Neuregulin-erbB signalling promotes microglial proliferation and chemotaxis contributing to microgliosis and pain following peripheral nerve injury

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Abstract
A key component in the response of the nervous system to injury is the proliferation and switch to a ‘pro-inflammatory’ phenotype by microglia (microgliosis). In situations where the blood brain barrier is intact microglial numbers increase via the proliferation and chemotaxis of resident microglia, however there is limited knowledge regarding the factors mediating this response. Following peripheral nerve injury a dorsal horn microgliosis develops which directly contributes to the development of neuropathic pain. Neuregulin-1 is a growth and differentiation factor with a well characterised role in neural and cardiac development. Microglia, express the NRG1 receptors erbB 2, 3 and 4 and NRG1 signalling via the erbB2 receptor stimulated microglial proliferation, chemotaxis, survival as well as interleukin-1β release in vitro. Intrathecal treatment with NRG1 resulted in microglial proliferation within the dorsal horn and these cells developed an activated morphology. This microglial response was associated with the development of both mechanical and cold pain related hypersensitivity. Primary afferents express NRG1 and following spinal nerve ligation (SNL) we observed both an increase in NRG1 within the dorsal horn as well as activation of erbB2 specifically within microglia. Blockade of the erbB2 receptor or sequestration of endogenous NRG following SNL reduced the proliferation, the number of microglia with an activated morphology and the expression of phospho-P38 by microglia. Furthermore consequent on such changes the mechanical pain related hypersensitivity and cold allodynia were reduced. NRG1-erbB signalling therefore represents a novel pathway regulating the injury response of microglia.

Keywords
microglia; proliferation/differentiation; apoptosis; chemotaxis; Neuregulin-1; pain

Microglia, are the resident myeloid cells of the CNS and respond rapidly to injury of the nervous system. This injury response (or ‘microgliosis’) has a number of different components including: microglial cell proliferation (Ajami et al., 2007;Echeverry et al., 2008), migration to the site of injury (Ifuku et al., 2007;Honda et al., 2001;Yao et al., 1990;Peterson et al., 1997), phagocytosis of cellular debris (Neumann et al., 2009), antigen
presentation (Fischer and Reichmann, 2001), adoption of an amoeboid morphology, and the release of a broad range of cytokines and chemokines (Milligan and Watkins, 2009). Microglia express receptors for and respond to a range of injury related molecules including cytokines, chemokines, complement components and purines. These molecules regulate different components of the injury response (a process which can be thought of as a continuum rather than an ‘all or nothing event’). The microglial response to peripheral nerve injury contributes to the generation of neuropathic pain and these cells are also implicated in disease processes as diverse as traumatic brain injury, neurodegeneration and demyelination (Hanisch and Kettenmann, 2007; Scholz and Woolf, 2007; Ransohoff and Perry, 2009). It has recently been demonstrated using chimeric animals obtained by parabiosis or bone marrow transplantation and selective irradiation that in situations where the blood brain barrier is intact the principal means by which microglial numbers increase is through the proliferation of resident microglia (rather than recruitment of blood born bone marrow derived microglial progenitors) (Ajami et al., 2007; Mildner et al., 2007). In this study we focus on the role of NRG1 in modulating microglial proliferation chemotaxis and cytokine release.

Neuregulin 1 (NRG1) is one of a family of growth factors (NRG1-4). NRG1 undergoes alternative splicing and differential promoter use to produce at least 15 isoforms which include both secreted and transmembrane forms (Esper et al., 2006; Falls, 2003). NRG1 has a key role in neural and cardiac development (Lee et al., 1995), it can modulate synaptic plasticity (Mei and Xiong, 2008) and it can stimulate the proliferation, survival and motility of a number of different cell types (Falls, 2003). All NRG1 isoforms contain an Epidermal Growth Factor (EGF)-like domain which is critical for mediating biologic activity and which binds to the tyrosine kinase receptors erbB3 and 4. These receptors subsequently heterodimerize with erbB2, which lacks a ligand binding domain but which is a key co-receptor in mediating signal transduction (Carraway, III and Cantley, 1994). ErbB3 lacks an active tyrosine kinase domain and so its interaction with erbB2 is essential for signalling. ErbB4 can signal via homodimers but dimerizes preferentially with erbB2 (Graus-Porta et al., 1997). Microglia are reported to express erbB 2, 3 and 4 receptors (Gerecke et al., 2001; Dimayuga et al., 2003)—however very little is known regarding the function of this signaling pathway within microglia. We therefore studied the effects of NRG1 on microglial survival, proliferation, chemotaxis and cytokine release in vitro and subsequently tested the hypothesis that this signalling pathway contributes to microgliosis and neuropathic pain following peripheral nerve injury in vivo.

Experimental procedures

Animals and surgery

Adult male Wistar rats were used in accordance with UK Home Office regulations. In one group of animals the left L5 spinal nerve was ligated and transected. Sham-operated animals were prepared in an identical manner but without injuring the nerve. To label dividing cells, rats were injected with 5-Bromo-2′-deoxyuridine (BrdU; Sigma dissolved in 0.007N NaOH/PBS, 100 mg/kg body weight i.p.) once daily for 3 consecutive days before perfusion and fixation.

Drugs and delivery

PD 168393 (Calbiochem) an irreversible erbB inhibitor was dissolved in 5% DMSO and delivered intrathecally at 1.25, 2.5, 5 or 10 μg/day by an Alzet osmotic pump (Cupertino, Model 2002). Control animals were given the same vehicle solution lacking the active compound. Anti-erbB2 antibodies (mAb 7.16.4 from Calbiochem and mAb-9 from Thermo Scientific, without Azide) were delivered intrathecally via lumbar puncture one hour before
nerve injury (2.5, 5 or 10 μg diluted in 25μL sterile PBS). For perineural application of mAB 7.16.4 to the site of nerve injury a 0.5 × 0.5 cm strip of oxidized regenerated cellulose (Surgicell, Ethicon) soaked in 10 μg of the antibody diluted in sterile PBS was applied directly over the site of spinal nerve ligation. Control animals received a non immune IgG2a antibody (Calbiochem) via the same method (i.t. or perineural respectively). To sequester endogenous NRG we used a fusion protein (HBD-S-H4) that was injected i.t every 3 days (3 μg in 20μl of sterile saline per injection). The drug doses were selected on the basis of previous reports and our preliminary studies. Neuregulin β1 EGF domain (rHRGβ1 aa177-244) was administered i.t. via lumbar puncture (0.4 or 4 ng in a volume of 20μl). To label dividing cells post NRG1 treatment, rats were injected with 5-Bromo-2′-deoxyuridine (BrdU; Sigma dissolved in 0.007N NaOH/PBS, 100 mg/kg body weight i.p.) 24 hr before perfusion and fixation. Before surgery animals were randomly allocated into experimental study groups (computer-generated randomization schedules). An independent investigator prepared the drugs individually and labelled them for each animal according to the randomization schedule. Operators and data analysts were blinded throughout the study.

**Behavioural testing**

Mechanical withdrawal thresholds were tested using a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy) which is an automated version of the von Frey hair assessment. A maximum cut-off of 50g was used. The withdrawal threshold is calculated as the average of three consecutive tests with at least 10 minutes between each test. To measure cold allodynia, we applied a drop of acetone to the plantar hindpaw and measured the time that the animal spent licking, shaking, or lifting the paw during the following 2 min (Kontinen and Dickenson, 2000).

**Histology**

After defined survival times, animals were terminally anaesthetized and transectardially perfused with 4% paraformaldehyde plus 1.5% picric acid in 0.1M phosphate buffer. The lumbar spinal cords and L5 dorsal root ganglia were excised, cryoprotected in 20% sucrose, cryostat cut (10 or 20 μm) and thaw-mounted onto glass slides. Spinal cord sections were incubated overnight with the primary antibody: rabbit anti-phospho-p38MAPK (1:100, Cell Signalling) or rabbit anti-phospho-erbB2 (p-Neu (Tyr 1248), 1:200, Santa Cruz BT), both of which were viewed by tyramide amplification (TSA™ Biotin System, Perkin Elmer) or with anti-rabbit Neuregulin-1 (H-210), Polyclonal Antibody (1:100, Santa Cruz Biotechnology). For co-localization studies the slides were then incubated with rabbit anti-Iba1 (1:1000, WAKO), mouse anti-OX42 (1:200, Serotec), rabbit anti-GFAP (1:1000, Dako), or mouse anti-NeuN (1:1000, Chemicon). DRGs sections were incubated overnight with the calcitonin gene-related peptide antibody (rabbit anti-CGRP, 1:2000, Sigma) and neurofilament 200 antibody (mouse anti-NF200, 1:1000, Chemicon, UK) or with anti-rabbit Neuregulin-1 (H-210). Following primary antibody incubation sections were washed and incubated for 1.5 h with corresponding secondary antibody solution. Isolectin B4 (IB4) detection was carried out using biotin-conjugated IB4 (Sigma, 1:50) and ExAvidin-AMCA (1:400, Vector Labs, UK). Slides were washed, cover-slipped with Vectashield mounting medium with or without DAPI (Vector Laboratories) and visualised under a Zeiss Axioplan 2 fluorescent microscope (Zeiss, U.K). The specificity of immunostaining was checked by the omission of the appropriate primary antibody or pre-incubation with the corresponding blocking peptide. Antibody detection of BrdU incorporated into DNA requires pre-treatment of the tissue to expose the BrdU epitope. For this purpose we used the antigen retrieval method described previously (Tang et al., 2007). Primary antibody solution contained mouse anti BrdU (1:200, BD;Biosciences) plus rabbit anti Iba-1 (1:1000, Wako) and the Secondary Antibody solution contained corresponding IgG-conjugated FITC 1:200 plus IgG-conjugated Cy3 1:400 (both from Stratech, UK).
Microglia cells and macrophages were 4% PFA fixed for immunohistochemistry. Microglia were double stained with erbB receptors antibodies (erbB 2/Neu(C-18), erbB3 (C-17) and erbB4 (C-18) rabbit, 1:100, Santa Cruz BT) and Isolectin B4 biotin conjugated (to label microglia, 1:50 Sigma) and visualized with corresponding secondary antibodies (Cy3 1:400 Stratech, UK for erbB receptors, extra-avidin FITC 1:200 Sigma, for IB4). For detecting MHC class II we used the monoclonal mouse anti-rat RT1B (clone OX-6) antibody from Serotec, UK (1:100). We used the ApopTag Fluorescein In Situ kit (Chemicon, USA) to identify apoptotic nuclei by terminal deoxynucleotidyl transferase-mediated X-dUTP nick end labelling (TUNEL). Microphages and microglial cultures purity was assessed staining cells with Iba1 and DAPI.

In Situ hybridization

ISH was performed after immunohistochemistry using 34-nucleotide long probes as previously described (Michael et al., 1997) The sequence and specificity of the NRG1 probe has been described before (Fricker et al., 2009) Oligonucleotides were chemically synthesized (Sigma-Genosys, UK) and radioactively end-labelled with 35S-dATP (Perkin-Elmer Life Sciences, Boston, MA, USA) using terminal deoxynucleotidyl transferase (Promega). Following pre-hybridization treatments (acetylation, dehydration, delipidation), the radioactive probe was added on the sections and hybridization was performed overnight at 37°C. The next day slides were washed in standard saline citrate solutions with increasing stringencies, rapidly dehydrated through graded alcohols, air-dried, dipped in autoradiographic emulsion (LM1, GE Healthcare, UK) and developed for 3–4 weeks.

Quantification and analysis

Quantitative assessment was carried out by determining the numbers of immunoreactive cells within 4 areas of 10,000 μm² in the superficial dorsal horn on 5-7 randomly selected L5 spinal sections from each animal. For BrdU staining the whole dorsal horn was analyzed. Microglia in which process length was less than double the soma diameter were classified as presenting an activated (or effector) morphology. Microglia in which the process length was double the soma diameter were classified as resting (or surveying) cells (Stence et al., 2001) (Figure 7). Cells were sampled only if the nucleus was visible within the plane of section and if cells profiles exhibited distinctly delineated borders. For quantification of dorsal root ganglia ISH, cells exhibiting grain density twice background levels were counted as exhibiting a positive hybridization signal. All analysis were performed with the operator blinded to treatment groups.

Detection of NRG1-β1 by ELISA

Ipsilateral dorsal horns were homogenized in lysate buffer (20 mM Tris, pH 8, 137 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, and protease inhibitor cocktail) and NRG1 was detected from lysates using a NRG1-β1 specific ELISA kit (detection range: 250-40000 pg/mL, DuoSet ELISA DY377, R&D Systems, UK). NRG1-β1 standards (250-40000 pg/mL) and 100 μl of dorsal horn lysate (50μg of protein) were run in duplicate following the manufacturer instructions. Samples were read at 450 nm with wavelength correction at 540 nm. Samples were considered NRG1-β1 positive if the signal was higher than background and within the range of the standard curve.

Primary microglia cell culture

Mixed glial cultures were isolated from cortex of P3 Wistar rats according to the method of Giulian and Baker (Giulian and Baker, 1986). After mechanical and chemical dissociation cells were seeded in DMEM with 10% FBS at a density of 500,000 cells/ml and cultured at 37°C in humidified 5% CO2/95% air. All reagents used were purchased from Invitrogen.
Medium was replaced every 2–3 days and confluency was achieved after 5 days in vitro. Confluent mixed glial cultures were manually shaken for 5 minutes and the floating cells were pelleted and subcultured. After 15 minutes of plating the medium was change to discharge all non adherent cells. This method resulted in 96-99% purity as assessed by Iba1 and DAPI staining. For proliferation assays GM-CSF (Granulocyte Monocyte Colony Stimulating Factor, Cellsciences, 1nM) was used as a positive control (Giulian and Ingeman, 1988). Neuregulin β1 EGF domain (rHRGβ1 aa177-244 Genentech) was used (0.5nM-10nM). NRG1, GM-CSF (1 nM) and negative control (only medium) were applied in triplicate. For blocking the erbB2 receptor we used PD168393 at 2.5, 5 or 10μM or mAb 7.16.4 at 1, 2 or 4μg/mL. BrdU 10μM was administered 15 hours before fixation to label proliferating cells.

**Peritoneal macrophage preparation**

Adult rats were killed with increasing CO2 concentrations and immediately after they were injected intraperitoneally with 20mL of sterile HBBS. The elicited peritoneal exudates cells were harvested and resuspended in DMEM supplemented or not with 10%FBS as required. Cells were plated at a density of 300,000 cells/ml and cultured at 37°C in humidified 5% CO2/95% air. For chemotaxis assays assays were used immediately after harvesting.

**Chemotaxis assay**

Chemotaxis was assessed using the Boyden chamber (Neuroprobe, Bethesda, MD). Polycarbonate filters (5 μm pore) were installed in the chamber, whose bottom wells were filled with serum-free DMEM or NRG1 at various concentrations. Freshly prepared microglia or macrophages were suspended in serum-free DMEM and were placed into the top wells (50000 cells/well). The chamber was kept in a CO2 incubator at 37°C for 3 hours. The filter was removed and stained with RapiDiffII (Biostain RRL,UK) The cells on the top side of the filter were wiped off, and the number of cells that had migrated to the bottom side were counted.

**RT-PCR**

was used for assessing mRNA expression of erbB receptors in microglial cultures. Total RNA was isolated using an RNeasy Mini Kit (Qiagen) and first strand cDNA was reverse-transcribed. The sequences for primers used in this study were: erbB2, CCTGcCCTCCACTTCAATCAT (forward), CAGGATCCCACTTCCGTAGA (reverse); erbB3 TGAAGATGTGTGAGCCTTGC (forward), GGTCCAGTGCGGGTATCTTA (reverse); erbB4 ATGGCCTTCCAACATGACTC (forward), CACCTGCCATCAGTCTTGTC (reverse), (all 5′–3′). Amplification of templates was detected using SYBR Green 1 dye (Roche, UK) on a Rotor-Gene thermal cycler (Corbett Life Science). Melting curve analysis and sequencing confirmed specificity of the products. PCR products were visualized in a 2% agarose gel.

**Detection of IL-1β by ELISA**

we used the Rat IL-1 beta/IL-1F2 Quantikine ELISA Kit (RLB00, R&D systems) which mainly detects the mature or released form of IL1β. IL1β standards (31.2-2000 pg/ml) and 10μl of microglia culture supernates were run in duplicate following manufacturer instructions. Samples were read at 450 nm with wavelength correction at 540 nm. Samples were considered IL1β positive if the signal was higher than background and within the range of the standard curve. The supernates of microglia were collected after the cells were primed with LPS (1 μg/ml, Sigma for 3 hours) and treated with ATP (1mM, Sigma) or NRG1 (10nM) for 30 minutes.
Western blots

Microglial and macrophages cultures or spinal cord dorsal horn were homogenized in lysate buffer (20 mM Tris, pH 8, 137 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, and protease inhibitor cocktail). The lysate were spun at 13,000 rpm at 4 °C for 15 minutes and the protein concentration of supernatant was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL). Proteins (50 μg/sample) were separated using 8% SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were then blocked in 10% skimmed milk for 1 hour at room temperature and washed in PBS-T for three times at 5 minutes each. Membranes were incubated with primary antibody, rabbit erbB2 (1:200), rabbit erbB3 (1:100) and rabbit erbB4 (1:200; all from Santa Cruz BT), overnight at 4°C. After several PBS-T washes as described before, membranes were incubated with donkey anti-rabbit HRP-conjugated secondary antibody (1:10,000; GE Healthcare) for 1 h at room temperature. As a loading control we used mouse β-actin (1:10000, Sigma) and the secondary antibody used was anti-mouse HRP-conjugated secondary antibody (1:10,000; GE Healthcare). Membranes were revealed using ECL-plus reagent for 5 min (GE Healthcare) for detection by autoradiography. Protein bands for all erbB receptors (185 kDa) and β-actin (42 kDa) were quantified using a model GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA). All quantifications were normalized against β-actin to ensure equal sample loading.

Statistical analysis

Sample sizes for experiments were based on results from pilot studies. Data sets were tested for normality using the Kolmogorov-Smirnov test and for homogeneity of variance using Levene's Test. Parametric or non parametric tests were used accordingly. Behavioural data was analysed using RM two way ANOVA. When the assumptions of sphericity were violated (Mauchly's test; p<0.05) the Greenhouse-Geisser correction was applied. P<0.05 was considered as significant. Data are presented as mean ± SEM.

RESULTS

NRG1 promotes the survival, proliferation and chemotaxis of microglia

Using reverse transcriptase-mediated polymerase chain reaction (RT-PCR) analysis, we found that cultured rat microglia express erbB2, 3 and 4 receptor mRNA and expression was confirmed by immunocytochemistry and western blot analysis (Fig1). When microglial cells are kept in serum free conditions, apoptotic cell death is observed within 24 hours (Koyama et al., 2000). We found that NRG1 significantly promoted the survival of microglia which had been cultured in such conditions for 72 hours (Fig 2 a-c). At NRG1 concentrations of 10nM the number of microglia was more than 5 times higher than seen in control (p=0.001, one way ANOVA, 4 independent experiments). NRG1 application (10 nM) resulted in a three fold reduction in the number of apoptotic microglial nuclei after 24 hours in serum free conditions, as seen by Terminal deoxynucleotidyl transferase dUTP nick end labeled staining (TUNEL) (p=0.008, one way ANOVA, 3 independent experiments fig 2 d-f). This indicates that inhibition of apoptosis was responsible for the survival promoting effects of NRG1. Because in many instances the erbB2 receptor is a key mediator of NRG1 signalling we tested the consequences of erbB2 receptor blockade. We used either an irreversible specific erbB inhibitor PD168393 (Bose et al., 2006) which blocks the ATP binding site of the receptor, or the monoclonal antibody (mAb) 7.16.4 which specifically binds to the extracellular domain of erbB2 (sharing antigenic epitopes with Herceptin®)(Katsumata et al., 1995;Zhang et al., 1999). Both of these agents which themselves had no effect on cell viability, could completely prevent the survival promoting effects of NRG1 in a dose dependent manner (there was no significant difference comparing treatment with NRG1
combined with PD168393 or mAb 7.16.4 and control, p=1, one way ANOVA, 3 independent experiments Fig 2 c, f, supplementary Fig 1).

We subsequently investigated the effects of NRG1 on microglial proliferation. In serum free conditions we observed virtually no basal proliferation and very little response to either GM-CSF or NRG1 (data not shown). Microglia were therefore cultured in medium supplemented with 5% foetal bovine serum (FBS) and cell proliferation in response to NRG1 was quantified using pulse labelling with BrdU. NRG1 treatment increased the proliferation of microglia in a dose dependent manner (Figure 2 g,i) such that a dose of 10nM increased the % of BrdU labelled nuclei from 11 to 60% (p<0.05, ANOVA on ranks, 3-4 independent experiments). This effect was again dependent on activation of the erbB2 receptor as it could be inhibited by PD168393 or mAb 7.16.4 in a dose dependent manner (both were not significantly different from control, p=0.215 and p=0.516 for NRG1 plus PD168393 or mAb 7.16.4 respectively, t-tests, 3 independent experiments, supplementary Fig 1). The effect of NRG1 was comparable to that elicited by GM-CSF a previously well described microglial mitogen (Giulian and Ingeman, 1988).

Microglia are motile cells and demonstrate directed migration towards regions of injury. Using a Boyden chamber in which microglia migrate through pores in a polycarbonate filter across a concentration gradient, NRG1 was found to significantly increase microglial chemotaxis in a dose dependant manner (Fig 2 j-l). A ‘chequerboard’ analysis (Martinet et al., 1994) in which different concentrations of NRG1 were used in the upper and lower wells was performed. This demonstrated that although there was migration when NRG1 was added to both upper and lower wells in equal concentrations migration was greatest when the concentration gradient between the lower and upper well was maximal (Fig 3). This is consistent with a true chemotactic (ie. directed migration along a concentration gradient) rather than solely a chemokinetic (ie. random migration) response produced by NRG1. The chemotactic action of NRG1 was dose dependently inhibited by erbB2 receptor blockade (NRG1 plus PD168393 or mAb 7.16.4, p=1 and p=0.36 versus control respectively, t-tests, 3 independent experiments, supplementary Fig 1). Note that neither of these erbB2 receptor blocking agents had any effect on basal migration (PD168393 vs control p=0.3, mAb 7.16.4 vs. control p=0.6, Mann-Whitney Rank Sum Test). Microglia are closely related to tissue macrophages and indeed we found that peritoneal macrophages also express erbB 2, 3 and 4 receptors and demonstrate enhanced motility in response to NRG1 (Supplementary Fig 2).

In response to CNS injury microglia adopt an effector (also termed activated) state which is associated with development of an amoeboid morphology, pro-inflammatory cytokine expression and expression of class 2 major histocompatibility complex (MHC-2) (Hanisch and Kettenmann, 2007). NRG1 had no effect on Interleukin-1β (IL-1β) release from naïve microglia however it significantly enhanced IL-1β release from microglia which had been primed with lipopolysaccharide (LPS, a molecule known to activate microglia via the Toll-like receptor 4 (Lehnardt et al., 2003), p<0.05 versus control, one way ANOVA on Ranks, Fig 4). The administration of LPS results in process retraction and increased OX6 immunoreactivity (a marker of MHC-2 expression) of microglia. NRG1 however did not elicit such changes (the percentage of OX6 immunoreactivity in LPS-treated cells was 5 times higher than in the control p<0.001, the NRG1-treated cells were no different from control p=1, one way ANOVA, 3 independent experiments) (Fig 4).

**Intrathecal NRG1 produces dorsal horn microgliosis associated with mechanical and cold pain related hypersensitivity**

To investigate the effects of NRG1 on microglia in vivo we administered this molecule intrathecally (0.4 or 4 ng given daily for 3 days), following which we assessed both the response of dorsal horn microglia as well as pain related behaviour. Intrathecal NRG1
produced a dose dependent increase in the number of microglia within the lumbar dorsal horn and many of these cells were noted to develop an activated morphology with hypertrophy of the cell body and process retraction (Fig 5a-c). Although a significant dorsal horn microgliosis was observed at day 1 (0.4 or 4 ng versus control p=0.02 and p=0.002 respectively, one way ANOVA, n=4 per group) it was much more apparent at day 3 (Fig 5a-c, 0.4 and 4 ng versus control p<0.05, ANOVA on Ranks, n=4). Furthermore, the number of microglia undergoing proliferation as assessed by pulse labelling with BrdU also significantly increased (Fig 5d-l, p<0.05 4ng versus control, ANOVA on Ranks, n=4 per group). The microglial response within the dorsal horn following NRG1 treatment was mirrored by the development of a dose dependent mechanical and cold pain related hypersensitivity (Fig 5m p=0.009 and p<0.001 at day 1 and 3 respectively for 4ng vs control; p=0.04 for 0.4ng vs control at day 3. Fig 5n p<0.001 for 4ng vs control at day 3, RM 2 way ANOVA, n=7-8 per group). As with the morphological changes in microglia pain related hypersensitivity was much more marked at day 3 than at day 1.

**ErbB2 receptor inhibition or sequestration of endogenous NRG reduces microgliosis following peripheral nerve injury**

We next explored the role of NRG1-erbB signalling in microglia in a nerve injury model (L5 Spinal Nerve Ligation (Kim and Chung, 1992)) which results in a robust microgliosis. To visualize the location of erbB2 in its activated state a phosphorylation state specific antibody was used (Guertin et al., 2005). Following L5 SNL a significant increase in expression of the phosphorylated (i.e. activated) form of erbB2 receptor was seen in the ipsilateral dorsal horn of the spinal cord (Fig 6 a). No immunostaining was observed following pre-incubation of the antibody with the peptide used as the immunogen. Phospho-erbB2 expression was entirely restricted to microglia; phospho-erbB2 co-localized with the microglial markers Iba-1 (ionized calcium binding adaptor molecule 1) and OX-42 (which recognizes complement receptor type 3). Of 518 phospho-erbB2 positive cells counted 99% co-expressed Iba-1. There was no co-localization with either the neuronal marker neuronal-specific nuclear protein (NeuN) or the astrocyte marker Glial Fibrillary Acidic Protein (GFAP) (Fig 6 b-g). The time course of p-erbB2 expression, which was maximal at days 3-7, closely mirrored the temporal profile of the development of microgliosis (Figure 7 d-h). Using Western Blot analysis we saw a significant increase in erbB3 receptor expression in the dorsal horn following SNL. No change was observed in erbB2 and erbB4 expression (p=0.02 naïve vs day 3 after SNL for erbB3, p= 0.34 and p=1 for erbB2 and erbB4 respectively, Mann-Whitney Rank Sum Test, n=4, supplementary Figure 3).

The principal isoforms of NRG1 expressed within the nervous system are those with a β EGF domain ((Meyer and Birchmeier, 1994; Shinoda et al., 1997). To identify cells expressing NRG1 in the adult we used an oligonucleotide probe which recognise a sequence common to all β isoforms (Fricker et al., 2009). In dorsal root ganglia as previously noted β EGF mRNA is expressed by both small diameter (identified through the expression of the neuropeptide CGRP or binding of Isolectin B4) and large diameter (identified by the expression of phosphorylated neurofilament heavy chain-NF200) DRG cells. Expression was highest in large diameter DRG cells consistent with the high level of expression of the type III β NRG isoform in these cells. In the naïve situation 67 ±3.7 % of DRG cells expressed β EGF mRNA and at 3 days post SNL (when microgliosis is well established) 62 ±1 % of DRG cells expressed β EGF mRNA (Figure 8a-i). This probe demonstrated neuronal expression within the spinal cord particularly in motoneurons and occasional neurons in the deeper laminae of the dorsal horn (data not shown). We did not see expression in astrocytes either in the naïve state or after injury. The most likely source of NRG within the dorsal horn is therefore likely to be primary afferent terminals.
The H 210 antibody that recognises the N terminus of NRG1 was used to localise NRG1 immunoreactivity. To confirm antibody specificity tissue from mice with conditional NRG1 gene ablation in sensory neurons was used (mice expressing Cre under control elements of the neurofilament heavy chain gene NFH-Cre (Mallucci et al., 2002) were crossed with mice with loxp sites flanking the essential EGF domain of NRG1 (NRG1 ^fl/fl^) Yang et al., 2001). In NFH Cre; NRG1 ^fl/fl^ mice NRG1 is ablated in sensory neurons in the late embryonic period resulting in a severe dysmyelinating neuropathy and shortened survival (DLHB unpublished observations). Note that normal NRG1 immunoreactivity is observed in DRG cells of NRG1 ^fl/fl^ (ie. control) animals while no immunoreactivity is present DRG cells of NFH Cre NRG1 ^fl/fl^ animals. (Figure 8 g-h). NRG1 immunoreactivity was observed within DRG cells of all cell sizes (in naïve situation: 61.9 ± 1.2 % of all cells were positive, at 3 days after SNL: 58.4 ± 2.1 %, figure 8 i-j). NRG1 immunoreactivity was also seen within the dorsal horn of the spinal cord (especially within superficial laminae, Figure 8k) and this increased at 3 days post SNL (ipsilateral to lesion). An ELISA which recognises NRG1 isoforms containing β EGF and IgG domains demonstrated a significant increase in the level of this protein within the dorsal horn at 3 days post SNL (Figure 8l, p=0.04, one way ANOVA Bonferroni post hoc test, n=3 per group).

To test whether activation of the erbB2 receptor contributes to microgliosis in vivo we inhibited it using PD168393, the erbB2 receptor blocking antibodies mAb7.16.4 (discussed above) or mAb-9 (B10) which causes erbB2 homodimerization without activation of downstream signalling pathways and accelerates receptor endocytosis (Pugatsch et al., 2006; Sawyer et al., 2002). At day 3 following SNL, when the microglial response is well established, we found that blocking the erbB2 receptor could dose dependently reduce the number of microglia within the ipsilateral dorsal horn with an activated morphology (using PD168393 5 μg/day i.t. infusion (p=0.002), mAb7.16.4 5 μg i.t. injection (p<0.001) or mAb-9 5 μg i.t. injection (p<0.001) respectively compared to control, one way ANOVA, n=3-4 per group, Fig 9 a-c, supplemental figure 1d). The number of microglia within the dorsal horn which were phospho-p38 MAPK positive was also significantly reduced following erbB2 inhibition (p<0.001, following treatment with PD168393 5 μg/day i.t. infusion, mAb7.16.4 5 μg i.t. injection, or mAb-9 5 μg i.t. injection compared to control, one way ANOVA, n=3-4 per group, Fig 9 f-j)).

To sequester endogenous NRG we used a fusion protein (HBD-S-H4) consisting of the soluble ectodomain of erbB4 which has high affinity for NRG and a heparin binding domain which helps target the molecule to the same heparan-sulfate rich cell surfaces which bind NRG. This has been shown to be highly effective in vitro and blocks the survival promoting activity of NRG on Schwann cells in vivo (Ma et al., 2009). Intrathecal administration of HBD-S-H4 (3 μg, i.t. injection) significantly reduced both the number of microglia with an activated morphology within the dorsal horn and phospho-p38 expression following SNL (in both cases p<0.01, following treatment with HBD-S-H4 versus control, unpaired t-test, n=3-4 per group, Figure 9).

Pulse labelling with BrdU showed a marked increase in the rate of cell proliferation within the dorsal horn of the spinal cord following SNL and, as previously reported (Echeverry et al., 2008), the vast majority of these cells (94.6 ± 1.2 %) were microglia. The number of proliferating microglia within the dorsal horn was reduced by approximately 60-70% following blockade of erbB2 (p<0.001, following treatment with PD168393 (5 μg/day i.t. infusion), mAb7.16.4 (5 μg/day i.t. infusion), or mAb-9 (5 μg/day i.t. infusion) compared to control, one way ANOVA, n=3-4 per group, Figure 10).

Inhibition of erbB2 significantly reduced the number of microglia with activated morphology, expression of phospho-p38 MAPK and microglial proliferation at a later time.
point following nerve injury ie. 7 days post SNL (Supplementary Fig 4). In a further set of experiments we assessed dorsal horn microgliosis at 7 days following pump emptying (ie. 21 days post SNL). We found no significant difference in dorsal horn microgliosis at this time point comparing intrathecal treatment with PD168393 and control (p=0.57, t-test, n=3-4, Supplementary Fig 5). Neuregulin is known to have a key role in signalling between axons and Schwann cells in peripheral nervous system (Chen et al., 2003; Fricker et al., 2009; Nave and Salzer 2006). There is therefore the theoretical possibility that these changes could be secondary to changes in axoglial signalling in the periphery if the inhibitor or antibody were to spread outside of the intrathecal space. To exclude this possibility we administered mAb7.16.4 directly to the site of spinal nerve ligation and found that in contrast to intrathecal treatment this peripheral administration had no effect on dorsal horn microgliosis (p=0.18, t-test, n=3-4, Supplementary Fig 6).

A week following SNL we observed a significant increase in astrocytes within the ipsilateral spinal cord (p=0.004, t-test, n=4), which is in accordance with previous reports of a delayed astrocytosis following nerve injury (Colburn et al., 1999). However, unlike the microgliosis this was not reduced by erbB2 inhibition (p=0.88, t-test, n=4, Supplementary Fig 7).

**ErbB2 receptor inhibition or sequestration of endogenous NRG reduces mechanical and cold pain related hypersensitivity following nerve injury**

There is increasing evidence that the microglial activation observed within the dorsal horn of the spinal cord following nerve injury contributes to the development of neuropathic pain (Tsuda et al., 2003; Milligan and Watkins, 2009). Intrathecal infusion of PD168393 over 7 days had no effect on mechanical withdrawal thresholds in naïve animals (p=0.25, RM 2 way ANOVA, PD168393 5µg/day versus vehicle, data not shown). We therefore monitored pain related behaviour over 14 days following L5 SNL in animals which were treated with the erbB2 inhibitor or vehicle delivered via continuous intrathecal infusion. In both groups of animals a clear mechanical hypersensitivity was observed at day 1 following SNL however at subsequent time points erbB2 receptor inhibition resulted in a significant dose dependent reduction in mechanical pain hypersensitivity on the side ipsilateral to the lesion (Fig 11a, p<0.05, RM 2 way ANOVA, PD168393 10 or 5 µg day versus vehicle n=7-11/group) with no effect on the contralateral paw (data not shown). Once the pump had emptied the mechanical withdrawal thresholds of the group receiving PD168393 5µg/day returned to those of control (Fig 11b). Delayed treatment with PD168393 5µg/day from day 3 onwards (by which time microgliosis is well established) was not effective at reversing mechanical pain related hypersensitivity (Fig 11c, p=0.75 RM 2 way ANOVA, n=7 per group). Cold alldynia (as assessed by nociceptive behaviour in response to acetone application to the paw) also developed following SNL and was significantly attenuated in a dose dependent fashion by erbB2 inhibition (Fig 11d p<0.05 PD168393 10 or 5 µg day versus vehicle RM 2 way ANOVA, n=7-11/group). Intrathecal injection of HBD-S-H4 (two 3µg i.t. injections administered on the day of surgery and on day 3 post surgery) which sequesters endogenous NRG also significantly attenuated mechanical pain related hypersensitivity (Fig 11e, p<0.001 versus control, RM 2 way ANOVA, n=8 per group) and cold alldynia (Fig 11f, p<0.05 versus control, RM 2 way ANOVA, n=8 per group).

**Discussion**

Microglia closely resemble tissue macrophages and are key sensors of injury to the central nervous system produced by many diverse pathologies. NRG1 has not previously been implicated in regulating microglial function. Here we demonstrate that NRG1 is a survival, proliferative and chemotactic factor for microglia in vitro and that NRG1-erbB signalling is activated specifically within microglia following peripheral nerve injury contributing to the development of microgliosis and consequently neuropathic pain.
Microglia, express the NRG1 receptors erbB2, 3 and 4. This factor promotes the survival of microglia in vitro through inhibition of apoptosis and is a potent proliferative factor for these cells in an erbB2 dependent manner. In addition to proliferation the other means by which microglia can accumulate at the site of injury is through directed migration in response to the release of chemotactic agents which include: purines (Honda et al., 2001) complement components (Yao et al., 1990), bradykinin (Ifuku et al., 2007) and chemokines (Peterson et al., 1997). We have used the Boyden chamber chemotaxis assay and by manipulating the concentration gradient in the upper and lower wells have shown that NRG1 is a chemotactic agent for microglia acting in an erbB2 dependent manner. Neuregulin has previously been shown to increase motility of a number of different cell types including malignant cell lines (Hijazi et al., 2000) keratinocytes (Schelfhout et al., 2002) and Schwann cells (Lyons et al., 2005). NRG1 could also enhance the release of interleukin-1β from LPS primed microglia. Unlike LPS however NRG1 application did not promote the development of amoeboid morphology or MHC class II expression in microglia. We have found that macrophages express erbB2, 3 and 4 receptors. Therefore, in situations where there is major disruption of the blood brain barrier (such as traumatic brain injury) resulting in macrophage infiltration into the CNS the function of macrophages may also be modulated by NRG1.

Because of the potent effects we observed of NRG1 on cultured microglia we explored the relevance of this signalling pathway in vivo. Intrathecal administration of NRG1 resulted in a dorsal horn microgliosis and also produced mechanical and cold pain related hypersensitivity (in agreement with a previous study, Lacroix-Fralish et al., 2008). Spinal nerve ligation provides a well characterized model of nerve injury associated with a robust microgliosis (Scholz and Woolf, 2007; Tsuda et al., 2003) and the development of neuropathic pain (Kim and Chung, 1992). We used an antibody which recognizes phosphorylated erbB2 as a measure of receptor activation (Guertin et al., 2005). In naïve and sham surgery animals there was only a low level of p-erbB2 expression in resting microglia. Following SNL p-erbB2 expression increased and it was localized specifically within microglia especially those with an activated morphology. The time course of p-erbB2 expression within the dorsal horn was coincident with the development of microgliosis being detectable at day 1 after injury and peaking at day 3-7 post SNL.

For NRG1 to act as a proliferative and chemotactic agent for microglia it needs to be released at the site of injury. All NRG1 isoforms contain an EGF domain which is required for biological activity and can be classified into sub groups according to the structure of their amino termini. Type I and II isoforms have Ig like domains and can either be directly secreted or alternatively transmembrane isoforms undergo metalloprotease mediated cleavage to be released as soluble proteins from the cell surface. These isoforms bind avidly to the extracellular matrix which can act as a source of localized release (Loeb and Fischbach, 1995). Type III isoforms possess a cysteine-rich domain (CRD) and undergo proteolytic cleavage by BACE (Willem et al., 2006) however, the EGF domain remains membrane tethered and so this molecule signals in a juxtacrine fashion. DRG cells have been reported to principally express type I and III isoforms (Bermingham-McDonogh et al., 1997). We found clear expression of NRG1 by small and large diameter DRG cells using in situ hybridization and a probe that recognises all β EGF isoforms (the nervous system principally expresses beta rather than alpha isoforms). β EGF mRNA was expressed at low levels by occasional dorsal horn neurons and so we propose that the most likely source of NRG1 within the dorsal horn is from primary afferent terminals. We did not observe a change in β EGF mRNA expression in DRG cells or the dorsal horn at 3 days following SNL a time point when microgliosis is well established. We did find significantly increased β EGF containing NRG1 protein within the dorsal horn using an ELISA following SNL. Cultured neurons have been shown to release soluble NRG1 in an activity dependent manner (Ozaki et al., 2004) and NRG1 activity has also been detected in human cerebrospinal fluid.
(Pankonin et al., 2009). In culture systems NRG1 activity has also been shown to be released from DRG cell axons (Esper and Loeb, 2004; Taveggia et al., 2005); this release is enhanced by BDNF (which is itself released by microglia following nerve injury (Coull et al., 2005)). The rapid response of microglia following SNL suggest that increased NRG1 activity within the dorsal horn following nerve injury is due to either: altered NRG1 protein trafficking or release from primary afferent terminals or possibly release from the extracellular matrix. We suggest that such NRG1 release provides an injury related signal from primary afferents triggering a microglial response within the dorsal horn.

We tested the hypothesis that inhibition of NRG-erbB signalling would reduce the microgliosis produced by SNL. We used two strategies for erbB2 receptor inhibition: intrathecal treatment with either an irreversible specific erbB inhibitor (PD168393) which blocks the ATP binding site of the receptor or monoclonal antibodies (mAb 7.16.4 and mAb-9). MAb 7.16.4 specifically binds to the extracellular domain of erbB2, down regulates surface expression of erbB2 and blocks activation of erbB2/3 and erbB2/4 heterodimers; this antibody has previously been shown to have efficacy in vivo (Yarden and Sliwkowski, 2001; Katsumata et al., 1995). Inhibition of the erbB2 receptor following nerve injury resulted in a significant reduction in the microgliosis within the dorsal horn as well as reducing the expression of Phosho-p38 (a component of the mitogen-activated protein kinase (MAPK) signalling cascade) which is specifically expressed within activated microglia (Jin et al., 2003; Kobayashi et al., 2008). This effect on p38 signaling is likely to be indirect as NRG1 did not stimulate the p38 MAPK pathway in cultured microglia although it does activate the MEK-ERK and PI-3 kinase-AKT pathways (MC, NZ and DB unpublished observations). Interestingly ERK has previously been shown to be activated in microglia following peripheral nerve injury (Zhuang et al., 2005). We have also employed a molecule (HBD-S-H4) to sequester endogenous NRG1. This is a fusion protein consisting of the soluble ectodomain of erbB4 which has high affinity for NRG1 and a heparin binding domain which helps target the molecule to the same heparan-sulfate rich cell surfaces which bind NRG1. This molecule could also effectively reduce dorsal horn microgliosis and pp38 expression following SNL.

Given the lack of major disruption to the blood brain barrier following SNL (Abram et al., 2006; Lu et al., 2009) proliferation of resident microglia is likely to be the principal means by which microglial numbers increase following SNL. We found a clear increase in microglial cell proliferation within the dorsal horn in the first 3 days following peripheral nerve injury. ErbB2 receptor blockade significantly reduced this proliferative response. Inhibition of microglial proliferation provides one explanation for the reduction in microgliosis observed following erbB2 receptor inhibition although impaired chemotaxis of local microglia may also play a role. ErbB2 inhibition specifically reduced microgliosis following nerve injury and did not prevent the development of astrocytosis.

There is now increasing evidence that glial cells (both microglia and astrocytes) play an important role in the generation of neuropathic pain. A number of molecules have been implicated in the recruitment and activation of microglia within the dorsal horn following nerve injury including cytokines, chemokines (such as CCL2 and Fractalkine), complement components and purines (Milligan and Watkins, 2009; Scholz and Woolf, 2007; Tsuda et al., 2003; Clark et al., 2007; Thacker et al., 2009; Zhang et al., 2007; Jin et al., 2003). Following activation microglia release cytokines and BDNF which enhance the excitability of dorsal horn neurons (Coull et al., 2005; Kawasaki et al., 2008). Inhibition of the microglial response to injury has been shown to effectively reduce the development of neuropathic pain (Tsuda et al., 2003; Coull et al., 2005; Ledeboer et al., 2005; Clark et al., 2007). ErbB2 receptor inhibition or sequestration of endogenous NRG1 could ameliorate (but not completely reverse) both mechanical and cold pain related hypersensitivity following SNL.
Following peripheral nerve injury the pro-inflammatory microglial response is clearly deleterious as it contributes to neuropathic pain however in other contexts there is evidence that these cells may be neuroprotective (Hanisch and Kettenmann, 2007). NRG-erbB signalling represents a novel pathway stimulating microglial proliferation and chemotaxis. We have shown that this contributes to the development neuropathic pain and it will be of great interest in the future to determine the role of NRG-erbB signalling in mediating the microglial response to other forms of CNS injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. erbB receptor expression in primary cultured microglia

a. Using reverse transcriptase-mediated polymerase chain reaction (RT-PCR) analysis we found that primary cultured rat microglia express the erbB2, 3 and 4 receptors. 2% agarose gels stained with ethidium bromide and photographed under ultraviolet light are shown for the PCR products of each of the genes amplified. Pos= positive control (E16 embryonic rat brain cDNA), MG= microglia, Neg= Negative control (water).

b. ErbB receptors expression is shown in microglial cells by IHC (IB4 labels microglia green, the erbB receptors are in red, scale bar: 20μm).

c. Western blot analysis showing a 185 kDa band for erbB2, 3 and 4 in lysates from microglial cells.
Figure 2. NRG1 stimulates microglial survival, proliferation and chemotaxis in vitro

a-c Survival of microglia in vitro was assessed by incubating cultured microglia for 3 days in serum-free medium which was supplemented with increasing doses of NRG1 (0.5-10nM or GM-CSF 1nM (as a positive control). Representative wells are shown in which microglia are identified by Iba1 immunostaining following no treatment (a) or addition of 10nM NRG1 (b). NRG1 promoted microglial survival and erbB2 inhibition using PD168393 (INH, 10μM) or mAb 7.16.4 (AB, 4μg/ml) blocked this effect. Quantification of microglial numbers is shown in (c)
d-f Cultured microglia were incubated for one day in serum-free medium with (e) or without (d) the addition of NRG1. DAPI staining (blue) demonstrated nuclei and TUNEL labeling (yellow) revealed those undergoing apoptosis. Treatment with NRG1 (10nM) significantly decreased apoptosis, an effect which was prevented when microglia were treated with the erbB inhibitor PD168393 in combination with NRG1. g-i Proliferation was assessed by incubating microglia in medium supplemented with 5% FBS for 3 days and pulse-labeling with BrdU (yellow). NRG1 treatment significantly increased the proportion of BrdU-positive microglial nuclei, an effect that was erbB2 dependant. j-l: The effects of NRG1 on microglial migration was studied using a Boyden chamber. The addition of NRG1 to the lower well of the chamber (k) increased microglial migration to the inner membrane surface compared to control (j). Inhibition of erbB2 blocked this action. Scale bars: 100μm. Error bars represent ± SEM (3-5 independent experiments). The statistical tests used were one way ANOVA with Bonferroni post hoc analysis for all comparisons except for the proliferation experiment in which the data was not normally distributed and ANOVA on ranks with Dunn’s post hoc test was used instead. ** p <0.005, * p <0.05. (CON: control, INH: inhibitor (PD168393), AB: neutralizing antibody (7.16.4).)
Figure 3. The migration of microglial cells in response to NRG1 is a true chemotactic response
In a Boyden chamber microglial cells migrate from an upper to a lower well through a polycarbonate filter in response to a concentration gradient of the putative chemotactic agent. A 'chequerboard' analysis of 3 independent experiments assessing the chemotactic response to NRG1 performed in triplicate and normalized to the unstimulated control is shown. Microglial cells were suspended in medium alone or with 0.1, 1 or 10 nM NRG1 and then allowed to migrate for 3 hours at 37°C towards different concentrations of NRG1 in the lower compartments. Highlighted in grey boxes are the results achieved by using the same concentration of NRG1 in upper and lower wells. Note that in this circumstance NRG1 increases microglial migration indicating chemokinesis. When there is an increasing concentration gradient from the upper to lower well (values in grey) migration is clearly enhanced indicating a true chemotactic response. (*p<0.05 comparing migration across a gradient versus migration when the NRG1 concentration is the same in both wells) Numbers represent the mean ± SEM.
Figure 4. NRG1 promotes the release of IL-1β from LPS primed microglia but does not stimulate MHC-2 expression

**a.** An ELISA was used to quantify IL-1β released into the medium of cultured microglia. Neither ATP (1 mM) nor NRG1 (10 nM) alone produced IL-1β release from naïve microglia however both significantly promoted IL-1β release from LPS (1 μg/ml) primed microglia in a dose dependent manner. (Error bars represent ± SEM, Statistical test: ANOVA on Ranks, *p<0.05, n=3).

**b.** Another aspect of the activation (or effector) response of microglia is that they increase expression of MHC-2. To investigate wherever NRG1 treatment leads to such a response in microglia we incubated primary cultures in serum-free medium and treated them with lipopolysaccharide (LPS 1 μg/ml), NRG1 (10 nM) or medium alone (CON). Microglia were immunostained with OX-6 (which labels MHC-2), Iba1 (microglial marker) and DAPI (nuclear staining). LPS treatment led to a 5-fold increase in OX-6 expression compared with control (p<0.001), whilst NRG1 treatment didn’t elicit such a response (p=1). Treatment with LPS also resulted in microglia adopting an amoeboid morphology unlike NRG1. Scale bars: 100 μm. Error bars represent ± SEM (3 independent experiments) Statistical test: one way ANOVA, Bonferroni post hoc analysis. **p<0.001
Figure 5. Intrathecal administration of NRG1 results in dorsal horn microgliosis associated with mechanical and cold pain related hypersensitivity

NRG1β was administered intrathecally (0.4 or 4 ng given daily for 3 days) a-b Dorsal horn of animals treated with saline (control in a) or NRG1 (4 ng in b) 3 days after the first injection, immunostained with Iba1. Note the increase in numbers of microglia with an activated morphology. c is shows quantification of this response at 1 and 3 days after NRG1 injections (*p<0.05, **p<0.001 comparing NRG1 doses vs control). In d-l we assessed proliferation (pulse labelling with BrdU) after NRG1 injections (Iba1 is shown in red, BrdU in green, DAPI in blue and in the last panel merged images are shown). D-g dorsal horn microglia from a saline treated animal (note that no BrdU is present). H-k dorsal horn microglia from a NRG1 (4 ng) treated animal. In l is shown the quantification of all BrdU positive microglia in the dorsal horn of all groups at day 1 and day 3 after injections were started (*p<0.05 comparing 4ng versus control). Mechanical (shown in m) and cold (shown in n) pain related hypersensitivity developed after NRG1 injections in a dose dependant manner (*p<0.05, **p<0.001 for 4ng versus control; #p<0.05 for 0.4ng versus control).

Scale bars: a-b: 100μm, d-k: 10 μm.
Figure 6. Peripheral nerve injury leads to increased phospho-erbB2 expression within dorsal horn microglia

Three days after L5 SNL, the expression of the phosphorylated form of the erbB2 receptor was increased in the ipsilateral dorsal horn (a). Phospho-erbB2 immunostaining (green) co-localized with Iba1 (microglial marker in red in b), but not with GFAP (astrocyte marker in red, c) or NeuN (neuronal marker in red, d). P-erbB2 (green in e) also co-localized with OX-42 (a marker of activated microglia red, f). Scale bars: 200 μm (a), 50 μm (b-d), 10 μm (e-g)
Figure 7. The time course of phospho-erbB2 expression is coincident with the development of microgliosis

The microglial response to nerve injury develops during the first week following injury, (demonstrated by immunostaining with the microglial marker Iba1) (a-c). An increase in microglial numbers is observed in the ipsilateral dorsal and ventral horn of the spinal cord of L5 SNL animals (a). A closer view shows that microglia change their morphology from having long processes and a small soma (b, naïve animal, termed surveying microglia) to having retracted processes and a hypertrophic soma (c, 3 days after L5 SNL termed an effector or activated morphology). Simultaneous with this microgliosis we observed an increased expression of the p-erbB2 receptor (green) in the ipsilateral dorsal horn microglia (labeled with Iba1 in red). d: naive, e: 1 day after SNL, f: 3 days after SNL, g: 21 days after SNL. There was a significant increase in both total microglial number and the proportion which were p-erbB2 positive within the dorsal horn following nerve injury, * p<0.05 (n: 3-4), one way ANOVA on ranks, post hoc Student-Newman-Keuls Method) Scale bars: 200 μm (a), 10 μm (b-c), 100 μm (d-g)
Figure 8. NRG1 expression after L5 Spinal Nerve Ligation

ISH: a-f In-situ hybridisation images using a probe directed against the βEGF domain of NRG1 in L5 DRG of naïve animals (a-c) and 3 days post SNL (d-f). Expression is highest in large diameter DRG cells positive for NF 200 (green) but is also observed in small DRG cells positive for either IB4 (blue) or CGRP (red). Arrows provide illustrative examples of double labelled cells. There was no significant change in the proportion of DRG cells expressing EGF mRNA at 3 days post SNL.

IHC: g-h. We used the H210 antibody which is raised against the N-terminal extracellular domain of NRG1. To confirm specificity we tested it in NRG1 knock out tissue (NFH Cre NRG1 flox/flox mice in which NRG1 is ablated in sensory neurons in the late embryonic period) Note that normal NRG1 immunoreactivity is observed in DRG cells of NRG1 flox/mice (ie. control) while no immunoreactivity is present DRG cells of NFH Cre NRG1 flox/mice. i-k Immunohistochemistry confirmed that NRG1 was present in around 60% of DRG cells (i-j) and within the dorsal horn of the spinal cord (especially within superficial laminae, k).

NRG1 expression was also assessed using a NRG1β specific ELISA (l). Lysate from dorsal horn spinal cord of naïve, 1 day and 3 days after SNL animals were run (50 µg of protein for each sample). All sample values were within the range of the ELISA detection. A significant increase in NRG1 was detected at 3 days after SNL (p=0.04, one way ANOVA, Bonferroni post hoc test, n=3) Scale bars: a-h: 50 µm, i: 200 µm.
Figure 9. ErbB2 receptor blockade or sequestration of endogenous NRG inhibits the development of microgliosis following spinal nerve ligation

The effect of erbB2 receptor blockers (PD168393, 5 μg/day i.t. or monoclonal antibodies mAb-9 or 7.16.4, 5 μg i.t.) or a NRG sequestering molecule (HBD-S-H4, 3 μg i.t.) was determined at 3 days post SNL. a-e ErbB2 receptor blockade (b and e low and high power photomicrographs respectively) resulted in a reduction in both p-erbB2 (green) expression and in the number of microglia in the dorsal horn (Iba1 immunostaining, red) compared with vehicle (a and d low and high power photomicrographs respectively). Quantification (c) demonstrates that SNL results in an increased number of microglia with activated morphology compared to sham surgery animals. This increase was significantly attenuated following treatment with either: erbB2 inhibitor, erbB2 receptor blocking antibodies or the NRG1 antagonist (HBD-S-H4) with respect to control. f-j SNL results in increased numbers of microglia expressing phospho-p38. The number of microglia (red) expressing p-p38 MAPK (green) post SNL was significantly reduced following erbB2 receptor blockade or sequestration of NRG using HBD-S-H4. Quantification in h. Scale bars for a-b and f-g: 100 μm, scale bars for d-e and i-j: 50 μm. Error bars represent ± SEM (n: 3-4 per group). SH: sham, VH: vehicle, INH: inhibitor (PD168393), IgG2a: non immune IgG (control), ANT: antagonist (HBD-S-H4). Statistical test: t-test (vehicle vs inhibitor and control vs antagonist) or one way ANOVA, Bonferroni post hoc (IgG2a vs erbB2 receptor blocking antibodies). **p<0.005. Scale bars:
Figure 10. Microglial proliferation after spinal nerve ligation is significantly reduced following erbB2 receptor inhibition

Three days after L5 SNL a proliferative response is seen in microglia within the dorsal horn as shown here by labelling newly dividing cells with BrdU (yellow) and microglia with Iba1 (red). DAPI is shown in blue to delineate nuclei. The BrdU labeled nuclei are almost exclusively within microglial cells (as seen in the merge image). Blocking the erbB2 receptor with PD168393 (5 μg/day i.t.) or with the blocking antibodies (mAb-9 or 7.16.4, 5 μg i.t.) resulted in a significant reduction in microglial proliferation. Scale bar: 100μm. Error bars represent ± SEM (n 3-4 per group). SH: sham, VH: vehicle, INH: inhibitor (PD168393), IgG2a: non immune IgG (control). Statistical test: one way ANOVA, Bonferroni post hoc. **p<0.005
Figure 11. erbB2 receptor inhibition or sequestration of endogenous NRG reduced mechanical pain hypersensitivity and cold allodynia following spinal nerve ligation

Animals underwent L5 SNL and received a continuous intrathecal infusion of the erbB2 inhibitor (PD168393, 1.25-10 μg/day) or vehicle for 14 days. 

a: Mechanical hypersensitivity developed at day 1 after SNL in both groups and after day 2 was significantly attenuated in animals receiving the erbB2 inhibitor in a dose dependent fashion (***p<0.001, *p<0.05 for 10 μg/day versus vehicle, # p<0.05 for 5 μg/day versus vehicle, n=7-11/group) 

b: Once the pump had emptied the withdrawal thresholds of the group receiving the erbB2 inhibitor (5 μg/day) returned to those of control. 

c: Delayed treatment with the erbB2 inhibitor (5 μg/day) from day 3 onwards (by which time microgliosis is well established) was not effective at reversing mechanical pain related hypersensitivity (p=0.75) 

d: Cold allodynia was also significantly reduced in a dose dependent fashion by inhibiting the erbB2 receptor (***p<0.001 for 10 μg/day versus vehicle, # p<0.05 for 5 μg/day versus vehicle, n=7-11/group).

e and f: Intrathecal administration of HBD-S-H4 (3 μg) at day 0 and 4 post SNL (shown by arrows) significantly reduced mechanical (p<0.001) (e) and cold (p<0.05) (f) pain related hypersensitivity. Error bars represent ±SEM. Statistical tests: two way ANOVA, Bonferroni or Fischer LSD post hoc analysis. When the assumptions of sphericity were violated (Mauchly's test; p<0.05) the Greenhouse-Geisser correction was applied and independent two-tailed t-tests were used to determine differences between groups. INH: inhibitor (PD168393), Nrg ANT: NRG antagonist (HBD-S-H4). The lines in a-d denote the period of pump infusion. The arrows in e-f denote the days of i.t. injections.