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NogoR1 and PirB Signaling Stimulates Neural Stem Cell Survival and Proliferation.

Srinivas Ramasamy [1], Fenggang Yu [2], Yuan Hong Yu [1], Hariharan Srivats[1], Gavin Stewart Dawe[3], Sohail Ahmed [1, 4,*]

[1]Institute of Medical Biology, 8 Biomedical Grove, #05-37 Immunos, Singapore 138648.; [2] Department of Otolaryngology, Yong Loo Lin School of Medicine, National University of Singapore.; [3] NUS Neurobiology Programme, CELS, National University of Singapore.; [4]Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore.

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ABSTRACT

Neural stem cells (NSCs) and neural progenitors (NPs) in the mammalian neocortex give rise to the main cell types of the nervous system. The biological behavior of these NSCs and NPs is regulated by extracellular niche derived autocrine-paracrine signaling factors on a developmental timeline. Our previous reports [1, 2] have shown that chondroitin sulfate proteoglycan (CSPG) and ApolipoproteinE (ApoE) are autocrine-paracrine survival factors for NSCs. NogoA, a myelin related protein, is expressed in the cortical ventricular zones where NSCs reside. However, the functional role of Nogo signaling proteins in NSC behavior is not completely understood. In this study, we show that NogoA receptors, NogoR1 and PirB, are expressed in the ventricular zone where NSCs reside between E10.5-14.5 but not at E15.5. Nogo ligands stimulate NSC survival and proliferation in a dosage dependent manner *in vitro*. NogoR1 and PirB, are low and high affinity Nogo receptors respectively, and are responsible for the effects of Nogo ligands on NSC behavior. Inhibition of autocrine-paracrine Nogo signaling blocks NSC survival and proliferation. In NSCs, NogoR1 functions through Rho whereas PirB uses Shp1/2 signaling pathways to control NSC behavior. Taken together, this work suggests that Nogo signaling is an important pathway for survival of NSCs.

INTRODUCTION

Neuroepithelial cells, the first neural stem cells (NSCs), are a single layer of cells that line the ventricles as early as E9.5 during mouse brain development [3]. NSCs are cells that give rise to astrocytes, neurons and oligodendrocytes *in vitro* and *in vivo*. The NSCs self-renew for a number of generations, while differentiating into the lineages of the neural tissue through the intermediate of neural progenitors (NPs). In the embryonic mouse brain NSCs reside in the ventricular zone (VZ) and sub-ventricular zone (SVZ; for review see [4]). NSCs and NPs are generated all along the dorsal and ventral VZs in the cortex (CX) and ganglionic eminences (GE). There is an increase in NSC number and neurogenesis at E14.5 with the expansion of the

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Corresponding author: Sohail Ahmed, PhD, Institute of Medical Biology , 8A Biomedical Grove, #05-37 Immunos, Singapore 138648, Tel: +65 64070165, Fax: +65 64642048, Email: sohail.ahmed@imb.a-star.edu.sg; Funding: This work was funded by Agency for Science, Technology and Research, Singapore.; Funding: This work was funded by Agency for Science, Technology and Research, Singapore. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.; Received July 26, 2013; accepted for publication December 11, 2013; 1066-5099/2014/$30.00/0 doi: 10.1002/stem.1645.

VZs, after which decline in neurogenesis is observed at E17.5 postnatal birth in mammalian brain [4-7].

The behavior of NSCs is regulated by extrinsic factors present in their niche during development [8]. The NSC niche includes factors such as cystatin C, Wnts, Sonic Hedgehog (Shh) and bone morphogenetic protein (BMP) [9-12]. The exact function of these niche factors in controlling NSC behavior is not understood [13-16]. The behavior and fate of NSCs can be followed *in vitro* using the neurosphere (nsph) assay [17]. We have developed a clonal nsph formation assay that allows quantitative determination of NSC numbers. Using this clonal assay we have shown that CSPG and ApoE are autocrine-paracrine factors that stimulate NSC survival and proliferation [1, 2].

NogoA and OmGP are present in the extracellular niche of the embryonic brain VZ where NSCs reside *in vivo* [18-22]. Nogo isoforms belong to the family of reticulon (RTN) domain proteins. NogoA is primarily expressed in the central nervous system (CNS), NogoB is found in various cell types, whereas NogoC is expressed mainly in muscle. NogoA was first identified as a protein inhibiting neurite outgrowth and signals through at least two receptors, NogoR1 and PirB, to mediate axonal outgrowth inhibition [23-27]. Nogo RTN homology domain proteins contain two transmembrane regions separated by a 66 amino acid loop, Nogo66, which is able to inhibit neurite outgrowth *in vitro*. To increase solubility and ease of purification Nogo66 can be reduced to Nogo54, which still inhibits neurite outgrowth. Like NogoA, Nogo66 and 54 function through Nogo66 receptor 1 (NogoR1) and leukocyte immunoglobulin-like receptor (LILRB3), also known as PirB [24, 25, 28-32]. NogoA is also important for maturation of oligodendrocytes and neuronal fasciculation and guidance during early brain development [33-36]. Soluble ectodomains generated from the receptors NogoR1 and PirB have been shown to block Nogo66 and improve neuronal sprouting in spinal cord injury [26, 27, 37]. Recent studies have shown that NogoR1 and NogoR3 receptors enable CSPG-dependent inhibition of neuronal outgrowth, providing evidence of a shared mechanism [38]. NogoA and NogoR1 have been shown to play distinct roles in adult NSC homeostasis [39]. Nogo has also been shown to regulate Nanog expression in embryonic stem cells (ESCs). Different regions of Nogo and Nogo 66 have been shown to play a role in differentiation of neural progenitors [40-42].

In the present study, we sought to understand the function of NogoA, NogoR1 and PirB in NSC behavior. We found NogoR1 and PirB to be expressed in the VZ/SVZ regions and GE at E14.5. However, the expression of these receptors is absent in the VZ/SVZ regions at E15.5. Interestingly, picomolar (pM) and nanomolar (nM) doses of Nogo66/54 stimulated NSC survival and proliferation via PirB and NogoR1, respectively. Blocking of endogenous NogoA signaling by using a combination of NogoR1 and PirB receptor knockout (KO) mice-derived NSCs/NPs and ectodomains led to decrease in nsph size and NSC numbers. Lastly, Nogo stimulation of NSC survival via NogoR1 required Rho proteins, while PirB required Shp1/2. Thus NogoA, acting through NogoR1/ Rho and PirB-Shp1/2 signaling pathways, is an autocrine-paracrine factor that stimulates NSC survival and proliferation.

MATERIALS AND METHODS

Animals and tissue collection

The animal procedures were performed in accordance with the IACUC and NACLAR guidelines and approved by the animal department (http://www.brc.astar.edu.sg/index.php?sectionID=11). The morning of plug detection was considered as embryonic day 0.5 (E0.5) and fetuses were removed from pregnant females at respective stages of development, euthanized in CO_2 chambers and individual brains were dissected before mounting in boats with cryo-mounting media and isopentane-dryice freezing the specimen. Sections of 12-14 μM thicknesses were obtained from the brains after mounting in chucks fitted in Leica cryostat. The sections were embedded in poly-L-lysine (PLL) coated

super frost slides and dried in room temperature for a few hours before proceeding for immunohistochemistry procedures.

Nogo54 protein purification, constructs and inhibitors

Constructs pGEX-GST, pGEX-GST Nogo54 were used for protein overexpression and purification as described previously [31, 43, 44]. Plasmids that have alkaline phosphatase fusions with 'His' tag (pAP, pAP-Nogo66, pAP-NogoR1 ectodomain (NogoR1ecto), pAP-PirB ectodomain(PirBecto), Phospho Tyrosine phosphatase (PTP) σ ectodomain(PTP σ ecto) constructs (PTP δ Lys ecto control) were transfected in HEK 293T cells for overexpression and purification was performed as described previously [26, 45, 46]. Receptor associated protein inhibitor (RAP) was used to inhibit ApoE receptors in NSCs/NPs as described previously [2].

Neuroblastoma cultures and neurite outgrowth assays

Neuroblastoma (NB) cultures for N1E-115 and ND7 cell lines were maintained and used in neurite outgrowth assays as described previously [43, 47].

Isolation and culturing of nsphs

NSCs/NPs were isolated from forebrain embryonic cortex at various developmental stages (E10.5-E15.5) from wild type C57BL/6 (+/+) mice. NSCs/NPs were also isolated from NogoR1-/-, PirB-/- (both from MMRC) and PirB-/- (Tohoku University) at E14.5. The characterization of the homozygote and heterozygote mice genotypes for NogoR1 and PirB was according to the protocols from (http://www.mmrrc.org/catalog/ sds.php? mmrrc_id=30668) and [48, 49]. Dissociated cortical tissue/cells were triturated and seeded at $1x10^5$ cells/ml (bulk density), $2x10^3$ cells/ml (low density), 50 cells/ml (clonal density) in NSC growth medium [Dulbecco's Modified Eagle's Medium (DMEM)/F-12, B27 supplement, 20ng/ml EGF, 10ng/ml bFGF(Peprotech) and 1% penicillin /streptomycin (Invitrogen)] [1, 2]. Cells were grown at 37°C in a 5% CO_2 atmosphere in a humidified incubator. Nsphs were passaged every 4-7 days. We estimate that under low-density culture conditions approximately 98% of nsphs are clonal [50]. Clonal cultures were cultured at 50-100 cells/well where aggregation is not observed [2]. In some experiments, dissociated NSCs/NPs were plated at 2.5 x 10^3 cells/ml in a 1.2% w/v hydrogel solution that was made by dissolving the arginine polymer (a gift from Dr. Kurisawa, IBN, Singapore) in NSC growth medium at 37ºC as previously described [51]. In adherent cultures, dissociated cells were plated at $1x10^4$ cells/ml on 0.1% poly-L-ornithine (PLO) (Sigma) coated plates. Nsph numbers were assessed wherein NSCs/NPs were cultured for 5-7 days with recombinant Nogo54 after cleavage from GST (Nogo54), Alkaline phosphatase (AP), AP-Nogo66 (Nogo66), NogoR1ecto, PirB ecto, Human receptor-associated protein (RAP; Innovative Research), PTP σ receptor ecto, PTP δ lys ecto control. For secondary nsph formation in self-renewal assays, nsphs were collected, triturated, re-seeded at respective clonal densities and grown for another seven days. Nsph numbers are expressed as nsph formation unit (NFU) which refers to the number of nsphs formed for every 100 cells plated.

Differentiation and immunocytochemistry

Single nsphs from low or clonal density cultures were transferred to each well of a 50 well cover glass (Sigma) coated with PLO (0.01%) and laminin (10 μg/ml; Invitrogen). Nsphs were differentiated for five days in differentiation medium [DMEM/F-12, B27 supplement, 1% penicillin/ streptomycin and 0.5% FBS (Invitrogen)]. After plating cells overnight or differentiating the nsphs, the coverslips were fixed with 4% paraformaldehyde (PFA) for 20 mins and washed several times with and without permeabilization (0.1% Triton X-100) before blocking with 3% BSA. NSCs/NPs were stained with mouse IgM anti-O4 (Chemicon), mouse IgG2a anti-Tuj1 (βIII-tubulin) (Covance) and rabbit IgG anti-GFAP (1:1000; Dako). The secondary antibodies were Alexa Fluor antibodies 488 anti-mouse IgM, 594 anti-mouse IgG2a and 647 anti-rabbit IgG (Invitrogen). NSCs/NPs were also stained with rabbit anti-NogoR1 and goat anti-LLIRB (PirB) or rat anti-

LLIRB. The cells were subsequently stained with 488 anti-rabbit IgG and 488 anti-rat IgG. The cells or the flattened nsphs were counterstained with DAPI and differentiated neurons, astrocytes and oligodendrocytes were scored based on staining and morphology.

Semi quantitative RT-PCR

Total RNA was isolated from nsphs at relevant days (E10.5-E15.5) according to manufacturer's instruction (TRIZOL) and RNA columns (Qiagen) were used to purify total RNA. Total RNA was used to reverse transcribe and obtain cDNA using first super script RTIII (Life Technologies). The cDNA was diluted and used for PCR reaction using Taq polymerase (NEB) with respective primers as mentioned in Table 1.

Western blotting

NogoR1 and PirB were detected by Western blotting. NSCs/NPs were lysed with RIPA lysis buffer [150 mM NaCl, 50 mM Tris HCl pH 7.8, 0.25 mM EDTA pH 8.0, 1% sodium deoxycholate, 1% Triton x-100] and cleared lysate was used for Western blotting receptor NogoR1 or immunoprecipitation of PirB. 100-200 µg of protein was loaded on SDS-PAGE gel and probed with the following antibodies: anti-NogoR1 (1:100), anti-LLIRB3 (1:100), anti-Sox2 (1:200; Santa Cruz), anti-Nestin (1:500; Millipore), anti-ß-actin (1:1000).

Immunohistochemistry

For immunohistochemistry, primary brain slices were embedded on PLL coated slides and dried for 4-6 hours at room temperature. The sections were washed with PBS before fixation with 50% cold methanol-acetone solvent (-20^{o}C). The sections were further washed with PBS several times with and without permeabilization (PBS containing 0.1% Triton X-100) and blocked with 5% normal goat serum (NGS) and 2% BSA mixture. The sections were then incubated with primary and secondary antibodies overnight and at room temperature, respectively, before counterstaining with DAPI. Immunoreagents were diluted in PBS containing NGS and BSA. The following primary and secondary antibodies were used in the immunohistochemistry procedure: NogoA (Santa Cruz), NogoR1 (Millipore), PirB (Santa Cruz; BD), Nestin (BD), Tuj1 (Covance) (1:100-500), Alexa Fluor 568 anti-rabbit, Alexa Fluor 488 anti-mouse, Alexa Fluor 568 anti-goat, Alexa Fluor 647 anti-rabbit and Alexa Fluor 568 anti-mouse (1:500).

TUNEL and BrdU assays

NSCs/NPs were dissociated and plated in PLO/laminin-coated coverslips. The reagents/purified protein samples were added to growth medium. Cells were monitored and fixed with 4% PFA at relevant time points. The cover slips were washed with PBS extensively to remove excess fixative before permeabilization of cell membrane with Triton X-100 (0.2-0.5%). Detergent treated cell samples were washed and the TUNEL reaction was performed at 37^{o}C as per the assay to label apoptotic nuclei (Promega). BrdU assays were performed by adding BrdU to cultures in growth medium after treatment with proteins/inhibitors for 2 hours as per the manufacturer (Neomarkers). The cells were later fixed using 4% PFA, washed and permeabilized. Cells were then blocked with (NGS/BSA) reagent and incubated with anti-BrdU antibody overnight and signal was detected using secondary Alexa Fluor 488 anti-mouse (Invitrogen). The nuclei were counterstained with DAPI. The TUNEL positive or BrdU positive NSCs/NPs were scored as percentage cells positive in comparison to total DAPI nuclei using Metamorph (cell scoring) imaging tool.

Data analysis

Results are represented as mean ± SEM where n means number of experiments or samples unless otherwise stated. Statistical significance was performed using One-way ANOVA with post-hoc comparisons using Bonferroni test, p-values denoted in the figures are *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. The phase images of NB cultures were taken with the Nikon Eclipse TS100 microscope and confocal images with Olympus point scanning FV-1000 confocal microscope by sequential scanning in order to avoid crosstalk of fluorophores.

RESULTS

NogoR1 and PirB are expressed in NSCs *in vitro* and in the VZ and GE of developing forebrain

NogoA is expressed in the VZ and SVZ of developing forebrain [19]. Using RT-PCR we show that NogoA, NogoR1 and PirB transcripts are expressed in developmental stages (E10.5-E14.5) using nsphs derived from embryonic mouse brain (Figure 1A). Interestingly, NogoR1 and PirB receptor expression is not seen at E15.5. There is a distinct localization pattern of endogenous NogoR1 and PirB receptors *in vitro* in embryo (E14.5) derived NSCs/NPs (Figure 1C, E). NogoR1 seems to be present in both plasma membrane and endoplasmic reticulum (ER) like compartments whereas PirB is asymmetrically localized in the membrane in 10-15% of NSCs/NPs as shown with arrow heads (Figure 1C, E). The antibodies against NogoR1 and PirB receptors are specific because there was only background staining from NSCs, NSC lysates and cortical coronal brain slices derived from respective NogoR1 (-/-) and PirB (-/-) KO mice (Figure S1).

To determine whether NogoR1 and PirB are expressed in NSCs and NPs *in vivo* we turned to *in situ* analysis of brain slices. E14.5 brain slices were probed with DAPI and nestin antibodies and the VZ and SVZ (VZ's) localized (Figure 2). The specificity of the antibodies was shown by using tissues from NogoR1-/- and PirB-/- knockout mice (see *in situ* in Figure S1A, B and western in Figure S1 C). Nestin is an early marker for NSCs/NPs and is turned off as cells differentiate into neurons, astrocytes and oligodendrocytes. We were able to colocalize NogoR1 (Figure 2A) and PirB (Figure 2B) with nestin in the VZ and SVZ. We looked in detail at two regions of the E14.5 brain – the CX (panels e-h) and the GE (i-l). The CX and GE are adjacent regions of the brain where NSCs/NPs differentiate and neurons and axons begin to migrate. Cellular colocalization of NogoR1/PirB with nestin can be seen in the inserts to panels f, g and h (CX) and j, k, and l (GE). The data show that both NogoR1 and PirB are expressed in NSCs/NPs but are not restricted to these cells. Interestingly, NogoR1 and PirB receptors are not expressed in the apical VZ/SVZ zones at E15.5. Nestin labeling is specific to the apical VZ/SVZs of the dorsal cortex at E15.5 (Figure 3 A, B) while Tuj1 is mostly present at the basal part of the cortex and does not co-localize with nestin. NogoR1 and PirB receptors follow Tuj1 expression and not Nestin at E15.5 (Figure 3). These results show a developmental change in expression and localization pattern of NogoR1 and PirB receptors during the transition from E14.5 to E15.5, which might signify a switch in functional roles of Nogo signaling. These results clearly show that NogoR1 and PirB are expressed in areas of embryonic brain where NSCs reside and are therefore able to play a role in NSC survival and proliferation.

Nogo66/54 increases nsph number and size

Since NogoA, NogoR1 and PirB are expressed in the VZ and SVZ at E14.5, where NSCs reside, we wanted to know whether NogoA regulates NSC behavior. For these experiments, we used Nogo66 and 54 polypeptides that mimic the NogoA ligand. Previous reports have shown that Nogo66 or Nogo54 can inhibit neurite outgrowth [27, 31, 52]. To confirm whether our Nogo polypeptides were active, we added them to differentiated NB cultures where we could score for neurite length. Both Nogo54 and Nogo66 significantly reduced neurite length (Figures S2 and S3). Specific ectodomains for NogoR1 and PirB receptors have been shown to reverse Nogo66-mediated neurite outgrowth inhibition [26, 27, 37, 53]. We confirmed that NogoR1 and PirB ectodomains were able to block the effect of Nogo54 and Nogo66 on neurite length (Figures S2 and S3). Next, we investigated the effect of Nogo ligands on nsph formation. We found that Nogo66 stimulated NFU by 1.7-2 fold over a wide range of concentration (100 pM-50 nM; Figure 4A) and this was correlated with a decrease in TUNEL positive cells and an increase in BrdU positive cells (Figures 4B and C).

In order to assess the role of NogoR1 and PirB receptors in Nogo66 stimulation, we repeated the nsph formation experiment with NSCs/NPs derived from NogoR1-/- or PirB-/- mice. Nogo

ligand between10-50 nM did not affect NFU in NogoR1-/- NSCs/NPs, but at pM doses was still stimulatory (Figure 4A). In contrast, Nogo ligand at 100-250 pM did not affect NFU from PirB-/- NSCs/NPs, but higher doses in the nM range were still stimulatory (Figure 4A). Nogo66 concentrations that gave 50% stimulation (SC50) of NFUs were 35 pM for PirB and 13 nM for NogoR1 (Figure S4). These results suggest that, (i) Nogo66 stimulation of NFU requires both NogoR1 and PirB and (ii) that NogoR1 and PirB are low and high affinity receptors for Nogo66, respectively. The Nogo54 C-terminal mutation (Lys50, Glu51, Arg53 and Arg54 changed to Ala) did not have any stimulatory effect on NSCs/NPs (data not shown; [31, 52]). The stimulation of NFU by Nogo66 and Nogo54 was found at both clonal (100 cells/ml) and low densities (2000 cells/well). Furthermore, similar results with Nogo66 and Nogo54 were obtained when the nsph formation assay was carried out in hydrogel (Figure S5A). The full-length NogoA (mouse and human) positive lysates increases nsph number in comparison to empty vector suggesting that the effect of Nogo on NSCs/NPs is not restricted to Nogo54/66 (Figure S5C). It is not an artificial effect produced by Nogo-66 since NogoA lysates have the same effect. The sphere size was analyzed as a measure of proliferation in clonal hydrogel experiments. There was 1.61 (50-100 μm) and 3.33 (>100 μm) folds increase in size compared to that of GST/AP controls (Figure S5B). Taken together, these results suggest that NogoA is important for NSC/NPs survival and proliferation.

Nogo54/66 increases NSC numbers

To determine whether Nogo ligand could affect NSC numbers we quantified the number multipotent nsphs generated (in clonal assays) in the presence and absence of different concentrations of Nogo66 (Figure 5). The NSC numbers are increased approximately 4-5 fold by the addition of Nogo66 at pM or nM concentrations (Figure 5H). Next we carried out nsph formation assays to see if Nogo66 influences self-renewal. In the absence of Nogo66 the stimulation of NFU was lost demonstrating the importance of Nogo signaling in self-renewal (Figure 5G).

Autocrine-paracrine nature of Nogo signaling on NSC survival

In order to investigate the autocrine-paracrine nature of Nogo signaling we blocked NogoR1 and PirB receptors and measured nsph size and NSC frequency in the absence of added ligand. Ectodomains for NogoR1 or PirB have been shown to functionally block these receptors in neurite outgrowth experiments (Figure S2 and S3). The ectodomains were added to NogoR1-/- and PirB-/- NSC/NP cultures and nsph size and NSC number measured. NogoR1 and PirB ecto are able to reduce nsph formation substantially in comparison to AP control (Figure 6A and B). The decrease in nsph number was associated with significant decrease in nsph size (Figure 6A-D). NSC numbers are also reduced by approximately 3.5-4 fold, when both PirB and NogoR1 receptors are blocked (Figure 6E and F). The decrease in nsph number and size in ectodomain assays was correlated to an increase in TUNEL positive nuclei and decrease in BrdU positive cells (Figures S6A, B). Thus Nogo is an autocrine-paracrine survival factor for NSCs.

Nsphs obtained from experiments where Nogo66 showed effect on NSC/NP survival, showed an increase in NSC and astro-glial NP frequency when differentiated. There was a 2.5-3 fold increase in the astro-glial lineage at both pM and nM doses of Nogo66 in comparison to AP control (Figure 5E, F). Moreover, autocrine/paracrine inhibition of Nogo signaling by ectodomains showed 3-4 fold reduction in number of astro-glial positive NPs (Figure 6E). These results suggest a role for Nogo signaling in promoting the generation of astro-glial NPs. Further work needs to be done to understand the precise role of Nogo signaling in the glial lineage. Lastly, Nogo did not affect or protect against general cell toxicity induced by Lipopolysaccharide, Acrylamide or Dicoflenac (Table 2) suggesting that the Nogo stimulation of NSC survival was specific.

Nogo, CSPG and ApoE and NSC survival

CSPG and ApoE found in E14.5 NSC/NP conditioned medium (CM) stimulate NSC proliferation and survival [1, 2] In order to study the role of Nogo, CSPG, and ApoE in NSC/NP survival we used antagonists to block signaling and measured nsph formation. To PirB-/- NSCs/NPs we added NogoR1ecto to block NogoR1, PTP σ receptor ecto to block CSPG, and receptor associated protein inhibitor (RAP) to block ApoE [2, 27, 45]. Inhibition of NogoR1 and CSPG in the absence of PirB receptor reduced nsph formation significantly in comparison to control (Figure S7). However, addition of RAP inhibitor in these experiments did not further reduce nsph number. These results suggest that basal signaling maintains nsph formation even when Nogo, CSPG and ApoE signaling are simultaneously inhibited.

NogoR1 and PirB signal NSC survival through distinct pathways.

NogoR1 receptor functions with cytoplasmic mediator Rho towards axonal plasticity and regeneration. We used signaling inhibitors to investigate the proteins involved downstream of NogoR1 and PirB receptors. The IC50 values for inhibitors obtained with nsph formation assays in the absence of Nogo66 ligand were used as a reference. The rationale for these signaling pathway experiments is as follows; we compare the dose response curves and IC50 values under three conditions in the presence of inhibitor. (i) with AP control, (ii) with pM Nogo66 (PirB) and (iii) with nM Nogo66 (NogoR1). If the Nogo66 curves/IC50 values follow AP controls, it suggests that the inhibitors have blocked the stimulation caused by Nogo66. In contrast if the dose response curves are shifted to the right and IC50 values higher, it suggests that the inhibitors are not blocking and the pathway is not involved. We used a similar strategy to investigate signaling pathways of NSC survival factors CSPG and ApoE [1, 2]. Inhibitor IC50 values were then measured in the presence of Nogo66 at pM or nM concentration; a decrease in IC50 suggested the protein was involved in Nogo signaling while an increase in IC50 suggested it was not.

PKC has also been shown to be important for Nogo signaling, and inhibition of PKC signaling using Go 6796 reduced IC50 at both nM and pM concentrations of Nogo66 (Figure 7A). The reference IC50 for Go 6796 was 3.83±0.07 and this value was reduced to 1.58±0.04 at nM Nogo66 and 1.49±0.11 at pM Nogo66 treatments. Rho and Rho kinase inhibitors, Y27632 and C3, reduced IC50 at nM but not at pM (Figure 7 B, C). The changes in IC50 from reference for Y27632 and C3 were 5.87±1.7 μM and 0.7±0.09 μg/ml, and reduced to 1.34±1.2 μM and 0.29±0.05 μg/ml with nM Nogo66, respectively.

Shp1/2 inhibitors, NSC878777 and PHPS1, reduced IC50 at pM Nogo66 but not at nM. The NSC 87877 reference IC50 value was 15±0.09 μM and decreased to 10±0.06 μM with pM Nogo66. With PHPS1 the reference IC50 value was 20±0.9 μM and decreased to 11±0.3μM with pM Nogo66. Taken together, these data suggest NogoR1 signals through Rho and Rho kinase (Figures 7 B, C) while PirB signals through Shp1/2 protein (Figures 7 D, E).

DISCUSSION

NogoA proteins were first identified as inhibitors of neurite regeneration[23]. Subsequent studies have revealed a role for NogoA in neuronal and oligodendrocyte differentiation, neuronal pruning and plasticity [18, 19, 36, 54] and induction of neural cell types in the early developing brain during neural plate formation [55]. NogoA is specifically expressed in the VZ/SVZ layers of mammalian embryonic brain [19]. However, the function of Nogo signaling in embryonic NSC behavior is not known. Here we have investigated the functional role for Nogo signaling in NSC/NP behavior.

Expression of NogoA receptors in NSCs

NogoA and NogoR1 transcripts are localized to human brain VZs and post-mitotic cells of the cortical plate by *in situ* hybridization [56, 57]. We find that NogoR1 and PirB are expressed between E10.5-E14.5 in NSCs/NPs *in vitro* but not at E15.5. *In vivo*, at E14.5, NogoR1 and PirB were found to be expressed in both VZ and SVZ

layers where NSCs reside. The presence of NogoA, NogoR1 and PirB in the VZ/SVZ zone suggests potential roles for Nogo signaling in NSC behavior and neurogenesis *in vivo* [19]. Moreover, Nogo receptors are also expressed in the GE. The expression of Nogo receptors in the ventral regions (GE) of the embryonic brain suggests roles in migrating neuronal and glial progenitor populations responsible for cortical expansion. By E15.5, both NogoR1 and PirB are found only in the non VZ/SVZ basal cortex, suggesting an interesting switch in Nogo function from E14.5. The regulation of Nogo receptors at E15.5 could be addressed by conditional gain of function studies in NSCs. Thus, expression pattern studies reveal important changes in expression and localization pattern of Nogo signaling receptors in the cortical neuroepithelium during early cortical expansion of the embryonic brain.

NogoR1 and PirB receptors are involved in NSC survival and proliferation.

To investigate the role of NogoR1 and PirB in NSC function we used Nogo ligands 66 and 54 and cell lysates expressing full-length NogoA. We found that over a wide range of concentrations, Nogo54/66 ligands stimulated NSC/NP survival, proliferation, potency and frequency. Using NSCs/NPs derived from KO mice, we were able to demonstrate that NogoR1 and PirB receptors sense nM and pM quantities of Nogo66 ligand. Thus, NogoR1 and PirB are low and high affinity Nogo receptors, respectively, involved in NSC behavior. Further work will be required to characterize the exact ligand receptor binding characteristics (Kd) of NogoR1 and PirB in NSCs. The high affinity of Nogo66- PirB activity in NSCs/NPs *in vitro* does not have its counterpart in Nogo inhibition neurite outgrowth inhibition. The presence of both pM and nM range of affinities suggest a wide range of sensitivity in NogoR1 and PirB receptor biological function *in vitro* in NSCs/NPs. It would be interesting to know whether gradient expression of NogoA at early development of brain dictates the affinity characteristics of NogoR1 and PirB receptors function *in vivo.* The NSC/NP assays have been studied under clonal conditions as a stringent requirement to report NSC activity distinct from NP activity *in vitro* [1, 2].

The increase in bipotential astro-glial positive nsphs (NPs) after differentiation at both pM and nM stimulatory doses suggests potential roles of Nogo signaling in promoting differentiation into the glial lineage. Interestingly, selection of NSCs/NPs positive for NogoR1 and PirB receptors by FACS analysis show a bias towards nsphs differentiating into oligodendrocytes (data not shown). Further studies are necessary to identify how Nogo signaling pathways may promote oligodendrocyte differentiation *in vitro* and *in vivo*.

NSC niche and autocrine-paracrine Nogo signaling in NSC survival

The function of the niche and its associated autocrine-paracrine factors, extracellular matrix (ECM) and membrane bound molecules, is important for survival and regulation of both embryonic and adult NSCs [58-61]. Our studies identified a number of proteins, e.g. DSD1-proteoglycan and ApoE, from CM of NSCs/NPs suggesting novel functions for myelin-related and lipid transporting proteins in NSC biology [1, 2, 62-64]. In an autocrine-paracrine context, the ectodomains of NogoR1 and PirB receptors have been used to antagonize Nogo ligand, in spinal cord injury [24, 26, 53, 65]. To test the role of autocrine-paracrine signaling for NogoA in NSC behavior, we used combinations of NogoR1 or PirB KO NSCs/NPs and ectodomains to inhibit the wild type receptor; NogoR1ecto with PirB KO cells and PirBecto with NogoR1 KO cells. When both NogoR1 and PirB receptors were blocked there was a significant reduction in NSC/NP survival, proliferation and NSC frequency. This is good evidence for NogoA as an autocrine paracrine factor and further supports the idea that NogoR1 and PirB are individually redundant in NSC survival.

We also assessed the roles of blocking ApoE signaling (using RAP) and CSPG receptor ectodomain PTP σ ecto in embryonic NSCs/NPs derived from PirB KO mice. We found that RAP and the PTP σ ecto did not reduce NSC/NP

survival further. These results indicate that EGF/FGF growth factors provide a basal survival of NSCs/NPs and that additional protein factors are stimulatory but not essential. However, we cannot rule out that additional as yet unidentified proteins support basal NSC/NP survival in addition to EGF/FGF. Mice that lack NogoA do not have any obvious NSC survival problems but have defective migration of BrdU positive neuronal precursor cells at E17.5 [22]. This suggests that more ligands or extracellular proteins have the potential to be redundant in NSC survival function *in vivo*. It is essential to investigate further the NSC survival, and related phenotypes, in mice that lack a combination of the abovementioned ligands or their respective receptors.

Nogo stimulates NSC survival in a dose dependent manner through NogoR1-Rho and PirB-Shp signaling pathways

Several survival signaling pathways including EGF/FGF, Notch, Wnt and Sonic Hedgehog (Shh) have been known to affect either NSC or NP survival [7, 10, 66-71]. Mice that lack Notch receptor or Shh/Smoothened (Smo) have decreased NSC survival *in vivo* [69, 70, 72]. Addition of Notch ligand Delta or Wnt3a has been shown to promote NSC survival in clonal assays [73, 74]. Our studies show that addition of CSPG, ApoE and Nogo increases NSC frequency by 3.5, 2.2 and 3.7 folds, respectively. Notch signaling has also been shown to regulate NSC survival through CBF-1 transcriptional regulation and Notch activation induces Hes3, Shh through rapid activation of serine/threonine kinase Akt, and STAT3 promotes the survival of NSCs [71, 73]. Our previous studies show the importance of EGFR and ERK survival signaling pathways for CSPG and ApoE in NSC survival [1, 2].

Nogo proteins have been shown to function through Rho-Rho kinase and protein kinase C (PKC) in axonal injury [75]. NogoR1 forms a complex with p75 co-receptor and signals through Rho and Rho kinase proteins [23, 32, 76]. PirB has been shown to associate with PTPN (Shp1/2) adaptor proteins in B cells and neurons [46, 77-79].

For NSCs/NPs we found that NogoR1 and PirB signal through independent cytoplasmic effector proteins RhoA and Shp, respectively. At pM doses of Nogo66, we found that receptor PirB activates Shp adaptor proteins and at nM doses, NogoR1 activates Rho kinase protein in a specific manner to regulate NSC survival.

CONCLUSION

Our studies on expression, characterization and *in vitro* activity of NogoR1 and PirB suggest novel roles for NogoA signaling in embryonic NSCs/NPs survival pathways through Rho and Shp1/2. Through these NSC studies an understanding of signaling pathways that control NSC/NP survival and proliferation is emerging. An interesting question for future studies is to evaluate the roles of NogoA and other signaling pathways in regulating NSCs *in vivo* and how they may be involved in disease states such as neurodevelopmental disorders and oligodendrogliomas [80-83].

ACKNOWLEDGMENTS

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Disclosure of potential conflicts of interests

The authors indicate no potential conflicts of interest.

REFERENCES

1. Tham M, Ramasamy S, Gan HT, et al. CSPG Is a Secreted Factor that Stimulates Neural Stem Cell Survival Possibly by Enhanced EGFR Signaling. PLoS ONE 2010;5:e15341.
2. Gan HT, Tham M, Hariharan S, et al. Identification of ApoE as an autocrine/paracrine factor that stimulates neural stem cell survival via MAPK/ERK signaling pathway. J Neurochem 2011;117:565-578.
3. Williams BP, Price J. Evidence for multiple precursor cell types in the embryonic rat cerebral cortex. Neuron 1995;14:1181-1188.
4. Temple S. The development of neural stem cells. Nature 2001;414:112-117.
5. Takahashi T, Goto T, Miyama S, et al. Sequence of neuron origin and neocortical laminar fate: relation to cell cycle of origin in the developing murine cerebral wall. J Neurosci 1999;19:10357-10371.
6. Takahashi T, Nowakowski RS, Caviness VS, Jr. Early ontogeny of the secondary proliferative population of the embryonic murine cerebral wall. J Neurosci 1995;15:6058-6068.
7. Ramasamy S, Narayanan G, Sankaran S, et al. Neural stem cell survival factors. Arch Biochem Biophys 2013.
8. Chang M, Park C, Lee S. Embryonic cortical stem cells secrete diffusible factors to enhance their survival. Neuroreport 2003;14:1191-1195.
9. Taupin P, Ray J, Fischer W, et al. FGF-2-responsive neural stem cell proliferation requires CCg, a novel autocrine/paracrine cofactor. Neuron 2000;28:385-397.
10. Kalani M, Cheshier S, Cord B, et al. Wnt-mediated self-renewal of neural stem/progenitor cells. Proc Natl Acad Sci U S A 2008;105:16970-16975.
11. Sousa VH, Fishell G. Sonic hedgehog functions through dynamic changes in temporal competence in the developing forebrain. Current Opinion in Genetics & Development 2010;20:391-399.
12. Lillien L, Raphael H. BMP and FGF regulate the development of EGF-responsive neural progenitor cells. Development 2000;127:4993-5005.
13. Theus MH, Ricard J, Bethea JR, et al. EphB3 limits the expansion of neural progenitor cells in the subventricular zone by regulating p53 during homeostasis and following traumatic brain injury. Stem Cells 2010;28:1231-1242.
14. Chen HL, Panchision DM. Concise review: bone morphogenetic protein pleiotropism in neural stem cells and their derivatives--alternative pathways, convergent signals. Stem Cells 2007;25:63-68.
15. Sakaguchi DS, Van Hoffelen SJ, Theusch E, et al. Transplantation of neural progenitor cells into the developing retina of the Brazilian opossum: an in vivo system for studying stem/progenitor cell plasticity. Dev Neurosci 2004;26:336-345.
16. del Valle K, Theus MH, Bethea JR, et al. Neural progenitors proliferation is inhibited by EphB3 in the developing subventricular zone. Int J Dev Neurosci 2011;29:9-14.
17. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992;255:1707-1710.
18. Mingorance A, Soriano-Garcia E, del Rio JA. [Nogo-A functions during the development of the central nervous system and in the adult]. Rev Neurol 2004;39:440-446.
19. Mingorance-Le Meur A, Zheng B, Soriano E, et al. Involvement of the myelin-associated inhibitor Nogo-A in early cortical development and neuronal maturation. Cereb Cortex 2007;17:2375-2386.
20. Mathis C, Schroter A, Thallmair M, et al. Nogo-a regulates neural precursor migration in the embryonic mouse cortex. Cereb Cortex;20:2380-2390.
21. Gil V, Bichler Z, Lee JK, et al. Developmental expression of the oligodendrocyte myelin glycoprotein in the mouse telencephalon. Cereb Cortex 2010;20:1769-1779.
22. Mathis C, Schroter A, Thallmair M, et al. Nogo-a regulates neural precursor migration in the embryonic mouse cortex. Cereb Cortex 2010;20:2380-2390.
23. Schwab ME. Functions of Nogo proteins and their receptors in the nervous system. Nat Rev Neurosci 2010;11:799-811.
24. Fournier AE, GrandPre T, Strittmatter SM. Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature 2001;409:341-346.
25. Fournier AE, Strittmatter SM. Repulsive factors and axon regeneration in the CNS. Curr Opin Neurobiol 2001;11:89-94.
26. Atwal JK, Pinkston-Gosse J, Syken J, et al. PirB is a functional receptor for myelin inhibitors of axonal regeneration. Science 2008;322:967-970.
27. Fournier AE, Gould GC, Liu BP, et al. Truncated soluble Nogo receptor binds Nogo-66 and blocks inhibition of axon growth by myelin. J Neurosci 2002;22:8876-8883.
28. Fournier AE, GrandPre T, Gould G, et al. Nogo and the Nogo-66 receptor. Prog Brain Res 2002;137:361-369.
29. Wong ST, Henley JR, Kanning KC, et al. A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. Nat Neurosci 2002;5:1302-1308.
30. Oertle T, van der Haar ME, Bandtlow CE, et al. Nogo-A inhibits neurite outgrowth and cell spreading with three discrete regions. J Neurosci 2003;23:5393-5406.
31. Li M, Li Y, Liao X, et al. Rational design, solution conformation and identification of functional residues of the soluble and structured Nogo-54, which mimics Nogo-66 in inhibiting the CNS neurite outgrowth. Biochem Biophys Res Commun 2008;373:498-503.
32. Wang KC, Kim JA, Sivasankaran R, et al. P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 2002;420:74-78.

33. Josephson A, Widenfalk J, Widmer HW, et al. NOGO mRNA expression in adult and fetal human and rat nervous tissue and in weight drop injury. Exp Neurol 2001;169:319-328.
34. Huber AB, Weinmann O, Brosamle C, et al. Patterns of Nogo mRNA and protein expression in the developing and adult rat and after CNS lesions. J Neurosci 2002;22:3553-3567.
35. Tozaki H, Kawasaki T, Takagi Y, et al. Expression of Nogo protein by growing axons in the developing nervous system. Brain Res Mol Brain Res 2002;104:111-119.
36. Petrinovic MM, Duncan CS, Bourikas D, et al. Neuronal Nogo-A regulates neurite fasciculation, branching and extension in the developing nervous system. Development 2010;137:2539-2550.
37. Wang X, Baughman KW, Basso DM, et al. Delayed Nogo receptor therapy improves recovery from spinal cord contusion. Ann Neurol 2006;60:540-549.
38. Dickendesher TL, Baldwin KT, Mironova YA, et al. NgR1 and NgR3 are receptors for chondroitin sulfate proteoglycans. Nat Neurosci 2012;15:703-712.
39. Rolando C, Parolisi R, Boda E, et al. Distinct roles of Nogo-a and Nogo receptor 1 in the homeostatic regulation of adult neural stem cell function and neuroblast migration. J Neurosci 2012 32:17788-17799.
40. Wang B, Xiao Z, Chen B, et al. Nogo-66 promotes the differentiation of neural progenitors into astroglial lineage cells through mTOR-STAT3 pathway. PLoS ONE 2008;3:e1856.
41. Gao Y, Wang B, Xiao Z, et al. Nogo-66 regulates nanog expression through stat3 pathway in murine embryonic stem cells. Stem Cells Dev 2010;19:53-60.
42. Gao Y, Xiao Z, Chen B, et al. Effect of different regions of Nogo-A on the differentiation of neural progenitors. Neurosci Lett 2009;458:132-135.
43. Govind S, Kozma R, Monfries C, et al. Cdc42Hs facilitates cytoskeletal reorganization and neurite outgrowth by localizing the 58-kD insulin receptor substrate to filamentous actin. J Cell Biol 2001;152:579-594.
44. Ramasamy S, Wang H, Quach HN, et al. Zebrafish Staufen1 and Staufen2 are required for the survival and migration of primordial germ cells. Dev Biol 2006;292:393-406.
45. Shen Y, Tenney AP, Busch SA, et al. PTPsigma is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. Science 2009;326:592-596.
46. Syken J, Grandpre T, Kanold PO, et al. PirB restricts ocular-dominance plasticity in visual cortex. Science 2006;313:1795-1800.
47. Wood JN, Bevan SJ, Coote PR, et al. Novel cell lines display properties of nociceptive sensory neurons. Proc Biol Sci 1990;241:187-194.
48. Ujike A, Takeda K, Nakamura A, et al. Impaired dendritic cell maturation and increased T(H)2 responses in PIR-B(-/-) mice. Nat Immunol 2002;3:542-548.
49. Zheng B, Atwal J, Ho C, et al. Genetic deletion of the Nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo. Proc Natl Acad Sci U S A 2005;102:1205-1210.
50. Coles-Takabe BL, Brain I, Purpura KA, et al. Don't look: growing clonal versus nonclonal neural stem cell colonies. Stem Cells 2008;26:2938-2944.
51. Wang H, Liu Z, Li D, et al. Injectable biodegradable hydrogels for embryonic stem cell transplantation: improved cardiac remodelling and function of myocardial infarction. J Cell Mol Med 2012;16:1310-1320.
52. Li M, Liu J, Song J. Nogo goes in the pure water: solution structure of Nogo-60 and design of the structured and buffer-soluble Nogo-54 for enhancing CNS regeneration. Protein Sci 2006;15:1835-1841.
53. He XL, Bazan JF, McDermott G, et al. Structure of the Nogo receptor ectodomain: a recognition module implicated in myelin inhibition. Neuron 2003;38:177-185.
54. Llorens F, Gil V, del Rio JA. Emerging functions of myelin-associated proteins during development, neuronal plasticity, and neurodegeneration. FASEB J 2011;25:463-475.
55. Caltharp SA, Pira CU, Mishima N, et al. NOGO-A induction and localization during chick brain development indicate a role disparate from neurite outgrowth inhibition. BMC Dev Biol 2007;7:32.
56. Buss A, Sellhaus B, Wolmsley A, et al. Expression pattern of NOGO-A protein in the human nervous system. Acta Neuropathol 2005;110:113-119.
57. Al Halabiah H, Delezoide AL, Cardona A, et al. Expression pattern of NOGO and NgR genes during human development. Gene Expr Patterns 2005;5:561-568.
58. Conover JC, Notti RQ. The neural stem cell niche. Cell Tissue Res 2008;331:211-224.
59. Kazanis I, ffrench-Constant C. Extracellular matrix and the neural stem cell niche. Dev Neurobiol 2011;71:1006-1017.
60. Sirko S, Akita K, Von Holst A, et al. Structural and functional analysis of chondroitin sulfate proteoglycans in the neural stem cell niche. Methods Enzymol 2010;479:37-71.
61. Sirko S, von Holst A, Wizenmann A, et al. Chondroitin sulfate glycosaminoglycans control proliferation, radial glia cell differentiation and neurogenesis in neural stem/progenitor cells. Development 2007;134:2727-2738.
62. Garcion E, Halilagic A, Faissner A, et al. Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin C. Development 2004;131:3423-3432.
63. von Holst A, Egbers U, Prochiantz A, et al. Neural stem/progenitor cells express 20 tenascin C isoforms that are differentially regulated by Pax6. J Biol Chem 2007;282:9172-9181.
64. von Holst A, Sirko S, Faissner A. The unique 473HD-Chondroitinsulfate epitope is expressed by radial glia

and involved in neural precursor cell proliferation. J Neurosci 2006;26:4082-4094.
65. Barton WA, Liu BP, Tzvetkova D, et al. Structure and axon outgrowth inhibitor binding of the Nogo-66 receptor and related proteins. EMBO J 2003;22:3291-3302.
66. Reynolds B, Tetzlaff W, Weiss S. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 1992;12:4565-4574.
67. Vescovi A, Reynolds B, Fraser D, et al. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. Neuron 1993;11:951-966.
68. Maric D, Fiorio Pla A, Chang YH, et al. Self-renewing and differentiating properties of cortical neural stem cells are selectively regulated by basic fibroblast growth factor (FGF) signaling via specific FGF receptors. J Neurosci 2007;27:1836-1852.
69. Komada M, Saitsu H, Kinboshi M, et al. Hedgehog signaling is involved in development of the neocortex. Development 2008;135:2717-2727.
70. Hitoshi S, Alexson T, Tropepe V, et al. Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes Dev 2002;16:846-858.
71. Mizutani K, Yoon K, Dang L, et al. Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. Nature 2007;449:351-355.
72. Hitoshi S, Seaberg RM, Koscik C, et al. Primitive neural stem cells from the mammalian epiblast differentiate to definitive neural stem cells under the control of Notch signaling. Genes Dev 2004;18:1806-1811.
73. Androutsellis-Theotokis A, Leker RR, Soldner F, et al. Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 2006;442:823-826.
74. Kalani MYS, Cheshier SH, Cord BJ, et al. Wnt-mediated self-renewal of neural stem/progenitor cells. Proceedings of the National Academy of Sciences 2008;105:16970-16975.
75. Sivasankaran R, Pei J, Wang KC, et al. PKC mediates inhibitory effects of myelin and chondroitin sulfate proteoglycans on axonal regeneration. Nat Neurosci 2004;7:261-268.
76. Yamashita T, Tohyama M. The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. Nat Neurosci 2003;6:461-467.
77. Maeda A, Kurosaki M, Ono M, et al. Requirement of SH2-containing protein tyrosine phosphatases SHP-1 and SHP-2 for paired immunoglobulin-like receptor B (PIR-B)-mediated inhibitory signal. J Exp Med 1998;187:1355-1360.
78. Takai T. Paired immunoglobulin-like receptors and their MHC class I recognition. Immunology 2005;115:433-440.
79. Takai T, Ono M. Activating and inhibitory nature of the murine paired immunoglobulin-like receptor family. Immunol Rev 2001;181:215-222.
80. Jung TY, Jung S, Lee KH, et al. Nogo-A expression in oligodendroglial tumors. Neuropathology 2011;31:11-19.
81. Marucci G, Di Oto E, Farnedi A, et al. Nogo-A: a useful marker for the diagnosis of oligodendroglioma and for identifying 1p19q codeletion. Hum Pathol 2011.
82. Hsu R, Woodroffe A, Lai WS, et al. Nogo Receptor 1 (RTN4R) as a candidate gene for schizophrenia: analysis using human and mouse genetic approaches. PLoS ONE 2007;2:e1234.
83. Gregorio SP, Mury FB, Ojopi EB, et al. Nogo CAA 3'UTR Insertion polymorphism is not associated with Schizophrenia nor with bipolar disorder. Schizophr Res 2005;75:5-9.

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Figure 1. NogoA, NogoR1 and PirB expression in mouse embryonic NSCs/NPs.
(A) mRNA was extracted from mouse brain derived nsphs at different development time points and analyzed by RT-PCR as described in the Materials and Methods section. Lanes show expression of Nogo-A, NogoR1, PirB from day E10.5 to E15.5 NSCs/NPs with RT minus (-) control and RT plus (+) with Gapdh as loading control. (B-E) NogoR1 and PirB receptor localization in mouse embryonic NSCs/NPs at day E14.5 using immunocytochemistry. NSCs/NPs derived from E14.5 (B) NogoR1-/-, (D) PirB -/-, (C) NogoR1 (+/+) and (E) PirB (+/+). NogoR1 and PirB (green) and DAPI (blue). Scale bar = 10 μm.

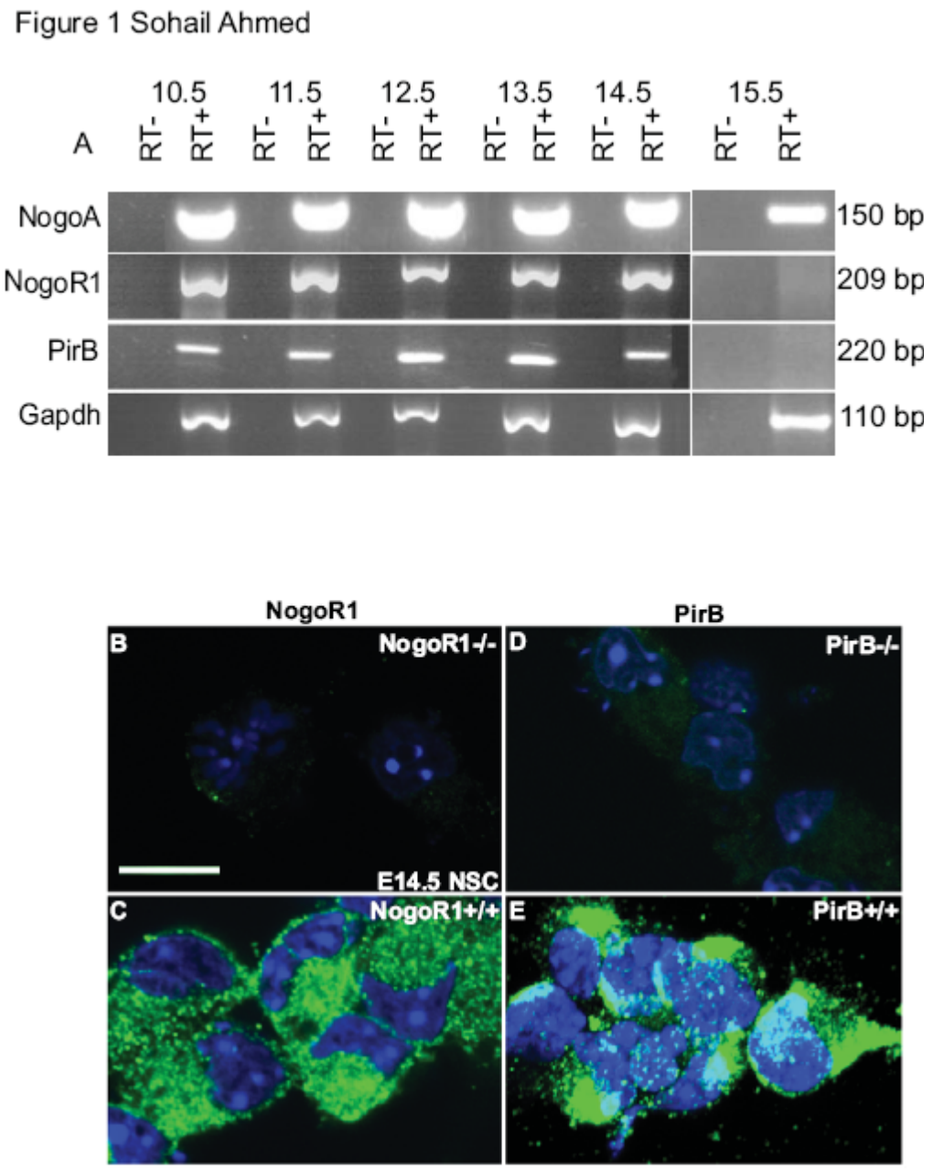


Figure 2. NogoR1 and PirB receptors are expressed in VZs of the mammalian embryonic cortex (CX) and the ganglionic eminence (GE) at day E14.5 and colocalizes with nestin.
(A) Embryo forebrain (E14.5) coronal sections were counterstained with DAPI (blue, panel a), nestin (green, panel b) and NogoR1 (red, panel c) as described in the Materials and Methods section. Merged is shown in panel d. In panel b arrowheads show the CX and the arrows show the GE in cortical VZs. The boxes in panel b span the VZ's from the apical to basal region and are shown at higher magnification in panels (e-h) for the CX and panels (i-l) for the GE. The boxes show the location of the inserts in panels f, g and h (CX) and panels j, k and l (GE). The inserts show optical zoom of the boxed areas. The signals of the panels g and k has been adjusted to visualize colocalization of nestin and NogoR1. DAPI (panels a, e and i), nestin (panels b, f and j), NogoR1 (panels c, g and k), merged (panels d, h, and l). Scale bar = 100 μm.
(B) Embryo forebrain (E14.5) coronal sections were counterstained with DAPI (blue, panel a), nestin (green, panel b) and PirB (red, panel c) as described in the Materials and Methods section. Merged is shown in panel d. In panel b arrowheads show the CX and the arrows show the GE in cortical VZs. The boxes in panel b span the VZ's from the apical to basal region and are shown at higher magnification in panels (e-h) for the CX and panels (i-l) for the GE. The boxes show the location of the inserts in panels f, g and h (CX) and panels j, k and l (GE). The inserts show optical zoom of the boxed areas. The signals of the panels g and k has been adjusted to visualize colocalization of nestin and PirB. DAPI (panels a, e and i), nestin (panels b, f and j), PirB (panels c, g and k), merged (panels d, h, and l). Scale bar = 100 μm.

Figure 2 Sohail Ahmed

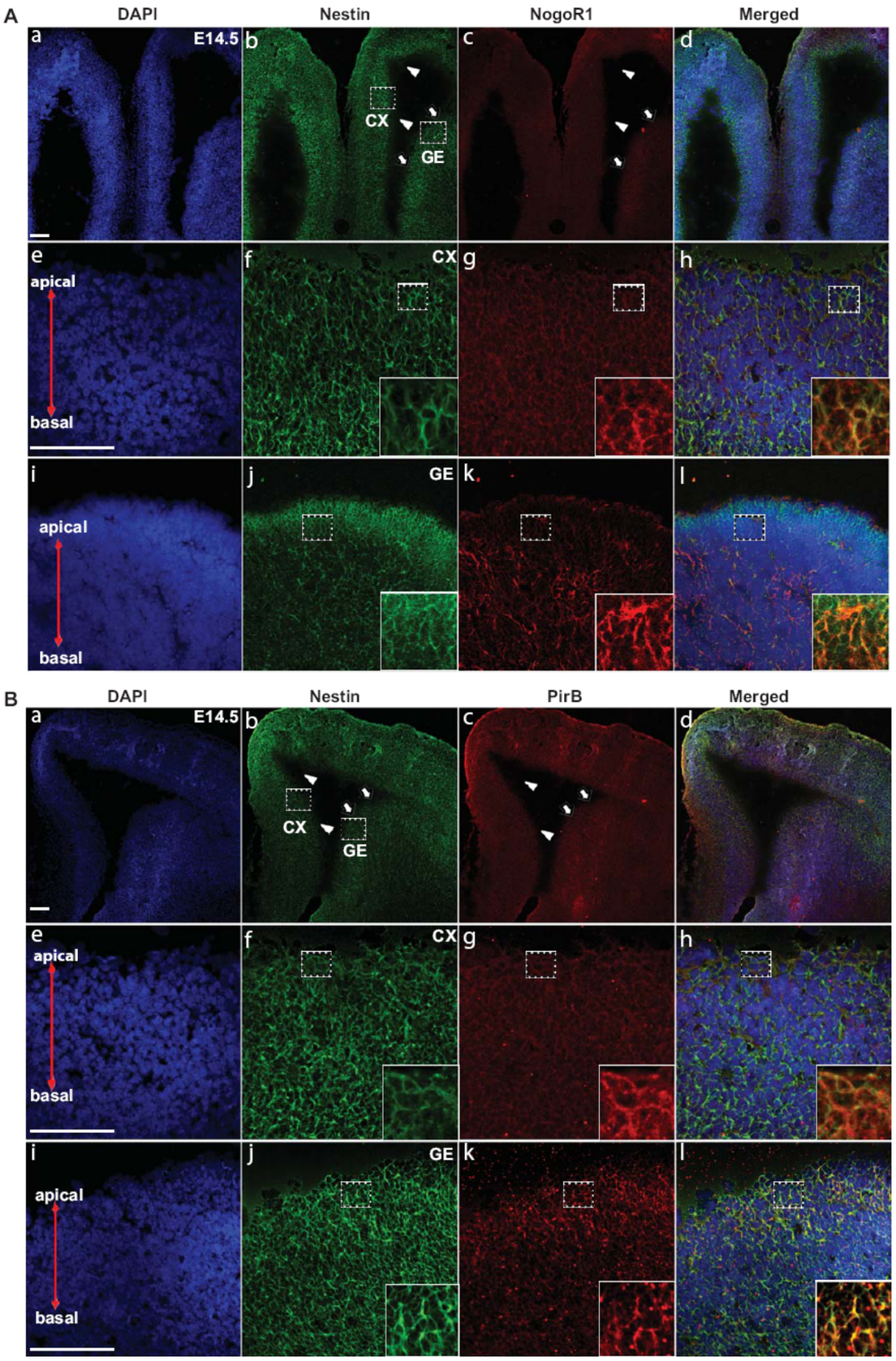


Figure 3. NogoR1 and PirB receptors are not expressed in the VZ or SVZ at day E15.5
Coronal sections (E15.5) shown in (A, B) were stained with DAPI (white), nestin (green) Tuj1 (red), NogoR1 and PirB (blue). The final panel right is the merge. The VZ and SVZ areas are shown by lines marked with VZ. Basal cortex is marked (CX) in the nestin panels. In the nestin panels the VZ/SVZ are highlighted by white arrowheads. Scale bar = 100 μm.

Figure 3 Sohail Ahmed

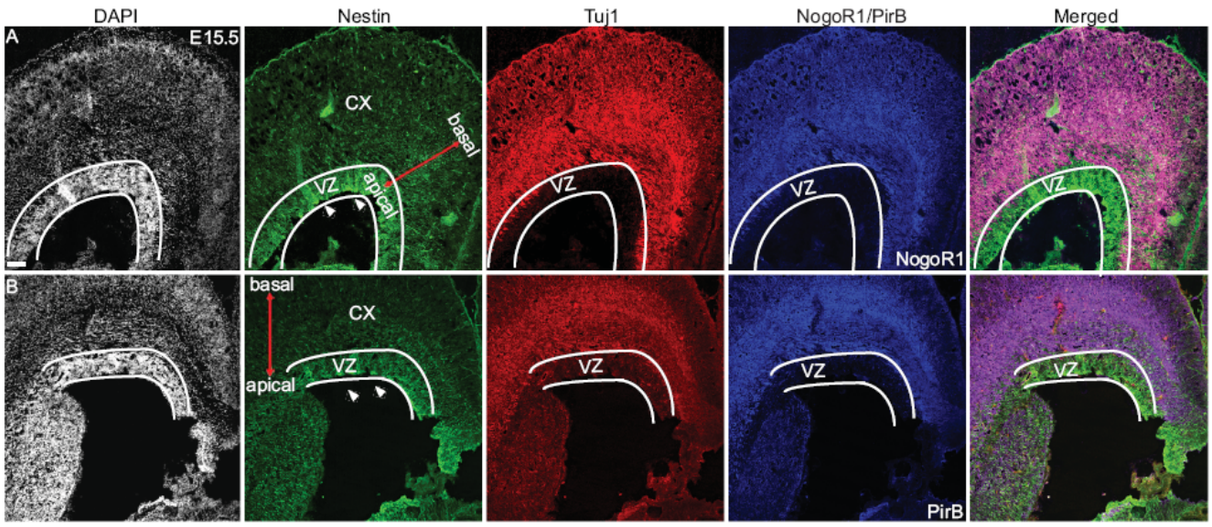


Figure 4. Effect of Nogo54 and Nogo66 on nsph formation in cells derived from WT, NogoR1 KO or PirB KO mice.
NSCs/NPs were plated at 50 cells/mL (clonal density) and nsph formation was followed for 5-7 days as described in the Materials and Methods section. (A) Addition of AP (1 μM) control or Nogo54/66 (100 pM -500 nM) to either WT cells or cells derived from KO mice. Data are presented as mean ± SEM, n≥6. (B) NSCs/NPs were assayed for the presence of TUNEL positive nuclei (cell death) in the presence of AP (1 μM) or Nogo66 at 100 pM and 50 nM. Data are presented as mean ± SEM, n=4. (C) NSCs/NPs were assayed for the presence of BrdU positive nuclei as a marker for proliferation in the presence of AP (1 μM) and Nogo66 at 100 pM and 50 nM. Data are presented as mean ± SEM, n=4. For both TUNEL and BrdU assays, unbiased fields of view in the coverslip (n≥70) were taken for each sample and statistical significance was assessed by ANOVA (*$p < 0.05$, **$p < 0.001$ and ***$p < 0.0001$). The NFU assay is described in detail in the materials and methods section. NB. In some experiments Nogo66 was seen to aggregate at concentrations greater than 50 nM.

Figure 4 Sohail Ahmed

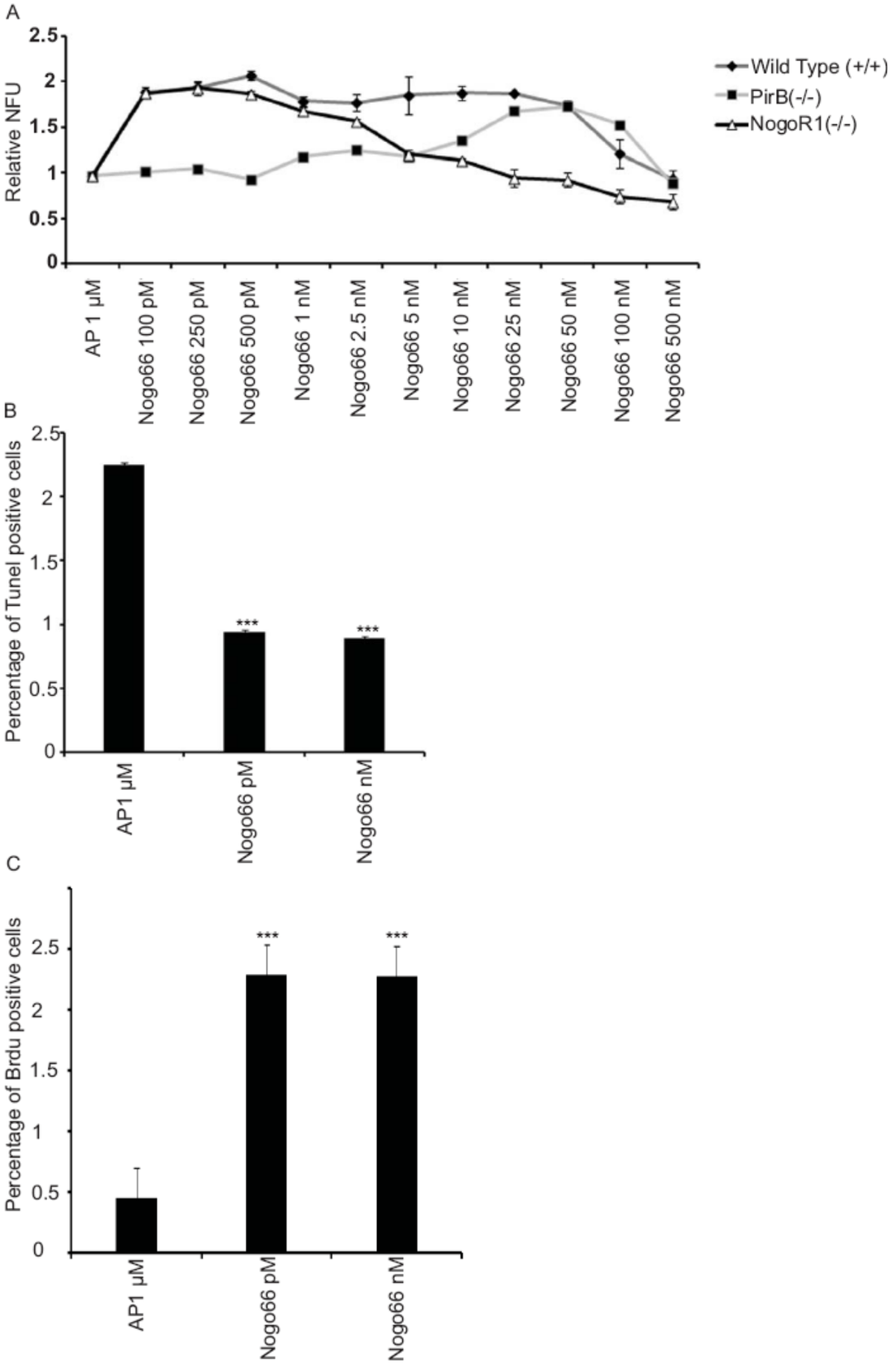
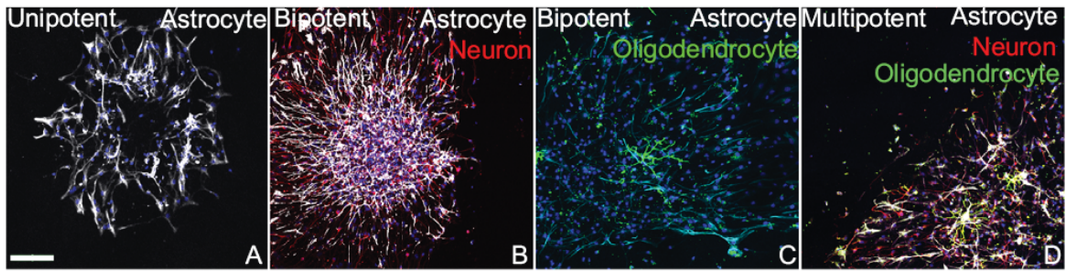
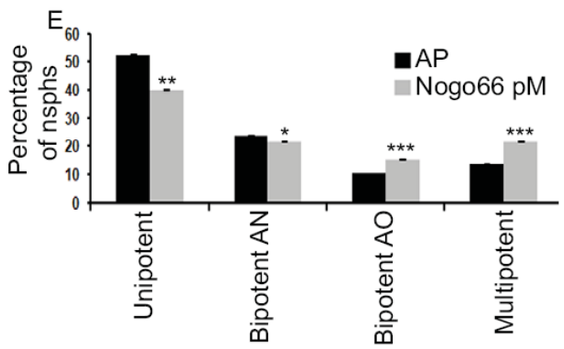
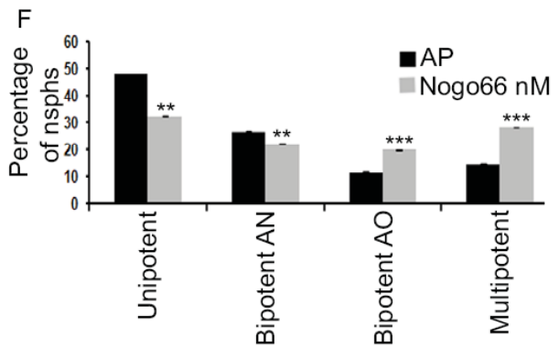


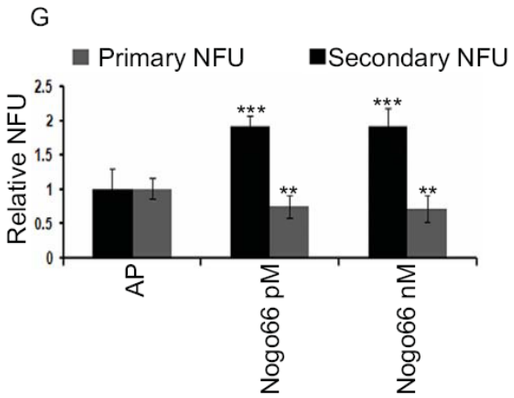
Figure 5. Effect of Nogo54/66 on potency of nsphs, NSC frequency and self-renewal
Nsph potency after differentiation under clonal conditions was scored as described in the Materials and Methods section. (A-D), shows examples of unipotent (A), bipotent (B,C) and multipotent (D) nsphs. Astrocytes stained by GFAP seen in grey, neurons stained by β-Tubulin III (clone Tuj1) seen in red and oligodendrocytes stained by O4 seen in green, Cells were counterstained with DAPI (blue). Scale bar = 50 µm. After differentiation the potency of the nsphs were scored in the presence of (E) Nogo66 pM or (F) Nogo66 nM with AP as control (E,F). For each concentration of Nogo66 approximately 270 clonally differentiated nsphs were analyzed. Bipotent nsphs with astrocytes and neurons (AN). Bipotent nsphs with astrocytes and oligodendrocyte (AO). (G) Nsphs were grown in the presence of either 100 pM or 50 nM Nogp66. Cells were then replated at clonal density in the absence of Nogo66 and scored for the number of secondary nsphs formed. AP was used as control. (H) NSC frequency was calculated by multiplying the absolute NFU and the percentage of multipotent nsphs. Data is presented as mean ± SEM with n ≥4 and statistical significance was assessed by ANOVA (*p <0.05, **p <0.001 and ***p<0.0001).

Figure 5 Sohail Ahmed









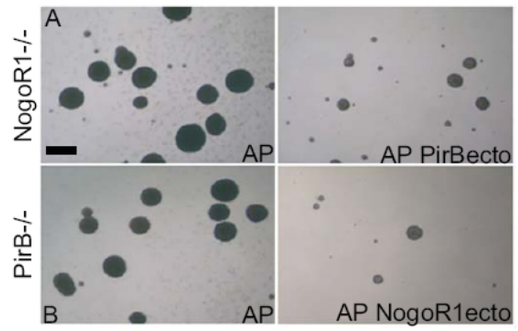
H

NSC frequency (%) =	NFU(%)	x	Multipotency (%)
Con	0.7	5.4±1.65[a]	13±1.74[b]
Nogo66 pM	2.68**	12.43±2.34[a]	21.69±2.34[b]
Nogo66 nM	3.19***	11.95±1.38[a]	25.98±1.23[c]

a from average of 6 experiments, b from Fig E, c from FigF

Figure 6. Effect of inhibition of Nogo ligand-receptor interactions on nsph formation
NSCs/NPs from +/+ and -/- (NogoR1 and PirB) were plated independently at low density and nsphs formation for 5-7 days followed as described in the Materials and Methods section. NogoR1-/- or PirB -/- NSC/NP cultures in the absence (A, B; left panels) or presence of PirBecto (A, right panel) or NogoR1ecto (B, right panel). (C) The nsph number is represented in relative NFU as seen in WT (+/+), NogoR1-/-, PirB-/- NSCs/NPs after addition of AP, NogoR1 /PirBecto individually or in combination. (D) Nsph diameter in μm was measured in WT (+/+), NogoR1-/-, PirB-/- NSCs/NPs after addition of AP, NogoR1/PirBecto individually or in combination. 172 nsphs were scored for each experiment. Data represented in (C and D) as mean ± SEM with n≥3, (E) The clonal nsphs generated in the presence or absence of ectodomains from (B) were scored for potency as described in the Materials and Methods section. 148 nsphs were analyzed for each experimental condition. Data represented in (E) as mean ± SEM with n=3. (F) NSC frequency was calculated by multiplying the absolute NFU and the percentage of multipotent nsphs. Significance was assessed by ANOVA (*p <0.05, **p <0.001 and ***p<0.0001). Scale bar = 100 μm (A, B).

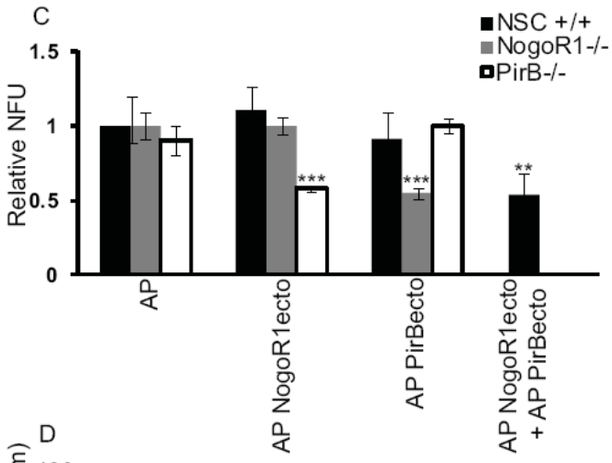
Figure 6 Sohail Ahmed

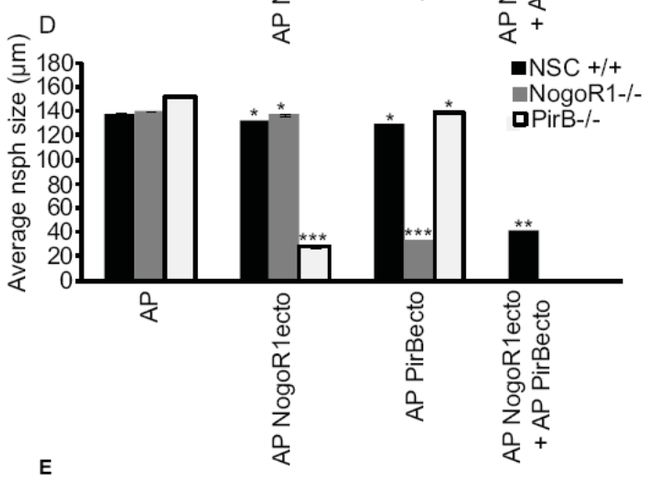


F

	NSC frequency (%) =	NFU(%) x	Multipotency (%)
AP (NogoR1-/- / PirB-/-)	0.7	5.4±1.65[a]	11±0.55[b]
AP NogoR1ecto (PirB-/-)	0.2**	2.03±0.12[a]	1.69±0.11[b]
AP PirBecto (NogoR1-/-)	0.3**	2.14±0.35[a]	1.8±0.2[b]

a from average of 5 experiments, b from Fig6E





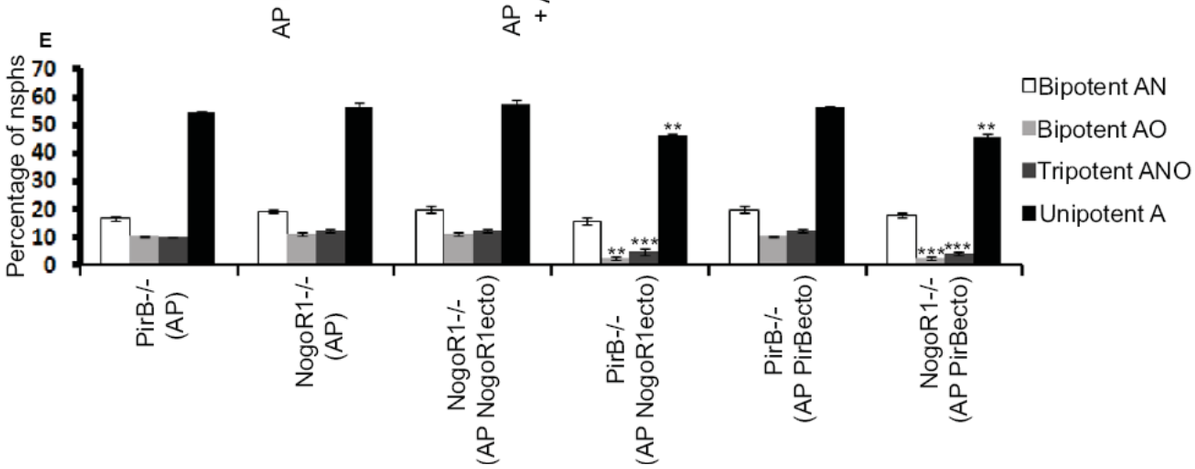


Figure 7. Nogo stimulates NSC survival through Rho and Shp1/2 signaling pathways.
NSCs/NPs were grown at low density and inhibitors for specific signaling pathways; Rho inhibitor (C3 toxin), Rho kinase inhibitor (Y27632), Shp inhibitors (PHPS1 and NSC87877) and aPKC inhibitor (GO6976) were added at different concentrations and nsph formation followed in the presence of control AP (1μM) peptide or Nogo66 at 100 pM or 50 nM doses. (A) Go 6796 concentration range (25 nM-50 μM). (B) Y27632 (100 nM-100 μM), (C) C3 toxin (25 ng/ml-25 μg/ml), (D) PHPS1 (50 nM-50 μM), (E) NSC87877 (50 nM-50 μM). Data are presented as mean ± SEM, n ≥ 3, all inhibitor concentrations in the x-axes for respective graphs (A-E) are represented in log scale.