Title: Developmental expression of the Oligodendrocyte Myelin Glycoprotein in the mouse telencephalon.

Authors: Vanessa Gil[1,2,6], Zoe Bichler[1,2,6], Jae K. Lee[3], Oscar Seira[1,2,6], Franc Llorens[1,2,6], Ana Bribian[1,2,6], Ricardo Morales[4], Enric Claverol-Tinture[4], Eduardo Soriano[5,6], Lauro Sumoy[7], Binhai Zheng[3] and Jose A. del Río[1,2,6,*]

Addresses: 1) Molecular and Cellular Neurobiotechnology laboratory, Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain **2)** Cellular and Molecular Basis of Neurodegeneration and Neurorepair (CMBNN), Department of Cell Biology, University of Barcelona, Spain **3)** Department of Neurosciences, University of California, San Diego, USA **4)** Neuroengineering group, Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain **5)** Neurobiology of Development and Regeneration Laboratory, Institute for Research in Biomedicine of Barcelona and Department of Cell Biology, University of Barcelona, Spain **6)** Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED) **7)** Institute of Predictive and Personalized Medicine of Cancer, Badalona, Spain

* **Correspondence to:**

Prof. J.A. del Río
Molecular and Cellular Neurobiotechnology
Institute for Bioengineering of Catalonia (IBEC)
Parc Científic de Barcelona
University of Barcelona
Baldiri Reixac 15-21; E-08028 Barcelona, Spain
Phone: +34-934035923/20296
Fax: +34-934020183
E-mail: jadelrio@ibec.pcb.ub.es

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ABSTRACT

The oligodendrocyte myelin glycoprotein is a glycosylphosphatidylinositol-anchored protein expressed by neurons and oligodendrocytes in the CNS. Attempts have been made to identify the functions of the myelin-associated inhibitory proteins (MAIPs) after axonal lesion or in neurodegeneration. However, the developmental roles of some of these proteins and their receptors remain elusive. Recent studies indicate that NgR1 and the recently discovered receptor PirB restrict cortical synaptic plasticity. However, the putative factors that trigger these effects are unknown. Since Nogo-A is mostly associated with the endoplasmic reticulum and MAG appears late during development, the putative participation of OMgp should be considered. Here we examine the pattern of development of OMgp immunoreactive elements during mouse telencephalic development. OMgp immunoreactivity in the developing cortex follows the establishment of the thalamo-cortical barrel-field. At cellular level, we located OMgp neuronal membranes in dendrites and axons as well as in brain synaptosome fractions and axon varicosities. Lastly, the analysis of the barrel-field in OMgp-deficient mice revealed that although thalamo-cortical connections were formed, their targeting in layer IV was altered and numerous axons ectopically invaded layer II-III. Our data support the idea that early-expressed MAIPs play an active role during development and point to OMgp participating in thalamo-cortical connections.

INTRODUCTION

The oligodendrocyte myelin glycoprotein (OMgp) is a glycosylphosphatidylinositol-anchored protein expressed by neurons and oligodendrocytes in the central nervous system (CNS) (Habib AA et al., 1998; Wang KC et al., 2002). Pioneer genomic studies reported that the *omgp* gene is located within intron 27b of the mouse *NF1* gene which encodes to Neurofibromin, a RasGAP protein, which, when mutated lead to neurofibromatosis type 1 (NF1) disease (Mikol DD, MJ Alexakos et al., 1990). NF1-deficient mice display deficits in cortical development (especially in the development of the neocortical barrel-field) (Lush ME et al., 2008). However, although function in adult in normal and neural degeneration is revealed, OMgp functions during development remain to be established.

OMgp belongs to a group of molecules located in CNS myelin protein fractions, with axon outgrowth inhibitory activity (Kottis V et al., 2002; Wang KC *et al.*, 2002). This group also includes, Nogo-A (GrandPre T et al., 2000; Huber AB and ME Schwab, 2000; Prinjha R et al., 2000) and MAG (McKerracher L et al., 1994; Mukhopadhyay G et al., 1994). All three proteins may act via the same receptor, the Nogo receptor (NgR1) (Fournier AE et al., 2001; Fujitani M et al., 2005) or its paralogues (NgR2 and/or NgR3) or the recently identified PirB (paired immunoglobulin-like receptor B) (Atwal JK et al., 2008; Barton WA et al., 2003; Lauren J et al., 2003; Pignot V et al., 2003; Venkatesh K et al., 2005). The participation and physiology of PirB is not fully known. However, NgR1 may form a complex with either $p75^{NGFR}$ (Domeniconi M et al., 2002; Hu WH et al., 2002) or TROY (Domeniconi M and MT Filbin, 2005; Shao Z et al., 2005) which would transduce intracellular signals by activating RhoA (Domeniconi M and MT Filbin, 2005; Shao Z *et al.*, 2005; Yamashita T and M Tohyama, 2003). In addition, NgR1 may also interact with another co-receptor, Lingo-1 (Llorens F et al., 2008; Mi S et al., 2004), which mediates intracellular signaling through the serine-threonine kinase WNK1 (Zhang Z et al.,

2009). Subsequent studies pointed out that ligands and their receptors may play crucial roles after lesion or in neurodegenerative diseases (e.g., (Fournier AE et al., 2002; Gil V et al., 2006; Jokic N et al., 2006; Karnezis T et al., 2004; Park JH et al., 2006; Teng FY and BL Tang, 2005)) or following alcohol abuse (Okamoto H et al., 2006). However, while these MAIPs are widely expressed in the adult CNS, emerging data indicate that some of them may play additional roles at early stages of brain development, since they are expressed before NgR1 and long before the onset of brain myelination. A recent example has been reported for Nogo-A with high neuronal expression and different roles during neuronal migration, neurite formation or oligodendrocyte maturation in the developing telencephalon (Mingorance-Le Meur A et al., 2007; Pernet V et al., 2008; Zhao XH et al., 2007). Another example is Lingo-1 (a co-receptor of NgR1, (Carim-Todd L et al., 2003; Mi S *et al.*, 2004)) which can also bind to the postmitotic neuron-specific zinc finger protein Myt1l (Llorens F *et al.*, 2008). In the studies of Habib et al., and Vourc'h et al., *omgp* expression was analyzed during postnatal development but earlier developmental stages were not studied.

Although oligodendrocyte expression of OMgp occurs at nodes of Ranvier with distinct roles in regulating nodal formation and function during CNS myelination (Apostolski S et al., 1994; Huang JK et al., 2005; Nie DY et al., 2006), several studies suggest that OMgp is mainly a neuronal protein, with is also expressed in oligodendrocytes (Habib AA *et al.*, 1998; Hunt D, RS Coffin et al., 2002; Koyama Y et al., 2008). However, the functions of neuronal OMgp during development have not been fully explored. Here we examined the pattern of OMgp expression in the embryonic mouse forebrain using a well characterized antibody, paying special attention to neurons. In addition, the cellular distribution and expression changes of neuronal OMgp protein were analyzed *in vivo* and *in vitro*. We report that neuronal OMgp is present at early stages of development (from E14), localized in the growing axons during axonal tract formation following the maturation of cortical connections (e.g., perforant pathway, thalamo-cortical

projection, etc). In addition, subsets of hippocampal interneurons express OMgp in the adult stages. At cellular level, OMgp is present in the neuronal membrane, synaptosomal fractions and axonal varicosities in primary hippocampal cultures. Lastly, the role of OMgp in the organization of thalamo-cortical connections was analyzed in *omgp -/-* mice. The barrel-field of *omgp -/-* mice was altered and ectopic thalamic axons were seen in layer II-III. Taken together, our data provide a detailed characterization of the OMgp protein expression in the embryonic mouse telencephalon and indicate that OMgp has a role in axonal target specification and synaptic plasticity.

MATERIALS AND METHODS

Animals

All animal experiments were carried out in accordance with the guidelines of the European Union (2003/65/CE) and current Spanish regulations (BOE 252/34367-91, 2005) for the use of laboratory animals. All experimental protocols were also approved by the local Ethical Committee. A total of 30 pregnant OF1 mice (Iffra Credo) were used. The morning of plug detection was considered as embryonic day 0 (E0) and the day of birth as postnatal day 0 (P0). Animals were killed at the following stages: E14, E16, P0, P5, P7, P10, P15, P21 and adults. Six to twelve animals (from two or more different litters) were used for each stage. In addition, 5 *omgp -/-* mice (stage P7) from two different litters were also used. *omgp -/-* mice were generated in the laboratory of Binhai Zheng (University of California, San Diego, USA). A detailed description of gene targeting at OMgp has recently been published (Lee JK et al., 2009). Briefly, the second exon, which contains all the coding sequence of the OMgp, is deleted, resulting in a null allele. This deletion does not interfere with NF1 expression (see below).

Antibodies

The following primary antibodies were used: OMgp (goat polyclonal, AF1674, R&D Systems, MN,1:3000 for IHC, 1:200 for IF and 1:1000 for Western blot), Nogo-A (rabbit polyclonal, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA), MBP (mouse monoclonal, 1:500, Chemicon, Temecula, CA), neuron-specific β-III Tubulin (mouse monoclonal,1:2000, Sigma, St. Louis, MO), Calbindin-28KD (CALB, rabbit polyclonal, 1:5000, Swant, Bellinzona, Switzerland), Calretinin (rabbit polyclonal, 1:500, Swant), CCK (rabbit polyclonal, 1:100, CRB, Cleveland, UK), Parvalbumin (rabbit polyclonal,1:250, Swant), Somatostatin (SOM, rabbit polyclonal, 1:5000, Swant), SNAP-25 (SMI81, mouse monoclonal, 1:5000, Covance, Princeton, NJ), Syntaxin 1

(mouse monoclonal, clone HPC-1, 1:5000, Sigma), Synaptophysin (mouse monoclonal, 1:1000, Dako, Glostrup, Denmark), Synapsin (rabbit polyclonal, 1:1000, Synaptics System, Goettingen, Germany), MAP2 (mouse monoclonal, 1:200, Sigma), Actin (mouse monoclonal, 1:1000, Chemicon), serotonin (5-HT) transporter (602-622) (5-HTT, rabbit polyclonal, 1:1000, Calbiochem, Gibbstown, NJ), HNK-1(412) (rat monoclonal,1:500, kindly provided by Prof. Melitta Schachner), HNK-1 (clone VC1.1, mouse monoclonal, 1:2000, Sigma) and Neurofibromin (NF1) (rabbit polyclonal, SC-67, 1:1000, Santa Cruz Biotechnology).

Preparation of adult brain myelin

CNS myelin was isolated following the procedure described by Norton and Poduslo (Norton WT and SE Poduslo, 1973). Briefly, adult Sprague-Dawley rat brains were homogenized in 0.32 M sucrose at 4º C in a Dounce homogenizer. This homogenate was layered over 0.85 M sucrose solution and centrifuged at 25,000 r.p.m for 30 min. The CNS myelin at the interface of the two sucrose layers was collected in water and centrifuged at 25,000 r.p.m for 15 min. The resultant pellet was obtained, collected in water and centrifuged at 10,000 r.p.m for 10 min twice. The white pellet was then suspended in 0.32 M sucrose and the initial gradient was replicated as described previously. Finally the myelin was removed from the interface and washed in water and spun at 25,000 r.p.m for 10 min to remove sucrose. The final pellet was freeze-dried overnight, and protein content was determined using the BCA protein assay kit (Pierce, Rockford, IL).

Cell transfection and OMgp detection

EBNA-293T cells were cultured with Dulbecco´s modified Eagle´s medium (DMEM), supplemented with 10% fetal bovine serum, glutamine, and antibiotics (all purchased from GIBCO Life Technologies, Paisley, UK). Cells were grown in 35-mm ø 6-well multiplates (Nunc, Roskilde, Denmark) containing 10-mm ø glass coverslips to 60-70% confluence and transfected

with pCMV-SPORT6-OMgp (full length cDNA clone IRAVp968C0766D purchased from RZPD (Germany)) using Lipofectamine-Plus reagents according to the manufacturer's instructions (GIBCO Life Technologies). Seventy-two hours later, cells were scraped and harvested in Laemmli sample buffer. Cell extracts were separated by 8% SDS-PAGE electrophoresis, electrotransferred to nitrocellulose membranes and immunoblotted with OMgp antibody. In parallel, protein samples of total adult brain and myelin extract were also included in the experiment as controls.

Immunohistochemical methods

For immunohistochemistry, fetuses were removed by caesarean section after deep anesthesia of the mother with chloral hydrate (3.5 mg/Kg i.p. injection) and transcardially perfused with 4% paraformaldehyde dissolved in phosphate buffered saline (PBS) 0.1 M. Postnatal mice were anesthetized with chloral hydrate and perfused. After perfusion, brains were removed and post-fixed in the same solution for 12 hours, cryoprotected in 30% sucrose and sectioned on a freezing microtome (Leica, Wetzlar, Germany) (50 µm thick for E16 and 30 µm for P0-adult). They were then processed for the immunocytochemical detection of OMgp following an immunoperoxidase protocol. Briefly, free-floating sections from different developmental stages were processed in parallel. Free-floating sections were rinsed in 0.1 M PBS and endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 and 10% methanol dissolved in 0.1 M PBS. After extensive rinsing, sections were incubated in 0.1 M PBS containing 0.2% gelatin, 10% normal goat serum, 0.2% glycine and 0.2% Triton-X 100 for 1 hour at room temperature. Afterwards, sections were incubated for 36 hours at 4° C with the primary antibody. Thereafter, sections were incubated with secondary biotinylated antibodies (2 hours, 1:200 diluted) and Streptavidin-Horseradish peroxidase complex (2 hours, 1:400 diluted). Peroxidase activity was revealed with 0.025% diaminobenzidine (DAB) and 0.003% hydrogen peroxide. After rinsing, sections were mounted onto slides, dehydrated, and coverslipped with Eukitt™ (Merck,

Darmstadt, Germany). In embryonic stages, the peroxidase activity was developed following the intensification method of Hancock (Hancock MB, 1986), using DAB-nickel ammonium sulfate as chromogen. Immunocytochemical controls, including omission of the primary antibody or its replacement by normal serum, prevented immunostaining.

To characterize OMgp expression in adult hippocampal interneurons, additional sections from p21-adult brains were processed for double immunofluorescence detection of OMgp and several markers of local circuit neurons such as Calbindin, Parvalbumin, and Calretinin; or neuropeptides (CCK and SOM) by using Alexa-Fluor 488 and Alexa-Fluor 568 tagged secondary antibodies (Molecular Probes, Eugene, OR). Sections were mounted on Fluoromount (Vector Labs, Burlingame, CA) and analyzed on an Olympus Fluoview SV 500 confocal microscope. All images were obtained in sequential scanning laser mode to avoid fluorochrome cross-excitation.

To determine differences between *omgp -/-* and *omgp +/+* cortical barrel-fields, coronal sections of P7 pups were processed in parallel in blind experiments. After genotype identification by Western blotting the parietal cortex was photodocumented using an Olympus BX61 microscope equipped with cooled digital DP72L camera. Pictures were densitometricaly analyzed using the Image-J software (NIH, USA). Brightness and contrast were calibrated in each picture using a pseudo-color lookup table (Rainbow RGB LUT) settled between 108 (background) and 248 (maximum) gray scale values.

Western blotting techniques

Mice were anesthetized, their brains were dissected out and the telencephalic portion was homogenized on ice in homogenization buffer containing 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, and 1x protease inhibitor cocktail. The homogenate was clarified by

centrifugation at 13,000 *g* for 15 min, and the protein content of soluble fractions was determined using the Bio-Rad detergent-compatible assay. Tissue extracts (30 µg) were boiled in Laemmli sample buffer at 100º C for 10 min, followed by 8% SDS-PAGE, and electro-transferred to nitrocellulose membranes (Amersham Biosciences, England, UK). Following transfer, membranes were incubated overnight at 4º C with α-OMgp antibody, α-Nogo-A or α-MBP and α-Tubulin to ensure equal amounts of protein in all samples. Membranes were subsequently incubated with peroxidase-tagged secondary antibodies (α-IgG raised in goat, rabbit or mouse respectively, Dako) and peroxidase activity was visualized using the ECL-plus kit (Amersham Biosciences). Cell extracts from OMgp transfected EBNA-293T cells were used as an internal control.

Primary neuronal cultures and immunocytochemical methods

E16 mouse brains were dissected in PBS containing 0.6% glucose and the hippocampus was dissected out. After gentle trypsinization, tissue pieces were dissociated by gentle sweeping. Cells were then counted and seeded onto poly-D-lysine-coated coverslips in Neurobasal medium containing B27 supplement (GIBCO Life Technologies). Cells were cultured for 7 days. Coverslips were fixed in 2% buffered paraformaldehyde, permeabilized with Triton X-100 in PBS 0.1 M and blocked with 10% normal serum in PBS 0.1 M. Cells were sequentially incubated overnight with primary antibodies at 4º C and then with Alexa Fluor-tagged secondary antibodies for 2 hours. After rinsing in PBS, cells were stained with Bisbenzimide (Hoescht 32444, 1 µM in PBS 0.1 M, for 10 min), rinsed, mounted on Fluoromount (Vector Labs) and analyzed with a confocal microscope (TCS SPII, Leica). To determine whether hippocampal neurons express OMgp, we incubated the cultures with α-OMgp and the neuronal marker α-MAP2 antibodies. To study the co-localization of OMgp with presynaptic markers, we labeled the cultures with α-OMgp and α-Synapsin antibodies.

Synaptosome subfractionation

Adult mouse forebrains were homogenized in 30 ml of Sol. A buffer (320 mM sucrose, 5 mM Na-HEPES/HCl, pH 7.4) with ten strokes at 600 r.p.m in a glass-Teflon homogenizer. The homogenate was centrifuged (5,000 r.p.m., SS34 rotor, for 5 min at 4º C). The resulting postnuclear supernatant was centrifuged twice at 11,000 r.p.m. (SS34, for 12 min at 4º C) and the crude synaptosomal fraction was resuspended in 4-8 ml of Sol. A buffer. This sample was layered on top of a discontinuous Ficoll gradient of 12% - 9% - 5%. After centrifugation for 35 min. at 22,500 r.p.m. in an SW28 rotor (Beckman Coulter Inc., Fullerton, CA) the synaptosomes were collected at the 5% - 9% and 9% - 12% interphases and resuspended in 15 ml of Sol. B buffer (10 mM glucose, 5 mM KCl, 140 mM NaCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4 and 20 mM Hepes/NaOH, pH 7.4). After centrifugation for 12 min at 11,000 r.p.m. the pellet was resuspended in 300 µl of Sol. B buffer and 2.7 ml of H_2O. This sample was layered on top of a discontinuous sucrose gradient (0.4 M, 0.6 M, 0.8 M, 1.0 M, 1.2 M, 1.4 M, 1.6 M, and 1.8 M). Gradients were centrifuged for 3 hours at 33,000 r.p.m. in an SW41 rotor (Beckman) and were collected in 0.5 ml fractions. The purity of the fractions was assessed with membrane markers by immunoblotting with α-Syntaxin 1, α-Synaptophysin and α-SNAP-25 antibodies.

RESULTS

Characterization of α-OMgp antibody

In this study we used a commercial α-OMgp antibody from R&D Systems. This antibody was produced in goat immunized with mouse OMgp. However, to further characterize the specificity of the α-OMgp antibody we transfected EBNA-293T cells with plasmid encoding mouse OMgp. Immunoblot analysis using the α-OMgp antibody (Fig.1A) exclusively detected a band of

approximately 120-130 kD in mouse brain extracts, myelin extracts (see material and methods for details) and lysates of OMgp-transfected EBNA-293T cells. Labeling was absent in mock transfected cells. It has been described that OMgp carries the HNK-1 epitope which is also present in other proteins such us NCAM or MAG (Mikol DD, JR Gulcher et al., 1990). To further corroborate that the OMgp antibody used in the present study does not recognize the HNK-1 carbohydrate, adult brain extracts were immunobloted using the OMgp antibody and the HNK1 antibody (clone VC1.1, Sigma) (Fig. 1B). The HNK-1(VC1.1) recognizes the HNK-1 epitope in several brain proteins such us NCAM, MAG and some chondroitin sulfate proteoglycans of different molecular weight. HNK-1(VC1.1) immunoblots renders a strong smear labeling at > 180-200 kD and an additional labeling of several bands of less than 150 kD which were not recognized by the OMgp antibody (Fig. 1B). In addition, to further corroborate these data in tissue sections, coronal brain sections from the same animal were immunostained using the HNK-1(412) and OMgp antibodies (Fig. 1C-D). The pattern of staining was completely different. Specific areas of the telencephalon (e.g., hippocampal fimbria, the anterior commissure or globus pallidus) were HNK-1(412)-positive. In contrast, although cortical layer IV was labeled with OMgp, did not show HNK-1(412) labeling (Fig. 1 C-D). Lastly, the OMgp antibody did not label any band in *omgp -/-* derived protein extracts (see below) nor in *omgp -/-* brain sections (not shown). We conclude that the goat α-OMgp used in the present study only recognized OMgp.

Developmental expression of OMgp during brain development

To determine the expression levels of OMgp during development, we first performed a Western blot analysis of protein extracts from developing telecephalon (Fig. 2). The results were compared with the developmental expression of Nogo-A and the Myelin Basic Protein (MBP) in parallel immunoblots. Immunoblot analysis using the rabbit α-Nogo-A antibody detected a band

of approximately 200-210 kD that decrease from E16 to adult stages (Fig. 2). In parallel immunoblots, a pale band of OMgp was first seen at E16. OMgp levels increased from P0 onwards reaching maximum levels in the adult. Similar postnatal results were reported by Vourc'h et al., using semi-quantitative RT-PCR (Vourc'h P et al., 2003). In contrast, MBP, a marker of the myelination, was detected only from P10 onwards in brain extracts. In a recent study (Mingorance A et al., 2005), we described that the first relevant expression of the MAG, a marker of myelinating oligodendrocytes, appeared at P8-P10 in the cortical white matter which correlates with MBP expression revealed by immunoblotting. Taken together, our data indicate that OMgp is expressed at embryonic stages long before the onset of the brain myelination, which suggests that OMgp may play additional roles during perinatal development as reported for other MAIPs (e.g., NogoA, (Mingorance-Le Meur A *et al.*, 2007; Mingorance A, E Soriano-Garcia et al., 2004; Montani L, B Gerrits, P Gehrig, L Dimou et al., 2009)).

Next, we aimed to corroborate these data by analyzing the protein expression pattern of OMgp during telencephalic development in brain sections, from E14 until adult stages (Fig. 3). Coronal or longitudinal sections from developmental series were immunohistochemicaly processed. The antibody mainly labeled neurons, although strong staining was also observed at late postnatal stages in white matter tracks, and labeled oligodendrocytes were identified in longitudinal spinal cord sections with similar morphologies and localization to those reported in oligodendroglial-like cells in paranodal sections (Huang JK *et al.*, 2005) (Fig. 3M, see also Fig. 2 of Huang et al., for details).

At E14, OMgp immunoreactivity was almost absent from the mouse telencephalon (Fig. 3A) except for mammillotegmental and mammillothalamic tracts and raphe dorsalis and dorsal thalamus nuclei (data not shown). At E16, OMgp labeling was prominent in the pyriform/entorhinal region (Fig. 3B), and after nickel-intensified DAB intensification, projecting

neurons in layer II-III were seen over the intense neuropil which expands to layer I and lower layers of the pyriform/entorhinal cortex. In addition, pale neuropil staining was seen in the *stratum lacunosum-moleculare* of the developing hippocampus at these stages. At P0, strong immunoreactivity was also observed in the amygdaloid complex and several hypothalamic nuclei (Fig. 3C-D). At P5, puncta-like immunocytochemical staining was observed in all neocortical areas with higher levels in lateral than medial cortical regions. Particularly, pale OMgp staining was observed in all cortical layers in contrast to layer IV where, in the parietal cortex, immunostaining elements were grouped in clusters corresponding to barrels while septa were clearly defined (Fig. 3D, G). In addition, Purkinje cells and axonal tracts of the cerebellum were also stained at P5 (not shown). At P8-P10, the barrel-field was clearly identifiable and increasing OMgp labeling was observed in subgranular cortical layers in the parietal cortex as well as in the formerly less immunoreactive regions of the cortex, striatum and dorsal thalamic nuclei (Fig. 3E, H-I). From P15-onwards the labeling of the cortical barrel-field was diluted with the intense OMgp immunoreactivity in the telencephalon (Fig. 3F, J). Particularly in the hippocampus, at P5 but especially at P8-P10, the *stratum lacunosum-moleculare* and the molecular layer of the dentate gyrus and the *stratum oriens* displayed strong OMgp immunoreactivity (Fig. 3E, K). From P15 to P21, the staining of the *stratum lacunosum-moleculare* gradually decreased to an intensity that was similar to that in the *stratum radiatum* but lower than in the *stratum oriens*. In the cerebellum, as indicated above, Purkinje cells were stained from P5 onwards (Fig. 3L).

OMgp expression by adult hippocampal interneurons of the CA1 region

Habib and co-workers reported OMgp in local and projection neurons (Habib AA *et al.*, 1998). In addition, in the Allen Brain Atlas, OMgp mRNA is expressed in neurons throughout the CNS. These data were also confirmed by Hunt and co-workers (Hunt et al., 2002) and recently by

Koyama *et al*., (Koyama Y *et al.*, 2008). In previous studies we analyzed the developmental expression of myelin associated proteins and receptors in the entorhino-hippocampal system in mouse, rat and human (Gil V *et al.*, 2006; Llorens F *et al.*, 2008; Mingorance A, X Fontana et al., 2004; Mingorance A *et al.*, 2005). Thus, to analyze the pattern of labeling of OMgp in the adult hippocampus, we processed horizontal and coronal sections of the adult hippocampal formation (Fig. 4). We found that OMgp was expressed by numerous interneurons mainly in the CA1 region (Fig. 4A). OMgp staining in hippocampal neurons delineated the complete neuron including dendrite and axon. Cell labeling was intense and several neuronal morphologies, ranking from multipolar to bipolar shapes, were observed scattered in plexiform layers (Fig. 4A-B). Double immunohistochemical labeling of OMgp and markers of local circuit neurons illustrated OMgp immunoreactivity in non-pyramidal cells expressing calcium-binding proteins (Parvalbumin-, Calretinin- and Calbindin-positive) (see Fig. 4C-E, for examples of double labeled Parvalbumin-OMgp interneurons) as well as some neuropeptides (CCK or SOM) (Fig. 4F-K). Although we were unable to establish a clear and specific co-localization of OMgp with particular subsets of hippocampal interneurons, the first appearance of OMgp staining in hippocampal interneurons coincided with the first appearance of inhibitory potentials in the hippocampus (see discussion).

OMgp co-localizes with presynaptic axonal markers *in vitro* and with presynaptic proteins in synaptosomal fractions *in vivo*

As indicated, OMgp is present during perinatal development in neocortical layer IV in puncta-like staining as well as in developmental axonal tracts. Thus, we next examined the putative presence of OMgp at the synapse (Fig. 5). First, OMgp localization was studied in primary hippocampal cultures after 7 days *in vitro* (Fig. 5A-H). In cultured neurons, OMgp completely labeled MAP2-positive hippocampal neurons (Fig. 5A-C) but was also present in axonal-like

varicosities close to neurites or the perikaryon of other cultured neurons. Further, double immunohistochemical studies showed that OMgp co-localized in axonal varicosities with presynaptic proteins such as Synapsin (Fig. 5D-H). To further confirm that OMgp was present in the presynaptic terminals, we analyzed the distribution of well-known presynaptic markers (Syntaxin 1 and SNAP-25) in adult sucrose-fractioned brain synaptosomes and we compared their distribution with OMgp by Western blotting. Fractionation of synaptosomal preparation showed that OMgp was present in the membrane and vesicular fractions (F11-F17), sharing distribution with synaptic markers but not with the cytosolic or mitochondrial fractions (F3-F9, F19) (Fig. 5I). Taken together, the data suggest that OMgp is localized in axons and synaptosomes in developing and adult neurons.

Altered thalamo-cortical targeting and barrel-field development in omgp -/- mice

As indicated, OMgp is present in developing axonal tracts. To further determine the role of OMgp in the development of the cortical barrel-field, we analyzed the distribution of 5-HTT immunoreactivity in coronal brain sections from 5 *omgp* -/- and 6 wild-type mice at P7 (Fig. 6). The experiments were conducted blind, with no knowledge of the genotype of the brain being processed and all free-floating sections were bulk processed during the immunolabeling. After the experiment and data acquisition, the genotype of each mouse was determined by immunoblotting (Fig. 6A). First, as indicated, the *omgp* gene is located within intron 27b of the mouse *NF1* gene, which encodes to Neurofibromin (Mikol DD, MJ Alexakos *et al.*, 1990). NF1-deficient mice display deficits in development of the somatosensory barrel-field (Lush ME *et al.*, 2008). Thus, we aimed to determine whether the pattern of NF1 expression is altered in *omgp -/-* mice compared to wild-type mice at the postnatal stages of barrel-field formation. After immunostaining, *omgp -/-* and wild-type mice showed similar patterns of immunostaining in the neocortex (Fig. 6B-C) and hippocampus (Suppl. Fig. 1). In addition, cortical layering was

maintained in adult *omgp -/-* mice compared to wild-type (Suppl. Fig. 2). Next, we determined that the thalamo-cortical connection is formed in *omgp -/-* mice. However, our results revealed that the distribution of the 5-HTT immunostaining in the barrel-field showed clear differences in *omgp -/-* compared to controls in neocortex. In *omgp -/-* mice, barrels were less defined in the first parietal cortex with numerous 5-HTT positive axons invading ectopically layer II-III (Fig. 6E). All the processed mutant mice showed these alterations. In Figure 6 we show the densitometric analysis in one of the analyzed mice and its parallel control littermate (Fig. 6F-G). In conclusion, *omgp -/-* mice showed altered distribution of thalamo-cortical axons in cortical layer IV, which indicates that OMgp is required to restrict correct thalamo-cortical axon targeting in the developing cortical barrel-field.

DISCUSSION

Neuronal OMgp expression during telencephalic development.

To date most studies have analyzed the pattern of OMgp expression during postnatal development (Habib AA *et al.*, 1998; Vourc'h P *et al.*, 2003) or in adult stages (Funahashi S et al., 2008; Hunt D, MR Mason et al., 2002; Lee JK *et al.*, 2009). Some studies reported that OMgp is expressed by oligodendrocytes (Funahashi S *et al.*, 2008), while others indicate a neuronal expression (Habib AA *et al.*, 1998; Hunt D, RS Coffin *et al.*, 2002; Koyama Y *et al.*, 2008; Lee JK *et al.*, 2009). These discrepancies in OMgp expression are very similar to those observed few years ago with the oligodendroglial and neuronal Nogo-A expression (see (Mingorance A, X Fontana *et al.*, 2004) for details). From a technical point of view, most authors used OMgp immunostaining since mRNA localization in oligodendrocytes is difficult and tissue treatments may underestimate the amount of mRNA in neurons (M. Schwab, personal communication, see also (Huber AB et al., 2002) for details). However, the available evidence indicates that OMgp is a neuronal protein which is also expressed by oligodendrocytes in healthy (Hunt D, RS Coffin *et al.*, 2002) or damaged CNS (Guo Q et al., 2007), as well as in cultured oligodendrocytes (Habib AA *et al.*, 1998). Interestingly, OMgp was found in the nodes of Ranvier, a non-myelinated axon region (Apostolski S *et al.*, 1994; Huang JK *et al.*, 2005; Nie DY *et al.*, 2006). Huang et al., reported that OMgp was not localized in compact myelin, but in oligodendroglial-like cells, whose processes converge to form a ring that completely encircles the nodes (Huang JK *et al.*, 2005).

Our results indicate that the goat α-OMgp antibody recognized endogenous and recombinant OMgp protein specifically. OMgp is present along non-myelinated axonal tracts during telencephalic development and expressed in cultured MAP2-positive hippocampal neurons and

adult hippocampal interneurons *in vivo*. Taken together, our results reinforce the notion that OMgp is expressed in neurons and oligodendrocytes. However, we did not observe OMgp-positive oligodendrocytes in the telencephalic regions due to the relevant neuropil staining of the sections from the second postnatal week onwards. However, in transversal sections of spinal cord a similar staining to those presented by Huang and coworkers was observed.

Early expression of OMgp during cortical development. A role in axon target specification?

We have determined that OMgp expression begins early in embryonic development long before the onset of brain myelination. This suggests that OMgp has additional roles other than the formation of the myelin sheath (Nie DY *et al.*, 2006) or preventing axon regrowth after injury (Ji B et al., 2008). OMgp immunostaining in developing neocortex follows the targeting of the thalamo-cortical projection in the layer IV in mice (Rebsam A et al., 2002, 2005; Rice FL and H Van der Loos, 1977). As described, the early cortical barrel in mice appears as a patch around P4 and septa become noticeable at P6 (Rice FL and H Van der Loos, 1977). Intrinsic cortical connections in the developing somatosensory barrel-field are detected from the first postnatal week after barrel formation (P8-P10) coinciding with the first appearance of spontaneous inhibitory potentials in middle cortical layers (Luhmann HJ and DA Prince, 1991). In our study, OMgp labeling in layer IV appeared during the first stage of barrel development (P4-P5).This suggests that OMgp plays an early role on the fine tuning of the thalamo-cortical axons in the developing cortex. This was corroborated by analyzing the parietal barrel-field in *omgp* -/- mice, which displays ectopic 5-HTT labeling in layer II-III. A disrupted barrel-field pattern was also reported in *nf1* -/- mice (Lush ME *et al.*, 2008) as well in *trkb* -/- mice or MAOA-trkB double knockout (Vitalis T et al., 2002). The *omgp* -/- mice used in the present study showed a normal pattern of NF1 protein compared to wild-type mice. Thus it is unlikely that NF1 is involved in

producing the present results. However, the phenotypes of the NF1-deficient mice and the OMgp knockout are different. As indicated by Lush and co-workers, NF1 knockout mice showed profound differences in cortical layer IV since patterning of cortical cells into barrels was strongly reduced compared to wild-type mice (Lush ME *et al.*, 2008). In contrast, the OMgp-deficient mice showed no apparent differences in the barrel formation and cortical layering (see Supl. Fig 2). The deficits observed in the NF1-deficient mice in the thalamo-cortical connection are stronger than those observed in the *omgp* -/- mice. Due to the particular location of the OMgp gene into the NF1 locus (see above) we cannot rule out an additional effect of the OMgp absence in the NF1 phenotype. However, *OMgp* expression was not determined in NF1 mice (Prof. Luis Parada, personal communication).

On the other hand, it has recently been reported that BDNF, the high-affinity ligand of trkB receptor, which plays key roles during cortical development (see e.g., (Alcantara S et al., 2006)), stimulates the phosphorylation of NgR1 by Casein kinase II, suppressing Nogo-dependent inhibition of neurite outgrowth in neuroblastoma-derived neural cells (Takei Y, 2009). Thus, the absence of TrkB may have a direct effect on NgR1-mediated axon inhibition and plasticity. It is no clear, whether OMgp expression is modulated by BDNF through TrkB receptor.

OMgp is located at the neuronal membrane (Habib AA *et al.*, 1998) and carries the HNK-1 epitope (Mikol et al., 1990), which is also present in well-characterized neural adhesion molecules such as NCAM, L1 or Tenascin R (see (Vourc'h and Andres, 2004), (Yamamoto S et al., 2002) or (Schachner M et al., 1995) for review). Although our OMgp antibody does not recognized HNK-1, it has been reported that similar CA1 adult hippocampal interneurons labeled with OMgp are HNK-1-positive. The HNK-1 epitope is involved in synaptic plasticity and neuronal physiology both during development and in adulthood (Schachner M *et al.*, 1995;

Yamamoto S *et al.*, 2002). Thus, a putative function of OMgp in neuronal physiology cannot be ruled out. On the other hand, a putative role of OMgp as an adhesion molecule during axonal development cannot be also discarded out either, even if we take into account the modifications of the distribution of 5-HTT axons in the *omgp -/-* mice. Furthemore, the absence of other neuronal MAIPs during development *in vitro* leads to increased neurite length and growth cone motility (Mingorance-Le Meur A *et al.*, 2007; Montani L, B Gerrits, P Gehrig, L Dimou *et al.*, 2009). Although not considered in the present study, we cannot discard a putative function of OMgp modulating cytoskeleton dynamics and neurite length.

To our knowledge, this is the first description of a putative function of MAIPs in barrel-field formation, and together with other studies (Martin I et al., 2009) the first step towards understanding the role of OMgp during cortical development. Although Nogo-A has been associated with neurite extension (Mingorance-Le Meur A *et al.*, 2007; Montani L, B Gerrits, P Gehrig, L Dimou *et al.*, 2009) its putative role in the development of the somatosensory barrel-field is unlikely, since cortical layering develops normally in Nogo mutant mice (McGee et al., 2005; Mingorance-Le Meur et al., 2007). In addition, Nogo-A expression levels do not change during the critical period, at least in the mouse visual cortex (P20-P26) (McGee AW et al., 2005) and other MAIPs, such as MAG, appear in the white matter of the somatosensory cortex at P5 (Mingorance A *et al.*, 2005). Indeed, numerous studies indicate that non-myelin related mechanisms may limit somatosensory barrel-field plasticity because the relevant critical period ends earlier in development (P1 to P4), before cortical myelination matures (McGee AW *et al.*, 2005). Thalamo-cortical axon targeting involves the participation of multiple lamina-specific molecules but relevantly its fine tuning via neural activity (see (Yamamoto N et al., 2007) for review). Thalamic axons grow and reach the cortex in the absence of OMgp and in the absence of other MAIPs. Moreover, myelination is absent during early barrel-field formation as indicated above, but a role of OMgp via NgR1 (expressed in layer IV neurons at these stages

(Mingorance A, X Fontana *et al.*, 2004)) or other receptors (see below) could take place. NgR1 has recently been implicated in activity-dependent synaptic strength (Lee H et al., 2008). In addition, NgR1-mediated signaling from myelin-derived proteins consolidates the neural circuitry established during experience-dependent plasticity (McGee AW *et al.*, 2005). Furthermore, a recent study described a new MAIPs receptor: PirB (Atwal JK *et al.*, 2008; Filbin MT, 2008) which has been implicated in restricting cortical plasticity in the visual cortex (Syken J et al., 2006). In this scenario, we cannot rule out the participation of OMgp together with others factors in restricting cortical plasticity.

Does OMgp play a role at the synapse?

As indicated, emerging descriptions indicate several roles for myelin protein ligands and receptors in functions very different from those reported above (for examples see (Mingorance-Le Meur et al., 2007), (Pernet V *et al.*, 2008), (Montani L, B Gerrits, P Gehrig, A Kempf et al., 2009) or (Wang YZ et al., 2006)). Nogo-A has been located at the neuronal synapse at the ultrastructural level in the postsynaptic active zone (Liu YY et al., 2003) as well as in developing axonal tracts (Mingorance-Le Meur A *et al.*, 2007; Tozaki H et al., 2002). Here we demonstrated, using biochemical and immunocytochemical methods, that OMgp is located in axonal tracts as well as in synaptosomal fractions and in axonal varicosities. Taken together, these data open up the field for a putative role of OMgp at the synapse. Whether these functions are structural or associated to neurotransmission warrants further study. Unfortunately, our antibody does not react with OMgp in post-embedding protocols so we cannot clearly define its location at the synaptic contact, as reported for Nogo-A (Liu YY *et al.*, 2003). However, its location in puncta-like structures or synaptosomal fractions, points to putative neuronal roles at the synapse, which would increase the new unexpected OMgp functions. For example, recent studies reported new functions for OMgp in controlling stem cell

physiology (Martin I *et al.*, 2009). Whether NgR1 or the recently discovered MAIPs receptor PirB or other unknown receptors mediate or participate in these new functions, including the targeting of thalamo-cortical axons, needs to be determined. In this respect, Lee and co-workers indicates that NgR1 modulates synaptic transmission by regulating FGF_2-FGFR-mediated signaling (Lee H *et al.*, 2008). It would be of interest to study whether OMgp-NgR1 or PirB interactions regulate FGF_2 roles in the developing telencephalon. In addition, several HNK-1-binding molecules located in perineural nets have been described, such as laminin, selectins, brevican or aggrecan which also contribute to corticogenesis (Domowicz MS et al., 2003; Hall H et al., 1993; Miura R et al., 2001; Needham LK and RL Schnaar, 1993). Interestingly, one of the most relevant compounds of the perineural nets is aggregan, which also showed profound alterations in expression and distributions after sensory deprivation (McRae PA et al., 2007). Further studies will help to answer these challenging questions.

FIGURE LEGENDS

Figure 1

Characterization of α-OMgp antibody.

A: Immunoblot of OMgp using the goat α-OMgp antibody in adult brain protein extracts, purified myelin and protein extracts of OMgp-transfected and Mock-transfected cells. For SDS-PAGE and Western blotting, 40 μg of adult and cell protein extracts and 15 and 30 μg of myelin extract were used.

B: Immunoblot in adult brain protein extracts using the goat α-OMgp antibody and the HNK-1(VC1.1). Blots with HNK-1 showed a pattern of staining (asterisks) different from those seen in parallel OMgp blots.

C-D: Low-power photomicrographs illustrating HNK-1 (C) and parallel OMgp-staining (C). Notice the different pattern of staining. Scale bar in C = 500 μm also pertains to D. Abbreviations: Fr, Frontal cortex; RS, Retrosplenial cortex; CA1-CA3 *cornus ammonis* 1-3; DG, dentate gyrus; F, Fimbria; Par1-2, Parietal cortex 1 and 2; CPu, Caudate putamen; GP, Globus palidus; Th, Thalamus; IC, Internal capsule; AC, anterior commissure; Hy, Hypothalamus; Pir, Pyriform cortex; Gu, Gustatory cortex.

Figure 2

Developmental expression of Nogo-A, OMgp and MBP in Western Blotting. α-OMgp antibody detected a band in brain samples with an apparent molecular weight of 120-130 kD. Membranes were reprobed with α-Tubulin antibody for protein standardization. Notice that Nogo-A expression started at E16 and continued until adult stages. A faint OMgp band can be seen at E16 long before the onset of myelination as marked by the first appearance of MBP labeling at P10.

Figure 3

Pattern of OMgp protein expression during telencephalic development.

A-F: General views of mouse brains at different stages during development: embryonic stage 14 (E14), E16, P0, P5, P10 and P21.

G-J: High magnifications of the primary somatosensory cortex of mice aged from P5 to P21. Notice the relevant staining of the barrel-field at layer IV between P5 and P10. A high magnification of an OMgp immunopositive barrel at P7 is shown in the insert.

K: High-power photomicrograph illustrating OMgp staining in P10 hippocampus.

L: OMgp staining in the cerebellum at P21. Purkinje cells are strongly labeled.

M: Transversal section of adult spinal cord labeled with anti-OMgp. An oligodendrocyte-like cell is OMgp-positive (arrow) as well as other longitudinal thin processes.

Abbreviations as in Figure 1 including: AC, amygdaloid complex; HL, hindlimb; H, hippocampus; NC, neocortex and I-VIb, cortical layers; gl, granular cell layer; h, hilus; ml, molecular layer; slm, stratum lacunosum-moleculare; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; GL, granular layer; ML, molecular layer; PCL, Purkinje cell layer; WM, white matter. Scale bars: A-F, 500 μm; G-K, 200 μm; L, 100 μm and M, 10 μm.

Figure 4

OMgp expression in the adult hippocampus

A: Low-power photomicrograph of OMgp labeling in the hippocampus.

B: High magnification of the boxed area in A, OMgp-positive cells in the CA1 region corresponded to hippocampal local circuit neurons.

C-H: Confocal microphotographs illustrating double-labeled (PARV/OMgp) interneurons in the stratum oriens (arrows in C-E); (CCK/OMgp) interneurons in the stratum radiatum (arrows in F-H). Note that some CCK-positive cells are not labeled with OMgp antibody (open arrow in F, H).

I-K: Confocal microphotographs illustrating double-labeled (SOM/OMgp) interneurons in the stratum radiatum (arrows in I-K). Abbreviations as in Figure 3. Scale bars: A, 200 μm; B, 100 μm and C-K, 25 μm.

Figure 5

OMgp expression in cultured MAP2-positive hippocampal neurons and Synapsin-labeled presynaptic terminals *in vitro*.

A-C: Confocal microphotographs illustrating hippocampal cultures (7DIV) incubated with antibodies α-OMgp and α-MAP2 to demonstrate the expression of OMgp in MAP2-positive neurons (arrows).

D-F: Parallel cultures were incubated with antibodies α-OMgp and α-Synapsin to demonstrate the presence of OMgp in axon terminals close to neuronal perikaryons (Synapsin-positive, arrows).

G-H: OMgp expression in Sinapsin-positive (arrows) axonal varicosities in cultured hippocampal neurons. Note that few OMgp-positive varicosities (arrowheads) are Synapsin negative.

I: OMgp expression in adult forebrain fractionated synaptosomes. Note that OMgp is detected in the same fractions (membrane/vesicles) which are immunoreactive for Syntaxin 1 and SNAP-25. Scale bars: (A,D,G) 25 μm pertains to (B, E); (C,F) and H, respectively.

Figure 6

OMgp immunostaining in the primary somatosensory cortex in *omgp -/-* mice.

A: Western blot corroboration of the presence of the OMgp protein in *omgp -/-* mice and wild-type controls.

B -C: Low-power photomicrographs illustrating representative sections of the parietal cortex of a wild-type (B) and *omgp -/-* (C) mouse, immunostained using the α-NF1 antibody.

D-G: Low-power photomicrographs illustrating representative sections of the somatosensory barrel-field in control (D,F) and *omgp -/-* (E,G) mice. Barrels in mutant mice (arrows in E) appeared less defined than in controls and numerous 5-HTT positive axons were seen ectopically in layer II-III (arrowheads). After application of the pseudocolor correlation the disorganization of the terminal thalamo-cortical field in the somatosensory cortex is better demonstrated. In the right, the LUT pseudocolour scale (Rainbow RGB) from the Image-J program indicating the grey scale value is shown. Abbreviations as in Figure 3. Scale bars: B and D, 100 μm pertains to C and E respectively. F-G, 100 μm.

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