Soluble LILRA3 promotes neurite outgrowth and synapses formation through a high-affinity interaction with Nogo 66

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ABSTRACT

Inhibitory proteins, particularly Nogo 66, a highly conserved 66-amino-acid loop of Nogo A (an isoform of RTN4), play key roles in limiting the intrinsic capacity of the central nervous system (CNS) to regenerate after injury. Ligation of surface Nogo receptors (NgRs) and/or leukocyte immunoglobulin-like receptor B2 (LILRB2) and its mouse orthologue the paired immunoglobulin-like receptor B (PIRB) by Nogo 66 transduces inhibitory signals that potently inhibit neurite outgrowth. Here, we show that soluble leukocyte immunoglobulin-like receptor A3 (LILRA3) is a high-affinity receptor for Nogo 66, suggesting that LILRA3 might be a competitive antagonist to these cell surface inhibitory receptors. Consistent with this, LILRA3 significantly reversed Nogo 66-mediated inhibition of neurite outgrowth and promoted synapse formation in primary cortical neurons through regulation of the ERK/MEK pathway. LILRA3 represents a new antagonist to Nogo 66-mediated inhibition of neurite outgrowth in the CNS, a function distinct from its immune-regulatory role in leukocytes. This report is also the first to demonstrate that a member of LILR family normally not expressed in rodents exerts functions on mouse neurons through the highly homologous Nogo 66 ligand.

KEY WORDS: Leukocyte immunoglobulin-like receptor A3, Nogo 66, PIRB, Cortical neuron, Neurite outgrowth, Synapse

INTRODUCTION

Leukocyte immunoglobulin-like receptor A3 (LILRA3) is a soluble member of a family of activating and inhibitory immune-regulatory cell surface receptors primarily expressed on leukocytes and that is increasingly recognised as a key regulator of the threshold and amplitude of leukocyte activation (Borges and Cosman, 2000; Brown et al., 2004; An et al., 2010; Tedla et al., 2011). LILRA3 is unique in that it lacks transmembrane and cytoplasmic domains, thus is an exclusively secreted protein (Borges and Cosman, 2000). Although LILRA3 functions have not been fully elucidated, it shares high structural similarity to cell surface LILRs, including the inhibitory LILRB2 (Borges and Cosman, 2000; Brown et al., 2004), and hence might act as a soluble antagonist through shared ligands. However, natural ligands for LILRA3 have not been fully identified; a knowledge gap that has severely hindered understanding of its functions. LILRs are selectively conserved in humans and primates and are the most conserved among genes located within the leukocyte receptor complex on chromosome 19, implying important functions (Martin et al., 2002). There are no rodent homologues, although paired immunoglobulin-like receptor B (PIRB), considered the murine orthologue of human LILRB2, regulates leukocyte functions through similar signalling cascades (Martin et al., 2002).

Nogo 66 is a highly conserved 66-amino-acid surface membrane loop of the reticulon family of proteins that include Nogo A, Nogo B and Nogo C (isoforms of RTN4), and is crucial for several vital inhibitory roles of Nogo proteins (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000; Fry et al., 2007; Rodriguez-Feo et al., 2007; David et al., 2008; Kritz et al., 2008; Schwab, 2010; Wright et al., 2010). In Nogo A, Nogo 66 is a potent inhibitor of axonal regeneration and neurite outgrowth after central nervous system (CNS) injury that acts through interaction with its traditional receptors (NgR1 and NgR2, also known as RTN4R and RTN4RL2, respectively) (Fournier et al., 2001; Schwab, 2010) and the newly discovered inhibitory immunoglobulin-like receptors, human LILRB2 and its murine orthologue PIRB (Atwal et al., 2008; Adelson et al., 2012). Although studies are limited, the more ubiquitously expressed Nogo B inhibits inflammation (Wright et al., 2010) and decreases vascular injury (Rodriguez-Feo et al., 2007; Kritz et al., 2008). Nogo C is expressed in the CNS and skeletal muscle, but functions are unknown (Schwab, 2010).

Limited studies show that LILRA3 interacts with some major histocompatibility complex (MHC) class I molecules, albeit with low-binding affinities (μM amounts), but there is a lack of functional data (Jones et al., 2011). These deficiencies might be due in part to the existence of an alternative high-affinity LILRA3 ligand(s) or due to the use of unsuitably glycosylated and/or truncated fusion LILRA3 proteins (Jones et al., 2011). Our aim was to identify cell surface proteins that interact with mammalian-produced, properly glycosylated, full-length recombinant LILRA3 (Lee et al., 2013) and define their functions. After initial screening of numerous primary cells and cell lines, we found human monocytes exhibited high-affinity binding to recombinant LILRA3 (Lee et al., 2013). Plasma membrane proteins from these cells were used in a new proteomic approach to identify two new [Nogo 66 and 67-kDa laminin receptor (also known as RPSA)] and one known MHC class I (HLA-B) molecules as candidate high-affinity LILRA3-binding proteins. Of particular interest was the interaction between Nogo 66 and LILRA3, as this was unexpected given that Nogo 66 is commonly associated with inhibition of neurite outgrowth in the CNS (GrandPré et al., 2000; Schwab, 2010). LILRA3 and Nogo 66 binding was therefore confirmed by

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several independent methods including surface plasmon resonance ( SPR), co-immunoprecipitation studies and biochemical assays. We show that LILRA3 binding to Nogo 66 was high affinity, saturable and specific. Importantly, LILRA3 potently reversed Nogo-66-mediated inhibition of neurite outgrowth in human and mouse primary cortical neurons in vitro and increased numbers of synaptic contacts, likely through regulation of the ERK/MEK pathway. This tantalising discovery points to new LILRA3 functions in the CNS, distinct from its proposed regulatory role in leukocytes. We propose that soluble LILRA3 might function as an antagonist against the closely related cell surface inhibitory receptors LILRB2 and PIRB by competitively binding to a shared Nogo 66 ligand. Reversal of Nogo-mediated inhibition of neurite outgrowth by endogenous LILRA3 might have implications in CNS disease states where Nogo A is a major contributor to the inhibitory microenvironment leading to failed regeneration after neuro-axonal damage.

RESULTS

Identification of Nogo 66 as a new ligand for soluble LILRA3
Nano liquid chromatography tandem mass spectrometry (LC-MS/MS) of in-gel trypsin digested peptides from co-immunoprecipitation experiments using placental alkaline phosphatase (AP)-tagged recombinant LILRA3 as bait identified three LILRA3-binding proteins with high MASCOT scores in three independent experiments (Fig. S1). Two proteins, Nogo A (four to seven peptide matches; mascot score of 189–277) and the 67-kDa laminin receptor (two to five peptide matches; mascot score of 112–115) were identified as potential new binding partners, whereas HLA-B (two to five peptide matches; mascot score of 109–113) is known to bind LILRA3 (Jones et al., 2011). At least three of the four to seven peptide sequences identified as Nogo proteins were part of the highly conserved 66-amino-acid surface membrane loop of the reticulon family of proteins that include Nogo A, Nogo B and Nogo C (Fig. S1). These proteins were not present in control samples when alkaline phosphatase control protein was used as bait, indicating specificity.

Nogo 66 is a high-affinity ligand for soluble LILRA3
High-affinity binding of LILRA3 to Nogo 66 was confirmed using four independent approaches. First, SPR steady-state equilibrium analysis indicated that soluble LILRA3 maintained picomolar affinity binding ($K_D=2.21\pm0.45\times10^{-10}$ M, $n=3$) to chip-immobilised recombinant Nogo 66 (500–600 relative units) (Fig. 1A). Second, recombinant Nogo 66 specifically bound to concanavalin A (ConA)–Sepharose-immobilised recombinant LILRA3 but not to control Sepharose (Fig. 1B). Third, His-tagged recombinant Nogo 66 bound plate-immobilised purified untagged recombinant LILRA3 in a concentration-dependent manner. His tag control protein did not bind the maximal concentration of plate-immobilised recombinant LILRA3, and Nogo 66 did not bind human IgG control confirming specificity (Fig. 1C). Fourth, LILRA3 binding was markedly reduced by pre-incubation with 10 µg/ml rabbit antibody against Nogo A and Nogo B for 30 min, confirming specificity ($n=4$).
Levels of Nogo A protein (identified by its molecular mass of ~180 kDa) in transfected cells (upper panel) and a summary densitometry (lower panel) of three independent western blot experiments showing 55.1±8.2% (mean±s.e.m.) reduction in Nogo A protein after specific gene silencing as compared to scrambled shRNA (**P<0.01; one-way ANOVA). (F) Nogo A shRNA gene silencing in primary cortical neurons significantly abrogated surface binding of LILRA3 (Fig. 1G), and strongly corroborated the SPR and co-immunoprecipitation studies.

**LILRA3 significantly reversed Nogo-66-mediated inhibition of neurite outgrowth in mouse and human cortical neurons**

Ligation of the inhibitory receptor PIRB/LILRB2 and NgR1 on neurons by Nogo 66 leads to inhibition of neurite outgrowth (GrandPré et al., 2000; Schwab, 2010). In Fig. 3A, we show a schematic where competitive binding of soluble LILRA3 with Nogo 66 reverses the inhibitory effects of Nogo 66 binding to the closely related inhibitory cell surface receptors PIRB and LILRB2. To address whether this occurs, we established an *in vitro* neurite outgrowth inhibition model by culturing primary mouse cortical neurons that constitutively express surface PIRB (Fig. 3B,C) on plate-immobilised recombinant Nogo 66, and assessed potential competitive effects of recombinant LILRA3. As expected, the neurite length of neurons plated on recombinant Nogo-66–His-coated coverslips were significantly shorter (170.7±73 µm; Fig. 4A,H) than neurons cultured on control His-tag peptide control-coated coverslips (593.3±37 µm) (Fig. 4E,H; mean±s.e.m.; n=7, P<0.001). Importantly, Nogo-66-mediated inhibition of neurite outgrowth was dramatically reversed by co-incubation with recombinant LILRA3; the average length of 773.9±38 µm was five times longer than neurons cultured on recombinant Nogo 66 (Fig. 4C,H; n=7, P<0.001). Consistent with these results, staining for βIII tubulin (a neuron-specific marker) and Tau1 (also known as microtubule-associated protein tau; a marker for axonal development) in neurons cultured on Nogo 66 was limited and patchy, likely due to a poorly developed neuronal network (Fig. 4B). In contrast, neurons cultured on recombinant Nogo 66 with recombinant LILRA3-coated coverslips retained extensive staining for both proteins (Fig. 4D), indicating that LILRA3 reversed inhibition of neurite and axonal growth outgrowth by Nogo 66 and promoted substantial dendrite and axonal development. Interestingly, neurons cultured on recombinant

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**Fig. 2. High-affinity interaction of recombinant LILRA3 to cell surface Nogo on primary mouse cortical neurons.** (A) Significant binding of LILRA3–AP but not alkaline phosphatase tag control to the surface of cortical neurons. Results are mean±s.e.m. (n=6). ***P<0.001 (one-way ANOVA). (B) Representative Scatchard analysis of LILRA3–AP binding to primary cortical neurons showing saturable high-affinity binding (K_d=6.70×10^-8 M, B_max=0.154×10^-8 M, R^2=0.93) (n=3). (C) Data in B presented as a Scatchard plot yielded a straight line plot, typical of a saturable receptor–ligand interaction (P=0.04, R^2=0.78). (D) Transient 5-day knockdown in primary cortical neurons using lentivirus-based shRNA reduced Nogo A mRNA by 78.3±6.3% (mean±s.e.m.) compared to neurons transfected with scrambled shRNA (n=3). ***P<0.001 (one-way ANOVA). (E) Representative western blot using antibody against Nogo A and Nogo B showing decreased levels of Nogo A protein (identified by its molecular mass of ~180 kDa) in transfected cells (upper panel) and a summary densitometry (lower panel) of three independent western blot experiments showing 55.1±8.2% (mean±s.e.m.) reduction in Nogo A protein after specific gene silencing as compared to scrambled shRNA. **P<0.01; one-way ANOVA). (F) Nogo A shRNA gene silencing in primary cortical neurons significantly abrogated surface binding of LILRA3–AP by 34.3±8.6% (mean±s.e.m.) compared to neurons treated with scrambled shRNA (n=3). *P<0.05 (one-way ANOVA).
LILRA3-coated coverslips were 15–25% longer than those with His tag alone (Fig. 4G,H, \( P<0.05 \)), suggesting that LILRA3 might also have partially reversed negative effects caused by native Nogo A expressed on these neurons (Fig. 1D).

To confirm that LILRA3 modulated Nogo-66-induced inhibition of neurite outgrowth in human neurons, LILRB2-expressing primary human fetal cortical neurons were cultured on Matrigel-coated plates with or without recombinant Nogo 66 and with or without recombinant LILRA3 for 8 days. As expected, Nogo 66 inhibited neurite outgrowth (average length 270.4±85.3 µm) (Fig. 5A,E) compared to the His tag control (average length of 466.3±50.7 µm) (Fig. 5C,E; mean±s.e.m.; \( n=4, P<0.01 \)). Importantly, co-treatment with recombinant LILRA3 significantly reversed Nogo-66-mediated inhibition, yielding neurites lengths of 530.2±36.7 µm (Fig. 5B,E; \( P<0.01 \)). This length was twice that of neurons grown on Nogo 66-coated plates and 1.3 times that of neurons on the His tag control (Fig. 5E). Furthermore, neurites of neurons grown on recombinant LILRA3 tended to be somewhat longer (544.8±110.1 µm) than those on the His tag control (Fig. 5E). This is the first demonstration that LILRA3 regulates neurite outgrowth in human and mouse cortical neurons through interaction with the highly homologous Nogo 66.

**LILRA3 increased numbers of synaptic contacts in mouse cortical neurons**

Mouse cortical neurons cultured on coverslips coated with recombinant Nogo 66 for 21 days had an average of 135.6±12.0 synaptophysin and PSD95 (also known as DLG4) double-positive spots (i.e. synaptic contacts) per field of view (77.5×77.5 µm area) (Fig. 6A–C,J). This number was significantly less than the number of synaptic contacts of neurons cultured on His tag control coverslips (324.4±9.6 per field; Fig. 6G–I,J; median±s.e.m., \( n=3, P<0.0001 \)). Importantly, co-coating with recombinant LILRA3 completely reversed recombinant Nogo 66-mediated inhibition and increased synaptic contacts to 337.6±7.8 per field, numbers similar to those in control neurons (Fig. 6D–F,J). Similarly, staining of neurons cultured for 14 days with synaptophysin and phalloidin, as pre-and post-synaptic markers, respectively, showed significantly less synaptic contacts in neurons grown on Nogo-66-coated coverslips as compared to those with Nogo 66 plus LILRA3 (Fig. S2).

**LILRA3 reverses Nogo-66-mediated de-phosphorylation of ERK and MEK**

Mouse cortical neurons cultured in growth-promoting neurobasal medium containing B-27 supplement were cultured for 1 h with recombinant Nogo 66 with or without recombinant LILRA3, and phosphorylation of potentially relevant kinases was assessed (Maeda et al., 1999; Nash et al., 2009). Neurons cultured on Nogo-66-coated dishes showed marked suppression of extracellular signal regulated kinases 1 and 2 (ERK1/2, also known as MAPK3 and MAPK1) and mitogen-activated protein tyrosine kinase kinase 1 and 2 (MEK1/2, also known as MAP2K1 and MAP2K2) phosphorylation compared to neurons cultured on control His-tag-coated dishes (Fig. 7; \( n=2 \)). Importantly, Nogo-66-mediated suppression ERK1/2 and MEK1/2 phosphorylation was completely reversed by co-culture with LILRA3 (Fig. 7). There was slight suppression of p38 MAPK phosphorylation in response to Nogo 66, that was also reversed by LILRA3, whereas AKT phosphorylation was minimally affected by any combination of Nogo 66 with or without recombinant LILRA3 (Fig. 7).

**DISCUSSION**

Clinical association studies (Koch et al., 2005; Kabalak et al., 2009; Ordóñez et al., 2009; An et al., 2010; Du et al., 2014) and limited in vitro experiments (Lee et al., 2013) suggest that soluble LILRA3 might have immune regulatory roles in chronic inflammation but its exact functions are unclear, primarily due to insufficient knowledge of its natural ligands. Here, we used mammalian-produced recombinant LILRA3, with a structure typical of the native protein (Lee et al., 2013), as bait, and reproducibly identified three binding proteins with high MASCOT scores using mass peptide sequencing. These corresponded to Nogo 66, 67-kDa laminin receptor and HLA-B. Identification of the MHC class I protein confirmed earlier findings showing that LILRA3 bound to...
single HLA-B- or HLA-C-coated beads (Jones et al., 2011). However, this is the first report to show concomitant binding of LILRA3 to an MHC-class I and two new non-MHC ligands, raising the possibility of multiple ligands of varying affinities. Consistent with this, the closely related cell surface receptor, LILRB2 has been shown to functionally interact with various MHC class I proteins (Cosman et al., 1997; Colonna et al., 1998; Shiroishi et al., 2003) and non-MHC ligands with varying affinities including interaction with angiopoietin-like proteins (Zheng et al., 2012) and β-amyloid (Kim et al., 2013). Of particular interest in our study was the high-affinity interaction of LILRA3 with Nogo 66, suggesting that LILRA3 might have new functions in the CNS, distinct from its expected immunoregulatory roles in leukocytes (Lee et al., 2013). We rigorously validated this interaction using several independent methods, including SPR, biochemical and immunochemical techniques (also see Fig. S4). Extensive ligand-binding studies showed that LILRA3 specifically bound to primary mouse cortical neurons with high affinity, and binding was saturable and specific. We confirmed that these neurons expressed high levels of Nogo A, and successful abrogation of binding by anti-Nogo antibody and following Nogo A gene silencing validated that LILRA3 binding was in the most part mediated by Nogo A. Interestingly, LILRB2, which has 82% sequence identity to LILRA3 (Borges and Cosman, 2000), and mouse PIRB also bind Nogo 66 (Atwal et al., 2008), suggesting a shared ligand.

Nogo 66 inhibits neurite outgrowth through interaction with PIRB (Atwal et al., 2008), although PIRB-mediated signalling events in neurons remain unclear. Based on our knowledge of PIRB-mediated inhibitory signalling pathways in leukocytes (Maeda et al., 1999), we propose that binding of Nogo 66 to PIRB might inhibit neurite outgrowth by de-phosphorylating growth-promoting protein tyrosine kinases (Figs 3A and 8A). Indeed, ERK1/2 and MEK1/2 phosphorylation in mouse cortical neurons cultured on recombinant Nogo-66-coated coverslips was profoundly suppressed and this was associated with reduced neurite outgrowth. Importantly, recombinant LILRA3 markedly reversed Nogo-66-mediated suppression of ERK1/2 and MEK1/2 phosphorylation and promoted neurite outgrowth. Effects of Nogo
66 on p38 MAPK phosphorylation were modest and there was little effect on AKT phosphorylation suggesting a more specific regulation of the ERK/MEK pathway. To our knowledge this is the first report showing Nogo-66-mediated suppression of ERK and MEK phosphorylation in primary neurons that was successfully reversed by LILRA3. Although further investigation is required, we speculate that suppression of ERK and MEK phosphorylation in Nogo-66-treated neurons occurs through its ligation of PIRB, which in turn promotes recruitment of SH2-domain-containing protein tyrosine phosphatases (SHP-1 and/or SHP-2, also known as PTPN6 and PTPN11, respectively) thereby deactivating signalling and causing neuronal growth arrest (Fig. 8A). Consistent with this, ligation of PIRB on the surface of neurons restricts plasticity in the visual cortex (Syken et al., 2006) and suppresses axonal regeneration (Fujita et al., 2011a) through recruitment of SHP-1 and/or SHP-2, and limited data indicates that the recruited SHP-1 and/or SHP dephosphorylate (deactivate) tropomyosin receptor kinase B (TrkB, also known as NTRK2) leading to suppression of downstream MAPK signalling (Nash et al., 2009).

In addition to PIRB, Nogo 66 can inhibit neurite outgrowth by interacting with NgR1 that forms a complex with transmembrane proteins LINGO1, and p75 (also known as NGFR) or TROY (also known as TNFRSF19) to increase intracellular Ca²⁺ and activate the small GTPase RhoA and its effector protein Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) eventually leading to growth arrest (Fournier et al., 2003; Nash et al., 2009; Schwab, 2010). LILRA3-mediated reversal of neurite growth might also in part be due to competitive blocking of the Nogo-66–NgR1 interaction by soluble LILRA3 thereby regulating this pathway (Fig. 8A,C), although this requires further research. Interestingly, p75 enhances the ability of PIRB to recruit SHP-1 and SHP-2 (Fujita et al., 2011b), suggesting a cross-talk between the NgR1 and PIRB signalling pathways.

The reversal of Nogo-66-mediated inhibition of neurite outgrowth by LILRA3 was similar in human and mouse cortical neurons, strongly validating our finding. It is possible that LILRA3 exerts positive effects in human neurons by competitively blocking the LILRBB2–Nogo-66 interaction, although this requires further investigation. Importantly, this study is the first to demonstrate a human LILR protein having cross-species functions in mice and man, despite lack of LILRs in rodents, likely due to the high similarity of the shared Nogo 66 ligand. This property would allow more thorough investigation of LILR functions using an unlimited source of cultured primary mouse neurons, and opens for the first time opportunities to explore in vivo LILR functions, such as the use of tissue-specific transgenic expression of human LILRA3 in rodents, to investigate its role in the CNS.

Here, we provide evidence that LILRA3 as a new high-affinity soluble receptor consistently and significantly reversed Nogo-66-mediated inhibition of neurite outgrowth and synapse formation in vitro by 60–70%. These effects are comparable to or better than other known inhibitors, including antibodies against Nogo A, Nogo A gene deletion, the use of soluble NgR fragments, NgR-blocking peptides, inhibitors of Rho-A and ROCK or inhibitors of intracellular Ca²⁺ influx (Schwab, 2004). Importantly, unlike these inhibitors, LILRA3 is an endogenous protein making it an attractive therapeutic candidate. We propose that LILRA3 might act as a broad-spectrum competitive antagonist to a range of inhibitory Nogo receptors including PIRB/LILRB2 and NgR1 (Fig. 8A–C). Although methodological differences should be accounted for and future concurrent comparative studies are required, our SPR results indicate that the binding affinity of Nogo 66 to LILRA3 is 10–100 times higher than that of Nogo A.
times higher than its binding to PIRB (Matsushita et al., 2011) or NgR1 (Lauren et al., 2007). These properties are consistent with a typical soluble competitive antagonist with broad functional specificity. Interestingly, we found constitutive expression of LILRA3 in neurons derived from fetal cortical brain and in normal adult brain tissue, suggesting physiological functions (Fig. S3). Future studies that systematically map expression patterns and identify cellular sources of LILRA3 and define its relationships to Nogo A in the brain during health and disease could provide new insights into its pathophysiological roles in the CNS.

MATERIALS AND METHODS

Production of recombinant proteins

C-terminal placental alkaline-phosphatase-tagged (LILRA3–AP), 6xHis-tagged (LILRA3–His) and untagged high-quality full-length LILRA3 recombinant proteins were produced in 293T HEK cells as described previously (Lee et al., 2013). Large-scale therapeutic grade LILRA3–His recombinant protein in PBS was custom-made in 293FT HEK cells in collaboration with the Commonwealth Scientific and Industrial Research Organisation (CSIRO, VIC, Australia). Purified human IgG1 was purchased from Jackson ImmunoResearch (PA, USA) and used as negative control to LILRA3. Human C-terminal 6xHis-tagged Nogo 66 (Nogo-66–His) was subcloned from Nogo 66 in pGEX2 (Pei-hua Lu, Shanghai University, China) into pET30 EK/LIC (Novagen, Darmstadt, Germany) using forward primer 5′-GACGACGACAGATGAGGATATACAAGGGT-3′ and reverse primer 5′-GAGGAGAAGCCCGGTTCACTTCAGAGAATC-3′ and protein was expressed in BL21 DES E. coli cells. Soluble recombinant Nogo-66–His protein was purified using fast protein liquid chromatography (FPLC, BioLogic DuoFlow, Bio-Rad, NSW, Australia) and reverse-phase high-pressure liquid chromatography 600S HPLC (Waters Corporation, MA, USA) on a C8 column (Sigma, MO, USA). Aliquots of purified Nogo-66–His were resuspended in water (1 mg/ml), tested for lipopolysaccharide (LPS) and used within 1 week. 6xHis-tag peptide in pET30 EK/LIC was expressed BL21 DES E. coli cells, purified as above and used as the relevant control.

Identification of LILRA3-binding proteins from peripheral blood mononuclear cells by mass spectrometry

Peripheral blood mononuclear cells (PBMCs) from healthy donors were lysed in a non-detergent lysis buffer using N2-cavitation (Hartgroves et al., 2003). In brief, 106 PBMCs were washed once in H-buffer (10 mM HEPES pH 7.2, 250 mM sucrose, 2 mM MgCl2, 10 mM NaF and 1 mM vanadate) and suspended in 3 ml H-buffer containing protease inhibitor cocktail (Roche, Australia), then dissociated using a Nitrogen cavitation chamber at 50 bar, 4°C for 10 min (Parr Instrument Company, IL, USA). Plasma membrane from cell homogenates was separated by sucrose gradient ultracentrifugation (Gaus et al., 2001). Protein concentrations were adjusted...
to 5 mg/ml in HBHA ligand-binding buffer (HBSS plus 0.5 mg/ml BSA, 0.1% NaN₃, 20 mM HEPES and protease inhibitors, pH 7.0), 500 µl (2.5 mg) membrane protein was incubated with 500 nM LILRA3–AP (experimental) or alkaline phosphatase (control) protein for 90 min at room temperature, and LILRA3–AP bait was co-immunoprecipitated with LILRA3-binding proteins by incubating samples with 20 µg (20 µl) Sepharose-conjugated anti-placental alkaline phosphatase monoclonal antibody (catalogue number Q332, GenHunter, TN) for 2 h at 4°C. Sepharose-bound proteins were precipitated by centrifugation and washed twice with Tris buffer (10 mM Tris-HCl, pH 8, 140 mM NaCl and 0.025% NaN₃) containing 0.1% Triton X-100 and 0.1% bovine haemoglobin (Sigma, NSW, Australia), followed by 4 washes with Tris buffer and a final wash with 50 mM Tris-HCl, pH 6.8. Sepharose bead pellets from experimental and control samples were resuspended in 30 µl SDS-PAGE gel loading buffer containing 10 mM DTT, boiled at 100°C for 5 min and loaded onto a 10% one-dimensional SDS-PAGE gel and run under reducing conditions. Gels were washed three times with Tris-buffered saline (TBS) then silver stained, and excised bands sent to the Bioanalytical Mass Spectrometry Facility at University of New South Wales for tryptic digest and peptide mass sequencing using Nano LC-MS/MS. Comparisons of experimental and theoretical tandem mass spectra were automatically performed by Mascot version 2.0 (http://www.matrixscience.com), which scored peptide matches and correlated with protein identifications against Homo sapiens proteins in the Swissprot database. Precursor tolerances were 4.0 ppm and product ion tolerances±0.4 Da; acceptable cut-off scores for individual MS/MS spectra were set to 20 (Lee et al., 2013).

Surface plasmon resonance

SPR experiments were performed using a BIAcore 2000 (BIAcore, Uppsala, Sweden) to determine the equilibrium dissociation constant (Kₐ) between Nogo 66 and LILRA3. Briefly, negatively charged recombinant Nogo 66 was ‘physiosorbed’ onto a research-grade positively charged untreated gold surface sensor chip (Sensor Chip AU, GE Healthcare, NSW, Australia) by a hydrophobic interaction at a flow rate of 5 µl/min to 500–600 response units (RU) as described previously (Melrose et al., 2006). After blocking sensor chips with BSA at a flow rate of 20 µl/min, chips were equilibrated in running buffer (PBS). Serially diluted recombinant LILRA3–His (20–200 nM) in PBS was injected over the immobilised flow cells at a rate of 20 µl/min for 3 min at 25°C. Binding responses with various concentrations were subtracted from the nonspecific responses to an empty flow cell. Kinetic constants were calculated with the BIA evaluation program (version 3.0.2; BIAcore).

Co-immunoprecipitation of Nogo 66 with LILRA3

Recombinant LILRA3–His (10 µg in 100 µl PBS) was immobilised onto 50 µl ConA-conjugated Sepharose (ConA, GE Healthcare, NSW, Australia) at 4°C overnight. After removing unbound protein by gentle centrifugation (200 g for 5 min at 4°C), beads were washed five times with cold 20 mM Tris-HCl, 150 mM NaCl, pH 7.4, and resuspended in 50 µl cold HBHA buffer. Recombinant Nogo-66–His (1 µg in 100 µl water) was then added to the bead slurry with or without immobilised LILRA3 and incubated for 2 h at 4°C. Unbound protein was then removed by centrifugation (800 g for 5 min at 4°C), and beads washed four times with Tris buffer and once with 0.1% NaN₃.
**Fig. 8. Summary of the proposed mechanisms of LILRA3-mediated promotion of neurite outgrowth and synapse formation.** (A) Soluble LILRA3 might block ligation of PIRB by Nogo 66 thereby preventing SHP-1 and/or SHP-2-mediated inhibitory signals by dephosphorylating (deactivating) key growth-promoting protein tyrosine kinases (left); similarly, LILRA3 might prevent ligation of NgR1 complex by Nogo 66 and the subsequent transduction of inhibitory signals (right). LILRA3 might therefore act as a soluble antagonist of PIRB and/or NgR by competitively binding to Nogo 66. (B, C) Representative immunofluorescence staining showing that both NgR and PIRB (green) are expressed on the surface of βIII-tubulin-positive (red) mouse cortical neurons cultured for 3 days (n≥6). Images acquired in 1524×1024 pixel array using Leica TCS SP5cw STED microscope and a 100× HCX Plan Apo NA 1.4 objective (Mannheim, Germany).

50 mM Tris-HCl, pH 6.8. Samples were separated by 10% SDS-PAGE under reducing conditions, transferred onto PVDF membranes and blocked with 5% low-fat milk plus 2% BSA in TBS for 2 h. Membranes were then immunoblotted with a rabbit antibody that recognises Nogo A and Nogo B (IMG-5346A, 1.5 μg/ml, Imgenex, CA, USA) at 4°C overnight followed by four washes with TBS + 0.1% Tween 20 (TBST) then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (172-1034, 1:5000 dilution; Bio-Rad, NSW, Australia) for 2 h at room temperature. After four washes with TBST, immunoreactive bands were detected using Western Lightning Plus chemiluminescent substrate (PerkinElmer, MA) and images were acquired with a ImageQuant™ LAS4000 (GE Healthcare Life Sciences). Membranes were then washed four times with TBST then reblotted with mouse anti-LILRA3 monoclonal antibody (1 μg/ml; Abnova, Taipei, Taiwan) 4°C overnight followed by HRP-conjugated goat anti-mouse antibody (1:10,000 dilution; Bio-Rad, NSW, Australia) for 2 h at room temperature, washed four times and immunoreactive bands detected as above.

**Binding of Nogo 66 to plate-immobilised LILRA3**

96-well flat bottom Nunc MaxiSorp ELISA plates were equilibrated with 100 μl 0.05 M carbonate-bicarbonate buffer, pH 9.6 for 30 min at room temperature, then coated with increasing concentrations (0–30 nM) of untagged recombinant LILRA3 or maximum amounts (30 nM) of human IgG control in 200 μl TBS and incubated overnight at 4°C. Unbound proteins were aspirated, wells washed twice in TBS then 20 nM recombinant Nogo-66–His or 20 nM His tag control in 200 μl TBS was added, and plates incubated for 2 h at room temperature. After removing unbound protein, wells were blocked with 5% BSA in TBS containing 0.05% Tween-20 (TBST) for 30 min at room temperature, washed three times with 200 μl/well TBST, then 50 μl mouse anti-His monoclonal antibody (0.5 μg/ml; Novagen, CA) diluted in TBST plus 5% BSA was added to each well and incubated at 4°C overnight. Wells were washed three times with TBST, incubated at room temperature for 2 h with 50 μl biotinylated goat anti-mouse antibody (1 μg/ml; DAKO, Glostrup, Denmark) in TBS, then washed three times with TBST and incubated with 50 μl Streptavidin–HRP (1:200 dilution; R&D Systems, MN) in TBS for 20 min at room temperature in the dark. After four washes with TBST, 100 μl of TMB substrate was added and plates were incubated in the dark for 1 h at room temperature, the reaction was stopped with 50 μl 1 M H2SO4 and optical density at 450 nm and 540 nm was measured (SpectraMax Plus plate reader, Molecular Devices, CA, USA).

**Primary mouse and human cortical neuron cultures**

Primary mouse cortical neurons were derived in vitro as described by us previously (Fath et al., 2009). Briefly, C57B6 mice and cerebral cortices carefully dissected, enzymatically digested and sequentially triturated using a wide tip then a narrow fire-polished glass pipette. Dissociated neurons in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS were then carefully seeded (105 cells) onto poly-D-lysine (PDL)-coated wells or 12-mm coverslips. After 2 h, the medium was changed to neurobasal medium supplemented with 2% B27 and 2 mM Glutamax (Life Technologies, VIC, Australia), incubated at 37°C in 5% CO2 air. The University of New South Wales Animal Ethics Committee approved the use of mouse fetal brain to derive cortical neurons in culture (reference number 13/119A).

Human fetal brains were obtained from 14–19-week fetuses after therapeutic termination. The collection of fetal brain tissue to derive human cortical neurons in culture was approved by Macquarie University Human Ethics Committee (reference number 5201300330). Informed consent was obtained from all donors and the investigations were conducted according to the principles expressed in the Declaration of Helsinki. Neurons were prepared and phenotyped according to our established protocol (Guillemin et al., 2005, 2007). Briefly, 1–2 g cortical brain tissue was washed in PBS with 2% antibiotics and antimitotics (Life Technologies, VIC, Australia) then dissociated using neural tissue dissociation kits (Miltenyl Biotec, NSW, Australia). Cells were filtered through 40 μm nylon mesh, centrifuged (350 g for 10 min, at room temperature) and resuspended in complete neurobasal medium containing 2% B27, 2 mM Glutamax, 50 mM HEPES, 200 IU/ml penicillin G, 200 μg/ml streptomycin sulfate, and 5 mM glucose (Life Technologies, VIC, Australia) at 2×105/ml, seeded onto PDL-treated Matrigel-coated (Life Technologies, VIC, Australia) 24-well plates or 12-mm coverslips and then incubated at 37°C in 5% CO2 and 95% air.

The LIVE/DEAD® assay kit that detects live cells that convert non-fluorescent Calcein AM into green fluorescent dye (fluorescence emission 510 nm) and dead cells that uptake EthD-1 (fluorescence emission at 645 nm) routinely showed >90% live cultured human neurons and >95% live mouse cortical neurons (Molecular Probes, OR). Treatment of these neurons with recombinant LILRA3 and/or recombinant Nogo 66, His tag control or anti-Nogo blocking antibody did not affect viability.

**Nogo A gene silencing**

Four Nogo shRNA constructs targeting Nogo A and B, and one scrambled shRNA in psi-LVRU6MP lentiviral vector containing mCherry fluorescent tag were custom-made by GeneCopoeia (MD). Nogo shRNA sequences were designed based on GeneBank accession number NM_024226.3. High-titre shRNA containing lentiviruses were produced in 293FT as described (Life Technologies, VIC, Australia). Freshly prepared viral supernatants were used to infect 105 primary mouse cortical neurons in six-well PDL-coated plates. In brief, neurons were cultured for 24 h in 3 ml complete neurobasal medium and then infected with 0.25 ml virus [multiplicity of infection (MOI)=1]. At 5 days after infection, neurons were used for RNA isolation, protein extraction or binding assays. After initial screening of all four shRNA constructs for Nogo A and B mRNA knockdown, we found that the Nogo A and B shRNA construct with target sequence 5′-GGCGCAGATAGATCATTATCT-3′ achieved the maximum Nogo A and B gene silencing (>70%). Gene silencing was validated by quantitative real-time PCR (qRT-PCR) using Nogo A primer sets (forward
and vector alone were used as controls.

Assessment of neurite outgrowth in cortical neurons

PDLC-coated or PDL plus Matrigel-coated 24-well plates were first spotted with 2.5 µl (10 nM/spot) recombinant Nogo-66·His (total 48 spots/well) or His-tag control in duplicates and dried at 37°C for 1 h as described (GrandPré et al., 2000; Fournier et al., 2001). Wells were then rinsed with PBS and incubated overnight at 4°C with 150 µl recombinant LILRA3·His or human IgG in PBS (each 100 nM). Unbound proteins were aspirated; wells rinsed with 500 µl neurobasal medium and seeded with primary mouse or human cortical neurons at 5×10³/well in 500 µl or 2×10³/well in 1 ml complete neurobasal medium, respectively. Seeded mouse and human neurons were cultured at 37°C, in 5% CO₂ in air in a humidified incubator for 4 or 8 days, respectively, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, rinsed and resuspended in PBS. Contiguous phase-contrast digital images of the entire well at 20× and 40× objective were acquired using a Olympus CKX41 microscope mounted with Q Imaging 3.3 RTV camera and Olympus CellSens software version 1.8 (Olympus, VIC, Australia). The neurite lengths of randomly selected neurons in each well were then assessed by an independent observer using computer-assisted imaging (NIH-Image2) as described previously (Curthoys et al., 2014). Neurite lengths of 30 neurons per treatment per experiment were measured for mouse neurons (n=7), and lengths of 20 neurons per condition in duplicates were measured for human neurons (n=4).

Binding of LILRA3-AP to mouse cortical neurons

In situ binding of LILRA3-AP or alkaline phosphatase control protein to mouse cortical neurons cultured on coverslips was assessed as described previously (Lee et al., 2013). For antibody-blocking, neurons on coverslips were pre-incubated for 30 min at room temperature with 10 µg/ml of the rabbit antibody against Nogo A and NogoB in HBHNA without BSA, rinsed and incubated with LILRA3-AP or alkaline phosphatase control as described previously (Lee et al., 2013). Positive blue-stained neurons counterstained with 1% neutral red were visualized using an Olympus BX51 light microscope (Olympus, VIC, Australia). For quantitative binding studies, units of alkaline phosphatase activity were calculated from an image using ImageJ software (NIH). The neurite lengths of 30 neurons per well were then assessed by an independent observer using computer-assisted imaging (NIH-Image2) as described previously (Curthoys et al., 2014). Neurite lengths of 30 neurons per treatment per experiment were measured for mouse neurons (n=7), and lengths of 20 neurons per condition in duplicates were measured for human neurons (n=4).

Immunofluorescence staining

To compare effects on synapse formation, mouse cortical neurons were cultured for 21 days on PDLC-coated 12-mm coverslips in four-well plates that were spotted (28 spots/coverslip; 10 nM/spot) with recombinant Nogo 66 with or without recombinant LILRA3·His with or without IgG control then fixed with 4% paraformaldehyde in PBS for 10 min. After 10 min permeabilisation with 0.1% Triton X-100 in PBS and incubated overnight at 4°C with rabbit anti-synaptophysin monoclonal antibody (2 µg/ml; Sigma, NSW, Australia) as a presynaptic marker and mouse-anti-PSD 95 (1 µg/ml; Chemicon, CA, USA) primary antibodies. This was followed by four washes and a 2-h incubation at room temperature with goat Alexa-Fluor-488-conjugated anti-rabbit-IgG and Alexa-Fluor-555-conjugated donkey anti-mouse-IgG antibodies (Molecular Probes, OR, USA) diluted in 0.1% Triton X-100 in PBS, respectively. Coverslips were then washed and incubated with chicken anti-β III tubulin Ab (1.2 µg/ml; Millipore, VIC, Australia) for 2 h at room temperature. After four washes, coverslips were incubated with goat anti-chicken Alexa-Fluor-647-conjugated secondary antibody (1:500 dilution; Molecular Probes, OR) for 2 h at room temperature, washed and wet mounted using ProLong® Gold Antifade reagent containing DAPI (Molecular Probes, OR). Images were acquired in 1024×1024 pixel array using a Leica TCS SP5eW STED microscope with a 100× HMX Plan Apo NA 1.4 objective (Mannheim, Germany). Numbers of synaptic contacts, defined as synaptophysin-positive pre-synaptic terminals (green) in direct contact with PSD95-positive post-synaptic protein (red) in 77.5±7.5 µm area =1 field of view), were quantified using a custom-written MATLAB code (available on request). In brief, images from both channels were normalised to the maximum pixel value, before their intensity ratio was calculated to extract regions of the image with high colocalisation. In each channel, only pixels with values of two standard deviations above the average background were counted. Intensity ratio images were then processed identifying numbers of synapse contacts by two approaches, first using a spot detection and, second, using a water-shedding algorithm to segment and identify individual clusters (Klotzsch et al., 2015); both yielded similar results. Six randomly selected fields per sample per treatment in three independent experiments were measured.

Western blot analysis of signalling molecules regulated in Nogo-66-mediated inhibition

Cortical neurons derived from E16.5 mouse embryos in neurobasal media supplemented with 2% B27 and 2 mM Glutamax were cultured for 1 h on PDLC-coated 6-cm dishes spotted with recombinant Nogo-66·His and control IgG, recombinant LILRA3·His and control IgG, His-tag control and LILRA3 or His-tag control and control IgG as described above. Dishes were then rinsed with 3 ml PBS and lysed in 300 µl cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA and 1% NP-40) containing 2 mg/ml protease inhibitors (Roche Applied Science) and 10 µM pervanadate (Sigma). Protein concentrations in cell lysates were determined using a BCA assay as described by the manufacturer (Thermo Scientific). Four sets of 20 µg cell lysate from each sample were separated by 10% SDS-PAGE under reducing conditions then transferred onto PVDF membranes. Membranes were blocked by 5% BSA in TBST (TBS with 0.1% Tween 20) for 2 h at room temperature then incubated with the primary rabbit antibodies against the following: anti-phosphorylated (p)-ERK1/2 [p-P44/42 MAPK (Erk 1/2) (Thr202/Tyr204), cat. no. 9101, 1:1000], anti-p-P38 MAPK (Thr180/Tyr182) (cat. no. 9215, clone 3D7, 1:1000), anti-p-MEK1/2 (Ser27/221) (cat. no. 9154, clone 41G9, 1:1000) or anti-p-AKT (Thr808) (cat. no. 2965, clone C315E, 1:1000) in TBST (Cell Signaling Technology, MA, USA) overnight at 4°C. Membranes were washed three times with TBST then incubated with goat HRP-conjugated anti-rabbit-IgG secondary antibody in TBST (Bio-Rad) for 2 h at room temperature, followed by three washes in TBST and immunoreactive bands detected using chemiluminescent substrate (Perkin Elmer). Images were acquired using ImageQuant™ LAS4000 on auto-setting (GE Healthcare Life Sciences). Membranes were then stripped using 62.5 mM Tris-HCl (pH 6.7), 100 mM β-mercaptoethanol (Sigma) in 2% SDS for 30 min at 50°C, rinsed thoroughly in TBS then blocked with the blocking buffer for 2 h at room temperature. Total ERK, p38, MEK or AKT was detected using rabbit anti-total ERK1/2 [p44/42 MAPK (Erk1/2, cat. no. 9102, 1:1000), anti-total p38 MAPK (cat. no. 9212, 1:1000), anti-total MEK1/2 (cat. no. 9122, 1:1000) or anti-total AKT antibodies (cat. no. 9272, 1:1000) (Cell Signaling Technology) as above. GAPDH protein as a loading control was detected using rat anti-GAPDH monoclonal antibody.


SUPPLEMENTARY FIGURES

Fig. S1. Nogo 66 is identified as LILRA3 binding protein by peptide mass spectrometry sequencing. (A) Isolation of LILRA3 binding molecules using recombinant LILRA3-APtag as bait and anti-AP mAb for co-immunoprecipitation of complexes from blood mononuclear cell membranes. Recombinant APtag protein control showed four major components (representative of three independent experiments). (B) In-gel trypsin digests and peptide sequencing by mass spectrometry of bands from the recombinant LILRA3-APtag bait yielded high-score matches with Nogo A, HLA-B and 67 kDa laminin receptor that were not detected in corresponding gels slices excised from the control recombinant APtag bait. As expected, the recombinant LILRA3-APtag bait was also detected in multiple bands when used as bait. Similarly, recombinant APtag was detected in all four major components in lane 1 (A) when recombinant APtag control was used as bait (n=3).
Fig. S2. LILRA3 increased numbers of synaptic contacts in mouse cortical neurons cultured for 14 days. (A, B) Representative primary mouse cortical neurons cultured for 14 days on PDL-coated coverslips that were spotted with recombinant Nogo 66 plus LILRA3 had increased numbers of pre-synaptic terminals stained with anti-synaptophysin Ab (green dots), post synaptic structures stained with anti-phalloidin-Alexa 555 (red dots) and synaptic contacts (red and green dots indicated by yellow arrows) along the neuronal dendrites stained with anti-β-III tubulin (purple) compared to significantly lower synaptic contacts in neurons cultured on Nogo 66 spotted coverslips (C). (D) His-tag control treated neurons showed similar numbers of synaptic contacts to (B). (E) Summary of numbers of synaptic contacts in neurons cultured under the different treatment conditions. Data are presented as means ± SEM of 5 independent experiments (**p<0.0, one way ANOVA).
Fig. S3. LILRA3 is expressed in cultured primary human cortical neurons and normal human brain. (A) Representative immunofluorescence staining showing LILRA3 expression on foetal primary human cortical neurons cultured for 10 days on coverslips, and co-stained with a neuron marker, MAP-2, and DAPI nuclear staining (n=4; 630x magnification). (B) Neurons incubated with corresponding negative control primary but the same secondary Abs as in A and B confirming absence of non-specific staining. (C) RT-PCR using mRNA from human primary cortical neuron 8 day cultures showing specific LILRA3 transcript; mRNA from PBMC was used as positive control, and no template RT-PCR was a negative control. (D) Quantitative RT-PCR shows measurable but significantly less LILRA3 mRNA in cultured primary cortical neurons compared to PBMC (n=3; ***p<0.001, one way ANOVA). LILRA3 primer sets (forward 5'-AATCAAAGCGCCAATCTC AT-3' and reverse primers 5'-GAGTCAGCAGGTAGGGGTTG-3') and β-actin housekeeping gene (forward: 5'-CATGTACGTTGCTATCCAGGC-3' and reverse primer: 5'-CTCCTTAATGTCACGCACGAT-3') were used and reactions were run on LightCycler 480 Real-Time PCR System (Roche, NSW, Australia). (E) Immunoprecipitation of LILRA3 from pooled normal brain tissue lysates using anti-LILRA3 showing specific band but not when isotype-matched control mAb was used (n=1). Cortical (ab30061) and cerebellar (ab30078) protein lysates (100 µg each) were purchased from Abcam (VIC, Australia). This is the first demonstration of LILRA3 mRNA and protein expression in tissue/cells other than leukocytes.
Fig. S4. Conformation of endogenous LILRA3 binding to endogenous cell surface Nogo.

Endogenous LILRA3 protein was enriched using protein A Sepharose from lysates of primary monocytes treated with recombinant IL-10 + Brefeldin A in vitro for 72 h. The enriched LILRA3 was used as bait to pulldown endogenous Nogo A/B from the surface of SKNSH neuronal cell line using anti-LILRA3 mAb; control IgG1 mAb was used as negative control. Immunoprecipitates were then sequential Western blotted using rabbit anti Nogo A/B (Imgenex) (top panel) and an in-house rabbit anti-LILRA3 Abs (bottom panel) and showed co-precipitation LILRA3 with Nogo A/B in samples pulled down with anti-LILRA3 mAb (lanes 2 and 3) but not when control IgG1 was used (lane 1) (n=1); lane 4 = LILRA3 in 2.5µl protein A Sepharose beads (total volume 50µl) prior to elution with 0.2M glycine. This confirmed successful LILRA3 binding to the beads; lane 5 = 15µl of the starting 1.5ml SKNSH lysate to determine input signal intensities and served as positive control for anti-Nogo A/B Western blot, lane 6 = blank. Lanes 7 and 8 are aliquots (15µl) of Sepharose-enriched LILRA3 that were eluted in 500µl of glycine prior to addition to SKNSH cell lysates. These confirmed successful elution of LILRA3 from the beads and sever as a positive control for anti-LILRA3 Western blotting.

Briefly, fresh peripheral blood monocytes (2x10^7/treatment) from a healthy blood bank donor
were cultured for 72 hours in the presence of 25 ng/ml recombinant IL-10 with 5µg/ml Brefeldin A. Monocytes were then non-detergent lysed in PBS by sonication; LILRA3 in the soluble fraction was bound to 50µl of protein A Sepharose beads and eluted with 0.2M glycine, pH 2.7 and collected directly into PBS pH 8.5-containing Eppendorf tubes. The enriched endogenous protein was then bound to the surface of SKNSH neuronal cell line (1x10⁷/treatment) that express endogenous Nogo for 90 min at RT. This was followed by 15 min incubation with mouse anti-LILRA3 mAb (Abnova) or negative control mouse IgG, both conjugated to sheep anti-mouse Dynal bead secondary Ab (Dynal) on ice and non-detergent lysis of cells using a N₂ cavitation chamber (J Biol Chem. 2003, 278, 20389). SKNSH membrane proteins that bound the LILRA3 bait that specifically reacted with anti-LILRA3 mAb or non-specifically bound to the control IgG were immunoprecipitated using a magnetic stand, resolved in 10% SDS PAGE under reducing conditions, transferred to a PVDF membrane and sequentially Western blotted using anti-Nogo A/B (Imgenex) and anti-LILRA3 (in-house) Abs.