LETTERS

BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain

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Neuropathic pain that occurs after peripheral nerve injury depends on the hyperexcitability of neurons in the dorsal horn of the spinal cord[1–3]. Spinal microglia stimulated by ATP contribute to tactile allodynia, a highly debilitating symptom of pain induced by nerve injury[4]. Signalling between microglia and neurons is therefore an essential link in neuropathic pain transmission, but how this signalling occurs is unknown. Here we show that ATP-stimulated microglia cause a depolarizing shift in the anion reversal potential (E_{anion}) in spinal lamina I neurons. This shift inverts the polarity of currents activated by GABA (γ-amino butyric acid), as has been shown to occur after peripheral nerve injury[5]. Applying brain-derived neurotrophic factor (BDNF) mimics the alteration in E_{anion}. Blocking signalling between BDNF and the receptor TrkB reverses the allodynia and the E_{anion} shift that follows both nerve injury and administration of ATP-stimulated microglia. ATP stimulation evokes the release of BDNF from microglia. Preventing BDNF release from microglia by pretreating them with interfering RNA directed against BDNF before ATP stimulation also inhibits the effects of these cells on the withdrawal threshold and E_{anion}. Our results show that ATP-stimulated microglia signal to lamina I neurons, causing a collapse of their transmembrane anion gradient, and that BDNF is a crucial signalling molecule between microglia and neurons. Blocking this microglia–neuron signalling pathway may represent a therapeutic strategy for treating neuropathic pain.

Peripheral nerve injury (PNI)-induced tactile allodynia depends on a depolarizing shift in the E_{anion} of spinal lamina I (LI) neurons in the dorsal spinal cord, causing disinhibition and, in some cases, converting the $GABA_A$-receptor- and glycine-receptor-mediated inhibition to excitation[5]. As microglia are implicated in the induction of neuropathic pain[4], we considered that these glia cells may affect E_{anion} in LI neurons. To investigate this possibility, we administered microglia to the lumbar spinal level of naive rats by an intrathecal catheter as described[4], and subsequently made perforated-patch and whole-cell recordings from LI neurons in acute spinal cord slices prepared from these rats. Prior to slice preparation, we determined the paw withdrawal threshold for each rat[4,5]. Administering control unstimulated microglia produced no change in paw withdrawal threshold, whereas microglia that had been stimulated with ATP caused a progressive decrease in paw withdrawal threshold over the 5 h of testing after injection (Fig. 1a). Cortically and spinally derived microglia produced a comparable decrease in paw withdrawal threshold. Owing to its larger size, the cortex yielded more microglia and was therefore used for all subsequent experiments.

Electrophysiological recordings were made from slices prepared 5 h after intrathecal microglia administration. Using voltage-clamp recording from LI neurons, we found that in spinal slices taken from rats injected with control microglia E_{anion} was -68.3 ± 1.8 mV (mean ± s.e.m; $n = 6$; Fig. 1b). By contrast, in LI neurons from rats that had been administered ATP-stimulated microglia, E_{anion} was -61.6 ± 1.1 mV ($n = 16$, $P < 0.0001$). In addition, using current-clamp recordings, we found that the GABA response switched from hyperpolarizing in control rats to depolarizing in rats treated with ATP-stimulated microglia (Fig. 1c).

We reasoned that, to effect the shift in E_{anion}, ATP-stimulated microglia must signal to the LI dorsal horn neurons. Activated microglia secrete various biologically active signalling molecules, one of which, BDNF[6], has been implicated in the hypersensitivity of dorsal horn neurons that follows sensitization and inflammation[7–9] and in anion gradient shifts in the hippocampus[10]. To test whether BDNF could trigger shifts in pain hypersensitivity and in LI neuronal E_{anion} similar to those resulting from the application of ATP-stimulated microglia, we administered recombinant BDNF intrathecally to naive rats. This locally delivered BDNF produced a significant and transient decrease in paw withdrawal threshold comparable to that produced by ATP-stimulated microglia (Fig. 2a).

To determine whether BDNF could cause a shift in E_{anion}, we bath-applied it to spinal slices taken from naive rats. E_{anion} of LI neurons ($n = 9$) in slices treated with BDNF (>90 min) was significantly less negative than that of LI neurons from control untreated slices ($n = 9$; $P < 0.005$; Fig. 2b). Thus, it seemed possible that responses to GABA might be excitatory, rather than inhibitory, during BDNF administration. We investigated this possibility by monitoring intracellular calcium ($[Ca^{2+}]_i$) after brief applications of GABA in LI neurons ($n = 96$). During perfusion with BDNF and in the presence of glutamate receptor blockers, the proportion of neurons responding to GABA with a rise in $[Ca^{2+}]_i$ increased over time, reaching 31% of neurons recorded between 80 and 120 min ($P < 0.05$; Fig. 2c). The rise in $[Ca^{2+}]_i$ was prevented by the $GABA_A$ receptor blocker bicuculline ($n = 18$; $P < 0.05$), confirming that the effect was mediated by $GABA_A$ receptors. Thus, acute administration of BDNF in slices caused a depolarizing shift in E_{anion} and, in about 30% of the cells, caused GABA to produce net excitation.

To determine the effects of sustained prolonged exposure to BDNF *in vivo*, we administered a BDNF-transducing recombinant adenovirus (adBDNF)[11] intrathecally to the rats ($n = 16$). After injection, adBDNF caused a progressive decrease in paw withdrawal threshold over the 4 d of testing. By contrast, a control adenovirus had no effect

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on paw withdrawal threshold over the same period ($n = 6$; $P < 0.005$; Fig. 2d). Because of the prolonged effect of the adBDNF treatment, we could test for changes in E_{anion} in slices taken from treated rats. E_{anion} in LI neurons from adBDNF-injected rats ($n = 7$) was significantly less negative than that in LI neurons from rats treated with the control adenovirus ($n = 4$; $P < 0.01$; Fig. 2e, f). Moreover, GABA application caused some LI neurons from adBDNF-injected rats to fire action potentials (2/7 cells tested), a feature that was not observed in control conditions. Thus, similar to acute administration of BDNF, sustained local release caused a decrease in paw withdrawal threshold, caused a depolarizing shift in E_{anion} and, in some neurons, could switch the action of GABA from inhibitory to excitatory.

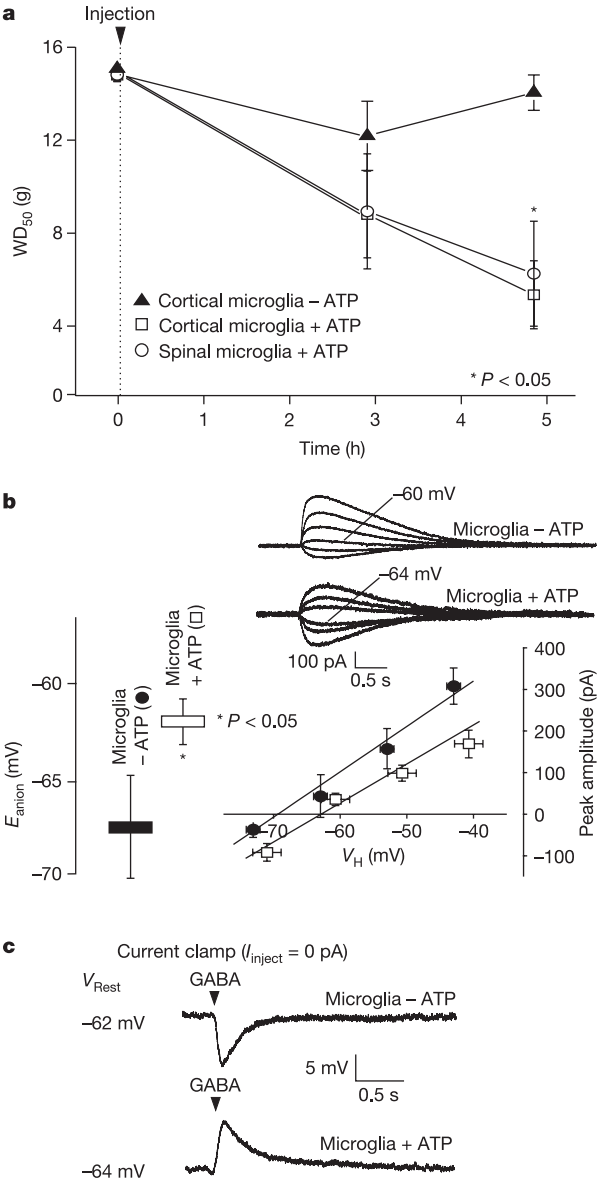

Figure 1 | Spinal delivery of ATP-stimulated microglia to rats evokes allodynia and a depolarizing shift in E_{anion} in spinal LI neurons. **a**, In naive rats, local spinal delivery of ATP-stimulated microglia (of cortical or spinal origin), but not resting microglia, by means of an intrathecal catheter caused a significant decrease in the mean paw withdrawal threshold (WD_{50}; vertical bars indicate the s.e.m.). **b**, Left, comparison of the mean ± s.e.m. E_{anion} recorded in LI neurons from rats injected with resting or ATP-stimulated microglia. Note that there were no significant changes in the resting membrane potential of the cells. Right, the mean ± s.e.m. peak current evoked by GABA, measured in LI neurons at various values of membrane potential (V_m) in slices taken from the rats in **a**. Horizontal error bars represent interneuron differences. Inset, representative raw traces. **c**, Representative traces in current-clamp recording mode showing that, at resting membrane potential, the postsynaptic response to GABA was depolarizing in a LI neuron taken from a rat with a WD_{50} of 3.4 g, in contrast to the response in a LI neuron taken from a rat with a WD_{50} of 12.6 g, where GABA was hyperpolarizing.

These results show that exogenous BDNF is sufficient to cause tactile allodynia and a shift in E_{anion}, raising the possibility that BDNF might be an endogenous mediator of the sequelae of PNI. To investigate this, we used a function-blocking antibody against the TrkB receptor (anti-TrkB) and a BDNF-sequestering fusion protein (TrkB–Fc), each of which blocks the effects of BDNF[7,9,12,13]. We administered anti-TrkB or TrkB–Fc intrathecally to rats that had developed allodynia 2 weeks after PNI. Both agents acutely reversed the decrease in paw withdrawal threshold ($n = 7$ and $n = 4$, respectively, $P < 0.05$; Fig. 3a). By contrast, vehicle administration to rats with PNI produced no change in withdrawal threshold (data not shown). To determine whether BDNF–TrkB signalling is necessary for the nerve injury-induced shift in E_{anion} in LI neurons, we examined the effect of anti-TrkB applied acutely to spinal cord slices taken from rats with allodynia 2 weeks after PNI. We found that E_{anion} of LI neurons in slices treated with anti-TrkB ($n = 7$) was significantly more negative than E_{anion} in vehicle-treated slices ($n = 6$; $P < 0.05$; Fig. 3b, c). Together, these findings indicate that endogenous BDNF is necessary to sustain both the tactile allodynia and the depolarizing shift in E_{anion} in LI neurons that result from PNI.

If microglia are the source of BDNF, then interfering with BDNF–TrkB signalling should prevent the tactile allodynia and the shift in LI neuronal E_{anion} produced by administering ATP-stimulated microglia (Fig. 4). We tested the first part of this prediction by delivering ATP-stimulated microglia together with either anti-TrkB or TrkB–Fc; the two cocktails produced no change in paw withdrawal threshold over the 5 h after intrathecal injection ($n = 8$ and $n = 7$, respectively; Fig. 4a). By contrast, allodynia developed progressively after the administration of ATP-stimulated microglia without these agents ($n = 8$). Notwithstanding these results, it remained possible that microglia stimulated with ATP might have provoked the release of BDNF from cells in the spinal cord and that the blockers interfered with the action of BDNF from this source rather than from the administered microglia *per se*.

To differentiate between these two possibilities, it was necessary to suppress BDNF production in the microglia that were subsequently stimulated with ATP and then administered intrathecally. We pretreated the cultured microglia with double-stranded short interfering RNA directed against BDNF (BDNF siRNA[14]). The microglia were then stimulated with ATP and, when injected intrathecally into naive rats, did not cause a change in withdrawal threshold ($n = 7$; Fig. 4a). To control for possible nonspecific effects of siRNA, we treated some microglia with a scrambled version of the BDNF siRNA before ATP stimulation; these microglia elicited a robust allodynia ($n = 4$, Fig. 4a). In addition, ATP-evoked Ca^{2+} responses in microglia treated with anti-TrkB or with BDNF siRNA did not differ from those of vehicle-treated control microglia, showing that anti-TrkB or BDNF siRNA treatment did not affect the response of the microglia to ATP (Fig. 4d). Yet, interfering with BDNF–TrkB signalling prevented microglia-induced tactile allodynia.

We then addressed the prediction that the depolarizing shift in E_{anion} produced by ATP-stimulated microglia could be prevented by interfering with BDNF–TrkB signalling. E_{anion} in LI neurons from rats receiving ATP-stimulated microglia together with anti-TrkB or after BDNF siRNA pretreatment did not differ significantly from that in LI neurons from rats receiving unstimulated microglia. However, E_{anion} in LI neurons from either of these groups of rats was significantly more negative than that in LI neurons from rats that had received ATP-stimulated microglia with vehicle (Fig. 4c). Thus, anti-TrkB and BDNF siRNA prevented the shift in E_{anion} induced by ATP-stimulated microglia. Moreover, ATP stimulation ($n = 3$), but

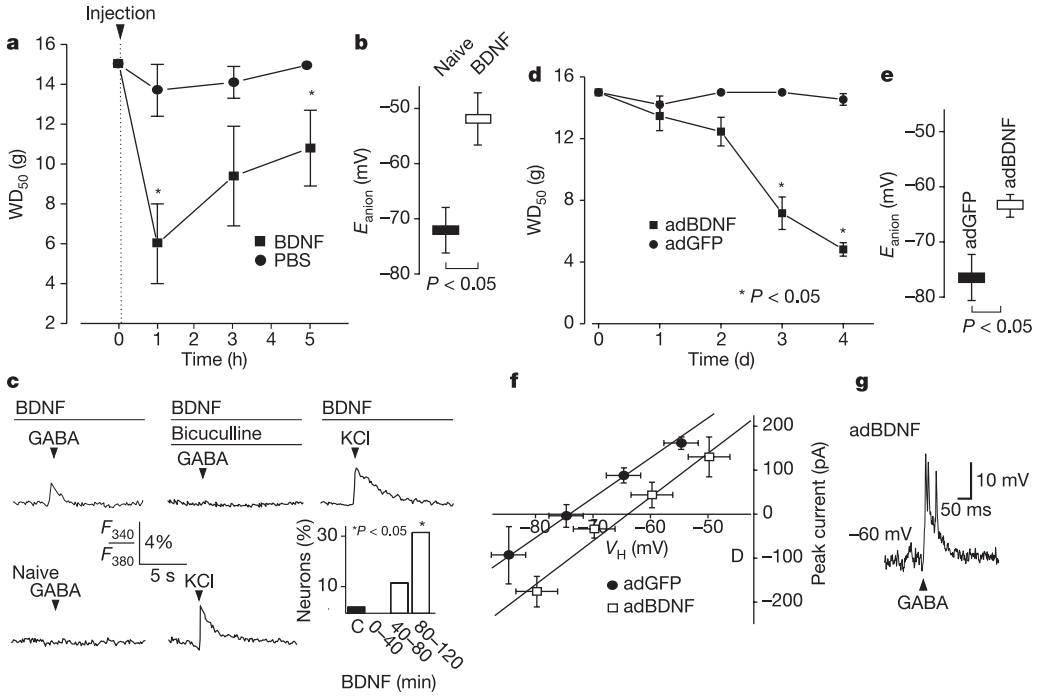

Figure 2 | Enhanced BDNF in the dorsal horn elicits nociceptive hypersensitivity and a depolarizing shift in E_{anion} in spinal LI neurons. **a**, Intrathecal delivery of recombinant human BDNF (20 μg) to the lumbar dorsal horn of intact rats led to a significant and transient decrease in the mean ± s.e.m. WD_{50} within 1 h, as compared with saline control. **b**, Significant depolarization of mean ± s.e.m. E_{anion} in LI neurons in slices treated with BDNF (50 ng ml^{-1}; for >90 min) as compared with slices in control ACSF (Naive). **c**, Representative traces of Ca^{2+} measurements from LI neurons showing that brief GABA application in slices superfused with BDNF caused a bicuculline-sensitive increase in $[Ca^{2+}]_i$. The viability of cells not responding to GABA was confirmed by KCl-mediated responses. Bottom right inset, the proportion of LI neurons showing a GABA-mediated rise in $[Ca^{2+}]_i$ increased progressively, reaching 31% between 80 and 120 min of continuous BDNF perfusion ($\chi^2_{corrected} = 5.15$). By contrast, only 2% of cells responded with a rise in $[Ca^{2+}]_i$ over a similar time period in the absence of BDNF (C; $\chi^2_{corrected} = 6.74$). **d**, Intrathecal administration of adBDNF[11] triggered a delayed and progressive decrease in the mean ± s.e.m. WD_{50} that persisted as long at 4 d after injection. By contrast, administration of a control adenovirus (adGFP) elicited no decrease in WD_{50}. **e**, Significant depolarization of the mean ± s.e.m. E_{anion} in LI neurons in slices taken from the rats in **d**. **f**, Mean ± s.e.m. peak current evoked by GABA measured in LI neurons at various values of V_m in slices taken from the rats in **d**. Horizontal standard error bars represent interneuron differences. **g**, Representative trace in current-clamp recording mode showing that brief GABA application to a LI neuron in a slice taken from an adBDNF-treated rat elicited action potentials.

not vehicle control ($n = 4$), caused release of BDNF from microglia in culture ($P < 0.001$; Fig. 4e). This effect of ATP was blocked by treating the cultures with the P2X receptor blocker TNP-ATP ($n = 3$; $P < 0.05$). In addition, pretreatment of the microglia with BDNF siRNA prevented release of BDNF by ATP stimulation ($n = 3$; $P < 0.001$). Together with our behavioural and electrophysiological results, these findings show that both the decrease in paw withdrawal threshold and the shift in E_{anion} in LI neurons caused by ATP-stimulated microglia require BDNF–TrkB signalling and that the source of BDNF is the microglia themselves.

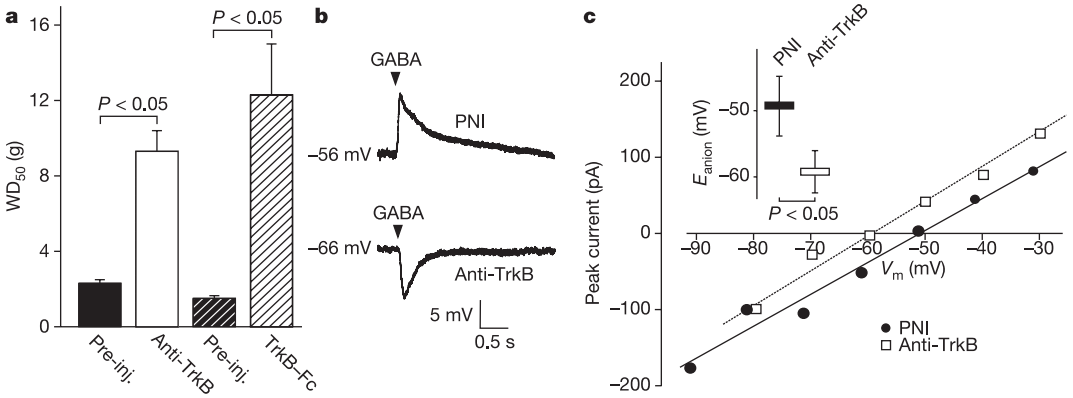

Figure 3 | Functional inhibition of BDNF–TrkB signalling reverses allodynia and the depolarizing shift in E_{anion} in spinal LI neurons in rats with PNI. **a**, Intrathecal administration of either anti-TrkB or TrkB–Fc to the lumbar dorsal horn of rats that showed a robust allodynia in response to PNI caused a significant increase in the mean ± s.e.m. WD_{50}. **b**, Representative traces in current-clamp recording mode showing the postsynaptic response to GABA in LI neurons taken from PNI rats in slices treated with or without anti-TrkB. **c**, Representative current–voltage plots in voltage-clamp recording mode of responses to brief (10-ms) local applications of GABA in two LI neurons in slices taken from PNI rats: one from a slice superfused with control ACSF, the other from a slice after anti-TrkB perfusion (1 μg ml^{-1}). Inset, pooled data showing that anti-TrkB perfusion of slices taken from rats with PNI elicited a significant hyperpolarization of the mean ± s.e.m. E_{anion} in LI neurons.

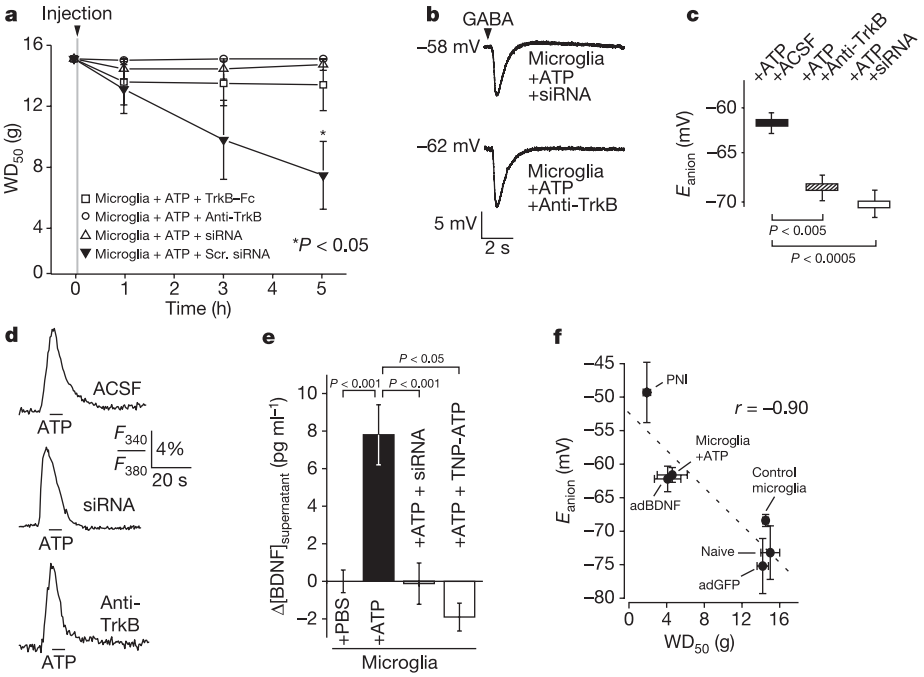

Figure 4 | Microglia-derived BDNF triggers both allodynia and the depolarizing shift in E_{anion} in LI neurons. **a**, Local spinal delivery of ATP-stimulated microglia either incubated with anti-TrkB or TrkB–Fc, or transfected with BDNF siRNA did not cause a significant change in the mean ± s.e.m. WD_{50}. By contrast, ATP-stimulated microglia transfected with a scrambled version of the interfering RNA (Scr. siRNA) caused the WD_{50} to drop significantly after 5 h. **b**, Representative traces in current-clamp recording mode showing postsynaptic responses to GABA in LI neurons from rats treated as in **a**. **c**, Pooled data showing the mean ± s.e.m. E_{anion} measured in LI neurons from rats treated as in **a**. **d**, Representative traces of Ca^{2+} measurements from microglia showing that responses to brief applications of ATP were not affected by exposure of microglia to anti-TrkB or to BDNF siRNA. **e**, ELISA-based measurement of the mean ± s.e.m. BDNF protein in the supernatant of cultured microglia 5 h after treatment with PBS vehicle, ATP, ATP plus TNP-ATP (10 μM) or ATP after pretreatment with BDNF siRNA. **f**, Correlation plot showing the relationship between E_{anion} and WD_{50} (error bars indicate the s.e.m.). The data shown include only those where both WD_{50} and E_{anion} were recorded in the same rat.

Our present findings suggest that, after nerve injury, ATP stimulation of microglia and the subsequent BDNF–TrkB signalling are crucial elements leading to the shift in E_{anion} of LI neurons. We therefore reasoned that inhibiting microglia ATP signalling should suppress the shift in E_{anion} caused by PNI. To test this, we bath-applied TNP-ATP (which reverses nerve-injury-induced tactile allodynia by acting on P2X receptors in microglia[4]) to spinal slices taken from allodynic rats 2 weeks after PNI. In the presence of TNP-ATP, E_{anion} of LI neurons was -59.3 ± 1.8 mV ($n = 6$), which was significantly more negative than that in LI neurons from untreated slices taken from nerve-injured rats (-49.3 ± 4.5 mV; $n = 6$; $P < 0.05$). Thus, P2X receptor activation is necessary to sustain the depolarizing shift in E_{anion} in rats with PNI. Moreover, we found an inverse correlation between paw withdrawal threshold and E_{anion} in LI neurons across all experimental conditions (Fig. 4f), suggesting that E_{anion} is an essential mechanistic link between microglia and allodynia.

Because acutely disrupting the microglia–BDNF–neuron signalling pathway reversed the change in E_{anion} and allodynia, ongoing activation of this pathway must be necessary to maintain nerve-injury-induced pain. Although BDNF has been implicated in spinal nociceptive hypersensitivity, the current framework of thinking on the role of BDNF is based on the hypothesis that the source of this neurotrophin is primary afferent neurons (reviewed in ref. 15). This source of BDNF has been brought into question in neuropathic pain, however, because of evidence indicating that there is a lack of primary-afferent-evoked release of BDNF in the spinal cord after PNI[16]. By showing that BDNF derives from microglia, our findings change the model for understanding how BDNF participates in the nociceptive hypersensitivity that underlies tactile allodynia. Our findings provide a new perspective from which to understand the aetiology of pain hypersensitivity and suggest that targeting microglia-derived BDNF may be a previously unsuspected avenue for treating neuropathic pain.

Our results define a biochemical pathway and a biophysical mechanism by which activated microglia affect neuronal function. Microglia become activated in many pathological processes in the central nervous system[17–20]. Given that depolarizing anion currents can gate plasticity and excitotoxicity[21], the microglia–neuron signalling mechanism that we have identified may be a substrate underlying these pathological disorders.

METHODS

Model of PNI and behavioural studies. PNI was induced as described[5,22]. In brief, a polyethylene cuff (~2 mm in length) was surgically implanted around the sciatic nerve of adult male Sprague–Dawley rats. The 50% paw withdrawal threshold to mechanical stimulation was assessed as described[5,23]. The withdrawal threshold was measured for each rat before use in slice experiments. In this model of PNI, there is microglia activation in the spinal cord ipsilateral to the nerve cuff, as indicated by a considerable increase in labelling for the microglia activation marker OX42 (by anti-CR3/CD11b, Cedarlane; diluted 1:1,000; data not shown).

Slice preparation. Parasagittal slices (300–350 μm) of spinal cord were prepared from ad ult male rats (aged >50 d) as described[24]. Slices were continually superfused (2–3 ml min^{-1}) with artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 26 $NaHCO_3$, 10 glucose, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$ and 1.25 NaH_2PO_4 buffer (bubbled with 95% O_2/5% CO_2; pH ≈ 7.4).

Recordings. The pipettes were filled with a solution containing (in mM): 130 potassium methyl sulphate ($KMeSO_4$), 5 CsCl, 2 $MgCl_2$, 11 BAPTA, 1 $CaCl_2$, 4 ATP, 0.4 GTP and 10 HEPES buffer (pH ≈ 7.4). For perforated-patch recordings, 25 μg ml^{-1} gramicidin D, from a stock solution of 10 mg ml^{-1} in dimethylsulphoxide (DMSO), was added to the above solution. GABA was applied locally for 10–50 ms by pressure ejection through a micropipette. Data acquisition and analysis of postsynaptic currents (PSCs) were done as

described[25]. Membrane potential measurements were corrected as described[26]. Neither input resistance nor resting membrane potential of LI neurons was affected significantly by any of the drugs or protocols used in this study. All data are given as the mean ± s.e.m., except where indicated. Statistical significance was tested by using Student's *t*-test for comparison of mean values, χ^2 tests for contingency tables, and mixed-design analyses of variance (*post hoc* Tukey's HSD test) for repeated measures.

Microglia cultures. Rat primary cultures were prepared from neonatal cortex or spinal cord as indicated, under standard conditions as described[4,27], and maintained for 10–14 d in DMEM medium with 10% fetal bovine serum. Microglia were separated from the primary culture by gentle shaking of the flask and were replated on plastic dishes. The cells were removed from the dish surface with a cell scraper and collected in 100 μl of PBS buffer. The density of microglia was measured by using a cell counter, and the volume of PBS was adjusted to give a final density of 1,000 cells per 10 μl. This method produces microglia cultures of >95% purity. For ATP stimulation, the purified microglia were incubated with 50 μM ATP for 1 h.

Intrathecal injections. At least 3 d before drug administration, rats were anaesthetized with sodium pentobarbital (65 mg per kg (body weight)), and a lumbar spinal catheter (PE-10 polyethylene tube) was inserted into the intrathecal space as described[28]. After drug or vehicle administration, rats were killed and their vertebral column was dissected to confirm visually that placement of the catheter was correct. Drugs included BDNF (10 μg per injection), anti-TrkB (30 μg per injection) and TrkB–Fc (5 μg per injection), all of which were prepared in saline plus 10% (v/v) DMSO. For virus-mediated transduction, adenoviral vectors encoding BDNF and enhanced green fluorescent protein (EGFP)[11] were administered once (20 μl, at 2.0×10^{10} plaque-forming units per ml). At the doses used, none of the compounds produced motor disturbances or sedation, as assessed by grasping, righting and placing reflexes and behavioural observations[29]. In all experiments, 30 μl of microglia plus supernatant were injected intrathecally in normal rats, and for all drugs and microglia tested the experimenter was blind to the treatment.

RNA interference. Microglia cultures were transfected with siRNA directed against BDNF or scrambled siRNA (Dharmacon) with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In brief, siRNA and Lipofectamine were diluted in serum-free medium, mixed and added to the microglia cultures. Transfection was allowed to occur for 5 h and the microglia were collected as described above for subsequent stimulation and intrathecal injection. The BDNF siRNA consisted of four pooled 21-nucleotide duplexes. The sequences of the four duplexes[14] were TCGAAGAGCTGCTGGATGA, TATGTACACTGACCATTAA, GAGCGTGTGTGACAGTATT and GAACTACCC AATCGTATGT.

Enzyme-linked immunosorbent assay. To measure BDNF secretion, we prepared microglia under the various experimental conditions described above and incubated them at 37 °C for 6 h to model the above *in vivo* experiments. Supernatants were removed and BDNF protein concentration was measured by ELISA (Chemicon).

Calcium imaging. Spinal cord slices were prepared for Ca^{2+} imaging and tested for responses to GABA as described[5]. Primary cultures of microglia were prepared as above, transferred to standard coverslips and incubated with 2.5 μM Fura-2-AM in HEPES-buffered saline plus 0.01% DMSO for 45 min. After fluorophore loading, changes in $[Ca^{2+}]_i$ in individual microglia were evoked with brief (~5 s) applications of ATP (10 μM) from a micropipette. $[Ca^{2+}]_i$ was fluorometrically measured with a 40× water-immersion objective on a Zeiss Axioscope equipped with epifluorescence optics. Images were acquired by using a TILL Photonics monochromator coupled to a CCD camera, and regions of interest (for ratio analysis) were drawn on clearly distinct cell bodies.

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