 ± 0.01; 381 cells analyzed) concluding, by this way, that PAR-1 is the receptor involved in the thrombin-induced $[Ca^{2+}]_i$ increase in SVZ-derived oligodendrocytes (Figure 4 B). In agreement with this role of PAR-1 in SVZ-derived oligodendrocytes we observed PAR-1 punctuated immunoreactivity in every O4-positive cell by immunocytochemical studies. Concerning the other cell phenotypes present in the mixed SVZ culture, we verified that MAP-2 neurons do not express PAR-1, nevertheless some of the GFAP expressing cells as well as scarce population of Nestin immature cells express PAR-1 (Figure 4 C), albeit not responding to thrombin stimulus.

Moreover, another set of SCCI experiments was performed in order to unravel the signaling mechanism of PAR-1-induced Ca^{2+} rise in SVZ cultures. All the experiments with this aim were performed in T3-treated SVZ cultures and the data were suppressed to the population containing oligodendrocytes, with Hist/KCl 0.9-1.1, as explained before.

As Figure 1 C shows, we replaced the normal Krebs by a 0 Ca^{2+} Krebs solution 2 minutes before TFLLR-NH_2 stimulation and during this pulse. In these conditions the increase of $[Ca^{2+}]_i$ was as high (TFLLR-NH_2 in 0 Ca^{2+}: 1.38 ± 0.01; 127 cells analyzed) as in normal Krebs, indicating that Ca^{2+} comes from intracellular stores and not from the extracellular solution (Figure 5).

Like other PARs, PAR-1 is a transmembrane metabotropic G protein-coupled receptor (GPCR). Particularly, PAR-1 can couple to members of the $G_{i/o}$, $G_{12/13}$ and $G_{q/11}$ families of

large heterotrimeric G proteins [16]. To disclose the role of large G proteins in the thrombin-induced $[Ca^{2+}]_i$ rise through PAR-1, we incubated cells with 200 ng/ml PTX, well-known to inactivate of $G_{i/o}$ proteins, the last 24 hours before the assay [11] (Figure 1 D). As shown in Figure 5, PTX incubation did not alter the Ca^{2+} mobilization in SVZ-derived oligodendrocytes, indicating that $G_{i/o}$ are not involved in the PAR-1-induced Ca^{2+} increase (1.39 ± 0.02; 149 cells analyzed). Therefore the signaling cascade triggered by TFLLR-NH_2 includes the PTX-insensitive proteins $G_{q/11}$ and/or $G_{12/13}$. The pathway upon PAR-1-coupled $G_{q/11}$ activation is quite well-described. $G_{q/11}$ activates phospholipase C isoform β (PLCβ) that catalyzes phosphoinositide hydrolysis, leading to Ca^{2+} recruitment from the intracellular compartments, and protein kinase C (PKC) activation. On the other hand, $G_{12/13}$ binds guanine-nucleotide exchange factors (RhoGEFs) initiating a Rho-associated protein kinase (ROCK) dependent pathway that leads to the activation of PLC isoform ε (PLCε), thus resulting in Ca^{2+} recruitment and PKC activation [17, 18, 19]. To search for the involvement of $G_{q/11}$ and/or $G_{12/13}$ in PAR-1-induced calcium oscillations in SVZ-derived oligodendrocytes, we pre-incubated a set of cells with 10 μM Y-27632, a ROCK inhibitor, for 24 hours, and then run the SCCI protocol consisting of KCl, histamine and TFLLR-NH_2 stimulations, as depicted in Figure 1 D. When ROCK was inhibited the TFLLR-NH_2 induced $[Ca^{2+}]_i$ increase was still present and in the same magnitude as compared to the peak evoked by TFLLR-NH_2 alone (1.39 ± 0.02; 284 cells analyzed). Furthermore, we developed a protocol that included 10 minutes incubation with 5 μM U73122, a PLC inhibitor (Figure 1 E). Accordingly, we demonstrate that U73122 abolish the cells response to TFLLR-NH_2, indicating that PLC activation is part of the triggered pathway (1.14 ± 0.01; 398 cells analyzed). In this protocol, the perfusion interval between the first and the last TFLLR-NH_2 stimulations was sufficient to wash the peptide applied firstly, exposing again the receptor-binding sites for a new stimulation. It is also noteworthy to refer that after both SCH79797

and U73122 incubations, there were cells responding to KCl application in the total population profiles (i.e. without data selection) demonstrating no alteration in functional properties of neurons.

Oligodendroglial differentiation can be assessed by measuring the variations of $[Ca^{2+}]_i$ upon stimulation with thrombin and histamine

To validate the method described in the present work, we functionally compared a non-treated SVZ culture with a T3-treated culture, both allowed to differentiate along 7-10 days following deposition in poly-D-lysine coverslips and growth factors withdrawal. Upon SCCI assay consisting on KCl, histamine and thrombin pulses (Figure 1 A), normalized peaks of fluorescence of all the individualized cells in the field of the microscope were measured and Throm/Hist ratio was calculated. Indeed, a control culture presented ~3% of cells responding with a Throm/Hist ratio above 1.3 (3.24 ± 1.06; 21 coverslips; 1790 cells analyzed) consistent with the normal oligodendrocyte differentiation in SVZ cultures [20, 21]. Upon T3 treatment, culture contained ~35% of these cells (35.07 ± 8.22; 10 coverslips; 626 cells analyzed) (Figure 6 A), which is in accordance with the well-described effect of T3 hormone increasing the OPCs number and promoting the differentiation in oligodendrocytes [22-26]. Furthermore, SVZ cultures pre-treated with the astrogliogenic factor CNTF [20, 26] or the neurogenic factor SCF [27], during the differentiation period, displayed a similar percentage of cells responding as oligodendrocytes as found in non-treated cultures (CNTF: 2.92 ± 1.19; 8 coverslips; 776 cells analyzed; SCF: 4.74 ± 1.91; 13 coverslips; 1304 cells analyzed). Representative profiles of calcium variations along SCCI protocol in the four different experimental conditions are depicted in Figure 6 B. As shown, oligodendrocyte-like responding cells were hardly found in control cultures. On the contrary, when treated with T3, most cells were insensitive to KCl or histamine, responding markedly to thrombin, thus

confirming the shift to an oligodendrocyte phenotype. On the other hand, in SCF-treated cultures displaying a neuronal tendency with many KCl-responding cells, and in CNTF-treated cultures exhibiting a typical astrocytic tendency with many non-responsive cells, oligodendrocyte population was unaffected and similar to levels in control cultures.

Additionally, SCCI experiments were performed in defined cultures of hippocampal neurons as well as in cultures of cortical astrocytes (Supplementary Figure 1). Both cultures presented no oligodendrocyte-like responding cells with the established ratio Throm/Hist>1.3 (hippocampal neurons: 0.00 ± 0.00, 8 coverslips; 243 cells analyzed; cortical astrocytes: 0.00 ± 0.00, 7 coverslips; 305 cells analyzed).

DISCUSSION

We here describe a novel method to functionally evaluate oligodendroglia differentiation in SVZ cultures. The method consists in measuring the intracellular calcium currents evoked by KCl, histamine and thrombin on the different cell types present in a SVZ culture, and is based on the observation that each cell type displays a distinguishable profile of Ca^{2+} oscillations during the stimulation protocol. The main objective of the present work was to disclose whether thrombin is a calcium-mobilizing stimulus for oligodendrocytes in SVZ cultures.

As oligodendrocytes are rare cells in a SVZ cell culture, we treated the SVZ spheres with T3 after deposition in poly-D-lysine coated coverslips and growth factors withdrawal, during 7-10 days, in order to increase the proportion of oligodendrocytes in culture, obtaining a SVZ cell-derived culture highly enriched in oligodendrocytes. The effect of T3 hormone as a pro-oligodendrogenic factor is quite well-described. Indeed, Barres *et al*. 1994 [22] concluded that, in the presence of mitogens, T3 stops cell division of bipotential precursors of both astrocytes type 2 and oligodendrocytes, so called oligodendrocyte type 2 astrocytes (O-2A)

allowing them to enter in the differentiation stage of oligodendrocytic lineage. Stem cells may differentiate into oligodendrocytes through an intermediate stage resembling an O-2A cell although there are still many controversies regarding the lineage paths arising from neural stem cells. Nevertheless, Johe *et al*. 1996 [23] demonstrated that T3 exposure leads rat embryonic and adult multipotent neural stem cells to a glial lineage and promotes oligodendrocyte differentiation if the treatment is prolonged, revealing an instructive mechanism of T3 action in cell fate decisions. Accordingly, T3 treatment was shown to increase the generation of oligodendrocytes from murine embryonic stem cells [24] as well as in human embryonic stem cells [25]. It seems that T3 not only accelerates the rate of oligodendrocyte precursor's generation but also the complexity of their morphology towards a mature oligodendrocyte [26] and the synthesis of myelin proteins [29, 30]. As expected, we observed much more O4 as well as NG2 positive oligodendrocytes in the T3 treated cultures as compared to the non-treated cultures.

Using microgrid coverslips we correlated the calcium profiles with subsequent phenotypic immunodetection. We were enable to show that 1) similarly to astrocytes, O4 oligodendrocytes do not respond to KCl or histamine; 2) $[Ca^{2+}]_i$ is not altered by thrombin in MAP-2 mature and DCX immature neurons, as well as in GFAP astrocytes; 3) however, O4 oligodendrocytes present an increase of $[Ca^{2+}]_i$ following thrombin perfusion. Therein, cells presenting a Hist/KCl ratio between 0.9-1.1 are glial cells as shown in our previous work, astrocytes or oligodendrocytes, the latest being identifiable by their specific rise of Ca^{2+} under thrombin perfusion and related throm/hist ratio above 1.3. According to these findings, thrombin stimulation was identified as a reliable tool to discriminate oligodendrocytes among other cells in SVZ cultures.

Using PAR-1 specific ligands, we concluded that thrombin triggered-Ca^{2+} increase in SVZ-derived oligodendrocytes is mediated by PAR-1 activation. Indeed, PAR-1 immunoreactivity

strongly co-localizes with O4 staining, labeling intensively in the oligodendrocyte cell body, and also appearing in a more dispersed manner on cell processes. Nevertheless, PAR-1 expression is not restricted to oligodendrocytes. Some astrocytes, and occasionally some Nestin immature cells, also express PAR-1. The scarce Nestin/PAR-1-positive cells presented a typic astrocytic morphology suggesting that expression of PAR-1 in astrocytes begins early in their development when they still express the Nestin marker of immaturity, but already committed to become astrocytes. In contrast with the robust PAR-1 labeling in oligodendrocytes cell body, these occasional Nestin and GFAP cells expressing the receptor presented only a dispersed punctuated PAR-1 staining indicating a minor expression in these cells. No GFAP or Nestin cells responding to thrombin were found in SCCI analysis, therefore suggesting that low expression of PAR-1 is insufficient to signal a detectable response by SCCI, or alternatively, that PAR-1 is not coupled to an increase of $[Ca^{2+}]_i$ in these cell types. On the contrary, mature neurons stained with MAP-2 did not express PAR-1. Previous studies have shown the presence of PAR-1 in motor neurons, olfactory neurons and postnatal hippocampal neurons [31, 32, 33]. Moreover, PAR-1-induced low calcium transients were observed in hippocampal neurons [33]. However, in our experiments we observed neither PAR-1 expression in SVZ-derived neurons, nor $[Ca^{2+}]_i$ changes in these cells, or in embryonic hippocampal neurons (see Supplementary Figure 1 A). It is worthwhile to refer that the observed calcium transients in hippocampal cultures reported by Gorbacheva *et al*. 2006 [33] is about 200 nM, a very modest response. Also, some authors report the expression of PAR-1 and $[Ca^{2+}]_i$-associated response upon PAR-1 stimulation of newborn rat or mice cortical astrocytes [34, 35]. We focused on cortical astrocytes from P7 mice and SVZ-derived astrocytes from newborn mice, the last having occasionally PAR-1 expression, but both being insensitive to thrombin stimulation (see Supplementary Figure 1 B).

Differences in culture conditions or animals age may be responsible for the different results in PAR-1 expression and thrombin response regarding cortical astrocytes.

Interestingly, the Throm/Hist ratio characteristic of O4 oligodendrocytes varies among high values and this can be explained by the wide range of oligodendrocyte differentiation stages where O4 sulfatide is present. Indeed, it seems that as oligodendrocyte maturation proceeds, the ratio increases, suggesting that immature oligodendrocytes expressing O4 respond to thrombin with less magnitude, decreasing the Throm/Hist ratio.

Concerning the mechanism of PAR-1 downstream signaling towards cytosolic calcium rise, our data demonstrate that PAR-1 binding leads to PLC activation through $G_{q/11}$ heteromeric protein activity. PLC hydrolyzes phosphoinositides generating inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), with consequent IP_3-dependent Ca^{2+} release from endoplasmic reticulum and PKC activation by DAG. This is consistent with previous studies examining the PAR-1 downstream signaling pathway in oligodendrocytes [11] although not so far characterized in SVZ-derived oligodendrocytes.

Finally, we validated the proposed method by comparing the percentage of cells with Throm/Hist above 1.3, thus oligodendrocytes, in non-treated SVZ cultures, T3-treated cultures, as well as in CNTF- and SCF-treated cultures. As compared to non-treated cultures, calcium profiles in treated cultures completely shifted differently reflecting the acquirement of different phenotypes. As expected, T3 induced an increase in the proportion of oligodendrocyte population, whereas treatment with CNTF or SCF conferred no changes in the proportion of oligodendrocytes further confirming the efficiency of the method.

CONCLUSION

In conclusion, we describe here a functional method to evaluate oligodendrocytic differentiation in SVZ cell cultures. This method is based on measurements of cellular Ca^{2+} concentration changes upon stimulation with thrombin, since this protease induces a specific increase of $[Ca^{2+}]_i$ in oligodendrocytes derived from SVZ cultures. It became possible, with this strategy, to analyze functional properties of the oligodendrocytic cell population in living SVZ cultures, a major interest for the development of efficient grafting strategies in the demyelinated brain. Moreover, this technique opens new perspectives for the search of new pro-oligodendrogenic factors.

The method described in this work is under patent protection (US Provisional 16/10/2074-PAT 2008100089504/0198).

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FIGURES

FIGURE LEGENDS (limit: 55 words)

Figure 1 – **Experimental protocols performed in SCCI analysis, aiming the functional identification of SVZ-derived oligodendrocytes by thrombin response (A), and the clarification of the thrombin-activated cellular mechanism (B-E).** SVZ cells loaded with the Ca^{2+} probe Fura-2AM were continuously perfused in Krebs solution and stimulated at different time intervals as shown by the time sequences. (54 words)

Figure 2 – **T3 increases the production of oligodendrocytes in SVZ cultures.** Representative fluorescence images of O4-positive oligodendrocytes (red; on top) and NG2-positive oligodendrocyte precursors (green; on bottom), counterstained with Hoechst 33342 (blue nucleus) in SVZ cells treated or not with 30 nM T3 along 7-10 days after spheres deposition. Scale bar = 50 m. (54 words)

Figure 3 – **$[Ca^{2+}]_i$ variations upon KCl, histamine and thrombin in SVZ-derived cells.** O4+ oligodendrocytes (A; red), MAP-2+ neurons (B; red), Nestin+ cells (C; green), GFAP+ astrocytes (D; red). Scale bar = 20 µm. (E) Throm/Hist ratios for each phenotype. ***p<0.001 using unpaired Student's t test, comparing to MAP-2, Nestin or GFAP. (51 words)

Figure 4 – **Thrombin-induced $[Ca^{2+}]_i$ increase in SVZ-derived oligodendrocytes is mediated by PAR-1 activation.** (A) Representative profiles upon perfusion protocol as in Figure 1 A (TFLLR-NH_2 as third pulse) and B. (B) Effect of PAR-1 agonist, and antagonist in oligodendrocytes response. ***p<0.001 using unpaired Student's t test, as compared to thrombin alone. (C) Expression of PAR-1 in SVZ-derived cell types. PAR-1 (in red, indicated

by arrows in top images) is expressed in O4-oligodendrocytes, and also in some Nestin+ and GFAP+ cells. Scale bar = 20 m, top images; 10 m, bottom images. (91 words)

Figure 5 – **PAR-1 signaling in SVZ-derived oligodendrocytes involves $G_{q/11}$ and PLC activity, with calcium recruitment from intracellular stores.** Means of peaks of normalized ratios of fluorescence at 340/380 displayed by SVZ-derived oligodendrocytes following perfusion protocols as in Figure 1 A (TFLLR-NH_2 as third pulse), C-E. ***$p<0.001$ using unpaired Student's t test, as compared to TFLLR-NH_2. (55 words)

Figure 6 – **Functional evaluation of oligodendrocyte differentiation in SVZ cultures.** (A) Percentage of oligodendrocyte-like responding cells in non-treated SVZ cultures and in cultures exposed to T3, or CNTF, or SCF for 7-10 days. *** $p<0.001$ using unpaired Student's t test, as compared to non-treated cultures. (B) Representative profiles of response elicited upon perfusion with KCl, histamine and thrombin. (58 words)

Supplementary Figure 1 - **$[Ca^{2+}]_i$ variations upon KCl, histamine and thrombin in neuronal (A) and astrocytic (B) cultures.** Oligodendrocyte-like responding cells, with typical thrombin response, are not found in either cultures (left). Immunostaining for MAP-2 (A) and GFAP (B) confirm the high purity of neuronal and astrocytic primary cultures, respectively (right). Scale bar = 50 m. (56 words)