Glutamate transporter dysfunction associated with nerve injury-induced pain in mice

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Abstract

Dysfunction at glutamatergic synapses has been proposed as a mechanism in the development of neuropathic pain. Here we sought to determine whether peripheral nerve injury-induced neuropathic pain results in functional changes to primary afferent synapses. Signs of neuropathic pain as well as an induction of glial fibrillary acidic protein in immunostained spinal cord sections 4 days after partial ligation of the sciatic nerve indicated the induction of neuropathic pain. We found that following nerve injury, no discernable change to kinetics of AMPA or NMDA receptor (NMDAR)-mediated evoked excitatory post synaptic currents (eEPSCs) could be observed in dorsal horn (lamina I/II) neurons compared to those of naive mice. However, we did find that nerve injury was accompanied by slowed decay of the early phase of eEPSCs in the presence of glutamate transporter inhibition by the competitive non-transportable inhibitor DL-threo-β-Benzyloxyaspartic acid (TBOA). Concomitantly, expression patterns for the two major glutamate transporters in spinal cord, EAAT1 and EAAT2, were found to be reduced at this time (4 days post-injury). We then sought to directly determine whether nerve injury results in glutamate spillover to NMDARs at dorsal horn synapses. By employing the use-dependent NMDAR blocker (±)MK-801 to block subsynaptic receptors, we found that although TBOA-induced spillover to extra-synaptic receptors trended to increased activation of these receptors after nerve injury, this was not significant compared to naive mice. Together, these results suggest the development of neuropathic pain involves subtle changes to glutamate transporter expression and function that could contribute to neuropathic pain during excessive synaptic activity.
Introduction

The synaptic mechanisms associated with development of chronic neuropathic pain remain poorly understood (Graham et al. 2007). An interplay of both an increase in primary afferent activity or glutamate release, as well as an impairment of glutamate re-uptake at primary afferent synapses in the dorsal horn have been proposed as possible mechanisms for the development of neuropathic pain (Tsuda et al. 2005; Sandkuhler 2007; Scholz and Woolf 2007).

One of the key components of normal sensory neurotransmission is the tight coupling of excitatory neurotransmitter release with rapid reuptake. Recovery of synaptic glutamate is mediated by a family of specific high-affinity membrane transporters (Danbolt 2001), the excitatory amino acid transporters (EAATs). Astrocytes express two such transporters, EAAT1 (GLAST, Slc1a3) and EAAT2 (GLT-1, Slc1a2), which are together responsible for the majority of synaptic glutamate re-uptake (Rothstein et al. 1994; Chaudhry et al. 1995; Rothstein et al. 1996). A third, neuronal isoform, known as EAAT3 (EAAC1, Slc1a1) is also found in the spinal cord, on primary afferents, interneurons and motor neurons of the ventral horn but plays only a limited role in synaptic glutamate re-uptake (Rothstein et al. 1994; Peghini et al. 1997; Stoffel et al. 2004; Sun et al. 2011). Intrathecal administration of EAAT inhibitors produces pain behaviours suggesting that dysfunction of these transporters may contribute to signs of neuropathic pain (Liaw et al. 2005; Weng et al. 2006). Findings of pathological changes to astroglia during development of neuropathic pain have raised the
possibility that EAAT function may be impaired in the vicinity of primary afferent synapses. Recent reports have indicated that spinal EAAT expression can both increase and decrease in the first 7 days following peripheral nerve injury in rodent neuropathic pain models (Sung et al. 2003; Wang et al. 2006; Sung et al. 2007; Tawfik et al. 2008; Xin et al. 2009) with substantial losses at day 7 and beyond. It has also been shown that these expression changes coincide with the development of neuropathic pain. However, exactly how these expression changes contribute to altered glutamatergic synaptic transmission has not yet been fully elucidated.

The aims of this study were therefore to determine if peripheral nerve injury influences the expression and uptake capacity of spinal glutamate transporters at functioning glutamatergic synapses in spinal cord of nerve injured mice during development of neuropathic pain. Although behavioural signs and markers of neuropathic pain such as astrocyte activation were observed after nerve injury, we found only a modest reduction in glutamate transporter expression after nerve injury and evidence for limited changes to glutamatergic transmission at primary afferent synapses that would be indicative of impaired glutamate uptake.

**Materials and Methods**

**Experimental animals**

Animals used in the experiments outlined below were approved for use by The University of Sydney and University of Technology Sydney Animal Ethics Committees. Experiments were
performed under the guidelines of the Australian code of practice for the care and use of animals for scientific purposes (National Health and Medical Research Council, Australia, 7th Edition). In all experiments unless otherwise stated, 6 – 8 week old inbred C57bl/6 male mice were used (91 in total). Animals were kept in 12hr light/dark cycles with food (standard rodent chow) and water provided ad libitum.

Induction and behavioural assessment of nerve injury

Partial sciatic nerve ligation (PNL) was performed as described previously (Seltzer et al. 1990). Briefly, under isoflurane (Aerrane, Baxter) anaesthesia (2.5% in oxygen), the biceps femoris muscle was blunt dissected to reveal the sciatic nerve proximal to its trifurcation. Connective tissue surrounding the nerve was carefully removed to improve access. A spinal hook was then used to lift the nerve without compression or stretching so that a single 6-0 silk suture could be threaded through approximately one-half of the nerve. This single suture was tied tightly before returning the nerve to its original position. The musculature was then sewn together with a single suture and the skin was brought together and closed with cyanoacrylate glue (Vetbond™, 3M™).

The impact of nerve injury on hind paw weight bearing was assessed with a Linton Incapacitance Tester (Linton Instrumentation, UK) prior to and 1, 4 and 7 days following nerve injury (Strickland et al. 2008). Measurements were taken by averaging the instantaneous force (measured as weight, in grams) applied to each hind-paw at pre-surgery (baseline) and each day post-surgery. Each datum is the average of 4 measurements taken...
at 10 second intervals. An incapacitance ratio was therefore derived as the force applied by
the injured hind paw / uninjured paw. Animals that failed to show a 25% or greater
reduction in their incapacitance ratio (7 of the 91 mice) were not used for biochemical or
electrophysiological experiments and were euthanized.

Immediately following incapacitance measurements, each animal was placed into a
plexiglass observation chamber (40cm x 20cm x 20cm) and was monitored for 5 min for
signs of spontaneous pain by scoring the number of events of hind-paw lifting, hind-paw
flicking or shaking, and hind paw tending. A composite score for each animal was calculated
by summing each score. As the mice were able to move freely about the enclosure, no
scoring bias was applied to the affected paw.

*Immunofluorescent detection of spinal glutamate transporters*

Following sodium pentobarbital administration, mice were transcardially perfused with 10
ml of a heparin containing (3000 IU /L) flush solution (in mM; 154 NaCl, 58.8 NaNO₃) before
perfusion with 100 ml of the fixation solution; 4% formaldehyde prepared in PBS (in mM;
25.3 NaH₂PO₄.H₂O, 108 Na₂HPO₄, 154 NaCl). Spinal cords were removed following fixation
and post-fixed for 1 hour, before transferring to 30% sucrose/PBS to cryoprotect the cords.
All steps used PBS as a buffer. Once sucrose had penetrated the cords (as determined by
cords sinking) they were snap-frozen in tissue support matrix (Tissue-Tek® O.C.T. compound,
Proscitech, Thuringowa, QLD, Australia) and stored at -80 °C until processed. Thirty
micrometer sections were then cut into PBS using a Leica cryostat (CM1850 UV) and stored at 4 °C. Only sections from the lumbar enlargement (L3 – L5) were used for staining.

For immunofluorescent staining, 6 sections from each of 4 mice per time point (or 4 naive mice, 12 mice in total) were permeabilised in 0.3% Triton-X100/PBS (wash buffer) for 10 minutes before blocking with 10% normal horse serum (NHS; EAAT1-2) or goat serum (for GFAP) for 30 minutes. Sections were then transferred into the appropriate primary antibody diluted in wash buffer (EAAT1 1:4,000 Millipore; EAAT2 1:400 Millipore; EAAT3 1:1000 Millipore; GFAP 1:1,000 Abcam) and incubated overnight at room temperature with gentle agitation. Excess primary antibody was removed with three 10 minutes washes of wash buffer before a 2 hour incubation in secondary antibody (EAAT1/2; anti-guinea pig A488, Jackson ImmunoResearch, GFAP; anti-rabbit A488, Invitrogen™, EAAT3; anti-mouse Cy3, Jackson ImmunoResearch). Finally, sections were washed again with three 10 minutes washes with wash buffer before mounting onto glass slides with fluoromount-G (Southern Biotech, Alabama, USA). Digital images of individual sections were taken at 10X optical magnification on an epifluorescent microscope. Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda USA). For high magnification of GFAP-stained sections, 4 10X confocal images comprising 30 1µm z-sections were stitched together using Photoshop® (Adobe, San Jose CA) to create one single image. Inlays are comprised of 9, 60X confocal images comprising 30 1µm z-sections stitched together.

In vitro electrophysiology
Spinal cord slices (340 µm) from the lumbar enlargement L3-L5 (Rigaud et al. 2008) were prepared from 24 nerve injured and 36 naive isoflurane anaesthetised mice on a Leica VT1200S vibrating blade microtome (Leica Microsystems, North Ryde, Australia) in ice-cold modified artificial cerebrospinal fluid (ACSF, in mM); choline chloride (120), glucose (11), NaHCO₃ (25), KCl (2.5), NaH₂PO₄ (1.4), CaCl₂ (0.5), MgCl₂ (7), atropine (0.001). Slices were allowed to recover for 1 hour at room temperature in ACSF (in mM); NaCl (125), KCl (2.5), NaH₂PO₄ (1.25), NaHCO₃ (25), glucose (11), MgCl₂ (1.2), CaCl₂ (2.5) before being transferred to a recording chamber where Dodt-contrast optics was used to identify lamina I/II superficial dorsal horn neurons for patch-clamp electrophysiology. The internal solution of the recording pipette contained (in mM); CsCl (140), EGTA (10), HEPES (5), CaCl₂ (2), MgATP (2), NaGTP (0.3), QX314 chloride (5) and had an osmolality of 290 mOsm. Drugs were superfused onto slices at a rate of 2 ml per minute in normal ACSF at a nominal 33°C.

Whole-cell voltage clamp was performed using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale CA) interfaced to an intel processor-based iMac computer (Apple, Cupertino CA; Mac OS X 10.5) via an ITC-18 digitiser (Heka Elektronik, Ludwigshafen Germany). Electrically-evoked EPSCs (eEPSCs) were elicited by stimulating dorsal roots attached to the slice at 0.03Hz with bipolar tungsten electrodes so that an appropriately sized current was produced in recorded neurons, in the range of 100 – 800 pA. eEPSCs were sampled at 10 kHz and filtered at 4 kHz using Axograph X (Axograph Scientific, Australia). AMPA receptor mediated currents were obtained by clamping the membrane potential at -60 mV in the presence of picrotoxin (100 µM), strychnine (5 µM) and DL-AP5 (100 µM). NMDA receptor mediated currents were obtained by clamping the membrane potential at
+40 mV in the presence of picrotoxin (100 µM), strychnine (5 µM) and CNQX (10 µM).

Miniature EPSCs were filtered (4 kHz low-pass filter) and sampled at 10 kHz for on-line and later off-line analysis, and were recorded in the presence of picrotoxin (100 µM), strychnine (5 µM) and tetrodotoxin (1 µM), to block GABA, glycine and sodium channels, respectively.

Miniature EPSCs above a preset threshold (4 standard deviations above baseline noise) were automatically detected by a sliding template algorithm, and then manually checked off-line.

Miniature EPSCs were then counted in 4 s epochs every 2 s to construct rate-time plots.

Data presented represent the average of at least 800 captured events over a minimum 5 minute interval for each condition (drug or vehicle) for each cell recorded (N = 4). Series resistance (< 20 MΩ) was compensated by 70-80% in all patch-clamp experiments.

Recordings were stopped if series resistance deviated by more than 20% of baseline.

Data analysis and statistics

Densitometry of immunohistochemistry was performed using imageJ (National Institutes of Health, Bethesda, USA). Behavioural testing was recorded by the observer in a notebook before being manually entered into Prism (Version 5 for Windows, GraphPad Software Inc. California, USA) for statistical analysis and generation of graphs. Illustrations were prepared using Photoshop® CS4 and Illustrator® CS4 for Windows. Kinetics of AMPAR and NMDAR evoked EPSCs were determined offline using the ‘fit exponential’ tool in Axograph on the assembled average of 10 consecutive eEPSCs for each condition. The kinetics of unperturbed (in the absence of uptake inhibitors) eEPSC decay were best described by a
single exponential. As with previous reports, the addition of a second exponential did not improve the quality of the fit (Grosskreutz et al. 2003). Rise and decay kinetics for each recording were only included for analysis if the assembled average trace was smooth enough to confidently measure these parameters. The area under the curve was also determined for each trace using Axograph. Statistical differences were determined using one-way ANOVA with Dunn’s multiple comparisons post-test (Western blots, behavioural tests) and student’s t-test (immunohistochemistry, electrophysiology) in Prism.

Drugs

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Disodium CNQX, picrotoxin, strychnine, DL-TBOA and DL-AP5 were purchased from Tocris (Bristol, UK). Tetrodotoxin and QX314 chloride were obtained from Alamone Labs Ltd. (Jerusalem, Israel).

Results

Nerve injury-induced signs of neuropathic pain and astrocyte activation

In agreement with previous reports, partial sciatic nerve ligation induced reliable signs of pain (Seltzer et al. 1990) as well as increased staining of GFAP (Coyle 1998; Narita et al. 2006). From as early as 24 hours post-surgery, nerve injured mice displayed a significant reduction in the force applied to the injured paw compared to baseline (Figure 1A. left/right ratio, baseline = 1.08 ± 0.03 vs. Day 1 = 0.44 ± 0.02, P< 0.0001; ANOVA). This change continued up to and beyond day 7 post-surgery (Day 4 = 0.51 ± 0.04, Day 7 = 0.58 ± 0.07, P<
Similarly, signs of paw lifting, flicking and guarding remained elevated during the 7 day post-surgical period (Figure 1B. Left vs. right Days 1, 4 and 7 P< 0.0001; ANOVA). We examined the well established activation of astrocytes following PNL by examining GFAP staining. As shown in Figure 1C the GFAP immunoreactivity was significantly elevated after nerve injury with the greatest effect being observed 4 days post-injury.

**Effect of PNL on expression of spinal glutamate transporters**

Following nerve injury, perfusion-fixed mouse spinal cord was sectioned for immunohistochemical staining of glutamate transporters. Previous reports have indicated that both glial and neuronal spinal glutamate transporter expression is perturbed by nerve injury and inflammation (Harris *et al.* 1996; Sung *et al.* 2003; Wang *et al.* 2006; Wang *et al.* 2008; Xin *et al.* 2009).

Modest decreases in astrocytic glutamate transporter expression were observed in immunohistochemically stained sections of lumbar spinal cord at days 4 and 7 post-injury. The glial transporters, EAAT1 and EAAT2 (Figure 2A and B, resp.) showed small, persistent decreases in ipsilateral expression when compared to contralateral dorsal horn. For EAAT1, the approx. 7% decrease in optical density in the dorsal horn was not significant at either day 4 or 7 after injury. However, when both post-injury days were pooled, the decrease was significant (P < 0.05). For EAAT2, the 9% decreases in optical density for ipsilateral versus contralateral dorsal horn were significantly reduced on both day 4 and day 7 after injury.
The small reduction in EAAT2 immunohistochemical staining was then further explored by Western blot on crudely isolated dorsal horn soluble protein extracts in a separate group of animals. In these experiments, no significant changes to total EAAT2 levels were found at either post-injury time point (data not shown). As shown in figure 2D, no trends for changed expression of EAAT3 were detected immunohistochemically. We should note however, that the antibody used to detect EAAT3 was from the same species (mouse) as the experimental animals and is most likely the cause of some non-specific staining, especially in the ventral horn. However, in concurrent experiments performed without primary antibody (negative controls), non-specific staining was not observed in the superficial dorsal horn where analysis was performed (data not shown). Collectively, these data suggest that nerve injury associated with development of neuropathic pain has only a modest impact on the expression of glutamate transporters in the spinal dorsal horn.

Effect of nerve injury on AMPAR synaptic current kinetics.

If the small reduction in immunohistochemical staining for the astrocyte glutamate transporters, EAAT1 and EAAT2, reflected reduced cell surface expression in the vicinity of excitatory synapses, then this could lead to reduced extrasynaptic glutamate reuptake. If this were to occur then kinetics of synaptic currents should be altered. We examined the kinetics of AMPAR and NMDAR mediated eEPSCs 4 days after injury because maximal effects on GFAP, as well as decreased expression of EAAT1 and EAAT2, were observed at that time.
Example AMPAR- and NMDAR mediated eEPSCs are shown in figures 3A and 3B.

AMPAR and NMDAR eEPSC decay time constants were best fit by single exponentials as previously described (Feldmeyer et al. 2002; Grosskreutz et al. 2003; Stubblefield and Benke 2010). Fitted time constants did not differ significantly for either the rise (naive; 0.95 ± 0.1 ms, N = 12, injured; 1.0 ± 0.15 ms, N = 10) or decay (naive; 6.15 ± 0.74 ms, N = 23, injured; 6.65 ± 0.95 ms, N = 20) of AMPAR-mediated eEPSCs in naive vs. injured animals, measured in the presence of DL-AP5 (100 µM), picrotoxin (100 µM) and strychnine (5 µM). Similarly, NMDAR rise time and decay kinetics, measured in the presence of CNQX (10 µM), picrotoxin (100 µM) and strychnine (5 µM), were not affected by nerve injury (rise; naive; 6.29 ± 1.09 ms N = 8, injured; 6.63 ± 1.69 ms N = 6, decay; naive; 195.6 ± 24.18, N = 7, injured; 187.8 ± 28.82 N = 7). These findings suggest that impaired glutamate transporter activity might not contribute greatly to primary afferent synaptic transmission after nerve injury.

Effect of nerve injury on sensitivity of glutamate transporters to inhibition

Measures of unperturbed decay time constants could be an insensitive measure of reduced glutamate reuptake in the vicinity of synapses because decay kinetics of both AMPAR and NMDAR mediated synaptic currents are dominated by channel kinetics rather than glutamate reuptake (Dingledine et al. 1999). Such measures could also be confounded by possible changes in subunit composition of AMPARs or NMDARs in pain states (Vikman et al. 2008) although our data suggest that no such changes occur in mouse dorsal horn, 4 days after nerve injury. Therefore, to further explore whether enhanced spillover of synaptic
glutamate develops after nerve injury, eEPSCs were recorded in the presence of a moderate concentration of a non-specific glutamate transport inhibitor, DL-threo-β-Benzylloxyaspartic acid (TBOA) (Shimamoto et al. 1998). By partially blocking glutamate reuptake to induce detectable spillover from excitatory synapses, any pathologically enhanced spillover of glutamate produced by nerve injury should be observed as an enhancement of these effects as previously reported at other CNS synapses (Nie and Weng 2010). As expected in naive animals, TBOA (30 and 100 µM) potentiated the duration but not amplitude of AMPAR eEPSCs in a concentration dependent manner by introducing the appearance of a second, late phase to the decay of the eEPSC without affecting the early decay phase (Figure 4A,B). As shown in figure 4B, 100µM TBOA significantly increased the eEPSC late time constant (P = 0.015, from 36.5 ± 8.1 ms to 125.4 ± 20.4 ms, n = 6), as well as the area under the curve (P < 0.05, from 1688 ± 373 pA.ms to 6013 ± 1269 pA.ms, n = 6) in naive animals. Both measures presumably reflect spillover of glutamate from subsynaptic to extrasynaptic sites and adjacent synapses. In dorsal horn neurons from untreated animals, transporter inhibition had no significant effect on the early decay time constant (control = 5.1 ± 0.5 ms, 30 µM = 5.6 ± 1.6 ms, 100 µM = 6.0 ± 1.2 ms), which is presumably dominated by decay kinetics of channel opening (see also below). In the presence of TBOA the addition of CNQX (10 µM in the continued presence of DL-AP5, picrotoxin and strychnine) blocked all slow synaptic currents and nearly abolished the fast component (n = 3). This suggests that the enhanced slow components of the eEPSC induced by TBOA are mediated exclusively by AMPARs without any contribution from mGluRs. mGluR-mediated slow eEPSCs have been reported in dorsal horn in the presence of
TBOA but only after high frequency stimulation of primary afferent fibres (Galik et al. 2008). TBOA (100 µM but not 30 µM) also produced a small but significant inhibition of the amplitude of eEPSCs (14 ± 1.8%), which could be mediated by spillover of glutamate to presynaptic mGluR (Drew et al. 2008) or KA receptors (Perrais et al. 2010). A 30 µM concentration of TBOA was therefore selected for subsequent experiments in nerve injured tissue because it produced only a moderate enhancement of the eEPSC late decay time constant without affecting amplitude.

If nerve injury does induce EAAT down-regulation in the vicinity of dorsal horn primary afferent synapses, glutamate spillover might be enhanced by moderate EAAT inhibition (30 µM TBOA) to a greater extent in nerve injured than control animals (Figure 3C). As shown in figure 3D, TBOA significantly increased the early decay time constant in dorsal horn neurons from nerve injured animals, suggesting that transporters in close proximity to glutamate release sites might be functionally modified to increase spillover. Alternatively, nerve injured mice could express more extra-synaptic AMPA receptors in close proximity to release sites. Nerve injury did not greatly influence distal extra-synaptic receptor activation as no change to area under the curve, or late decay time constant was observed following transporter inhibition. These findings suggest that reduced EAAT activity in the vicinity of primary afferent synapses, if it occurs after nerve injury, is not due to a widespread reduction in glutamate uptake capacity but is restricted to proximal extrasynaptic sites.
To ensure that the enhancing effects of TBOA on AMPAR kinetics were not due to presynaptic actions, miniature EPSCs (mEPSCs) were examined in naive animals. In the presence of 1µM tetrodotoxin (TTX), no significant changes to mEPSC decay, amplitude or frequency were observed during glutamate transporter inhibition in naive animals (100 µM TBOA, N = 4) (Figure 5). This is consistent with the finding that the early decay time constant of the eEPSC in uninjured mice was unaffected by TBOA and the expectation that mEPSCs, being sporadic quantal events should produce little spillover to extrasynaptic AMPARs.

If reduced perisynaptic glutamate transporter activity does indeed explain the increased early decay time constant of AMPAR-mediated synaptic events via increased spillover, then it might also be possible to detect increased spillover to NMDA receptors. To address this, we utilised the use-dependent NMDA receptor blocker (+)-MK-801 to block subsynaptic NMDARs before enhancing glutamate spillover with TBOA, as reported at this synapse (Nie and Weng 2009). In this experiment, stable baseline NMDAR-mediated eEPSCs (in the presence of CNQX) for dorsal horn neurons in whole-cell voltage-clamp (+40mV) were established by electrically stimulating the dorsal roots at 0.03Hz (Figure 6Ai). Then active/open NMDARs were blocked during a ten minute superfusion of 50 µM MK-801. At this time, subsynaptic NMDAR eEPSCs were completely blocked (Figure 6Aii). A ten minute wash was then performed to remove unbound MK-801 whilst stimulation continued (Figure 6Aii). Following this, 100µM TBOA was superfused onto slices to generate glutamate
spillover (Figure 6Aiii). As shown in figure 6A, this approach successfully induced glutamate spillover and activation of extra-synaptic NMDARs. When performed in slices from nerve injured mice, no change in time to peak was observed (Figure 6B). However a small non-significant trend for increase in peak amplitude and area under the curve were observed. Collectively however, these findings suggest that no significant increase of spillover to extra-synaptic NMDA receptors takes place following nerve injury. However, this does not rule out the possibility of increased spillover combined with reduced NMDAR expression.

Discussion

The present study suggests that nerve injury associated with allodynia and astroglial activation produces only small changes to expression and function of glutamate transporters in the vicinity of primary afferent synapses. Our findings therefore argue against a major role for a contribution of glutamate transporter dysfunction and glutamate spillover in the development of neuropathic pain. It is well established that peripheral nerve injury leads to changes in the expression and function of markers of glutamatergic neurotransmission in the spinal dorsal horn. These changes are also thought to be responsible, in part, for the development and maintenance of neuropathic pain (Tsuda et al. 2005; Sandkuhler 2007; Scholz and Woolf 2007; Vikman et al. 2008). One of the ways in which this has been proposed to occur is a generalized down-regulation of glutamate transporters in the vicinity of synapses and a concomitant increase in the expression and function of glutamate receptors (Harris et al. 1996; Popratiloff et al. 1998; Garry et al. 2003; Sung et al. 2003; Yang et al. 2004; Wang et al. 2006; Sung et al. 2007; Tawfik et al. 2008;
Wang et al. 2008). For example, in rats, PNL induced an approximately 51% and 40% reduction in EAAT1 (Xin et al. 2009) and EAAT2 (Maeda et al. 2008; Xin et al. 2009), respectively, expression at day 7. In another model of neuropathic pain, chronic constriction injury (CCI), these losses are preceded by an increase in EAAT1-3 at day 4 (Sung et al. 2003; Wang et al. 2006). However, despite an overall increase in total protein at this time, glutamate reuptake activity was in fact lower than in sham-operated animals (Sung et al. 2003). In contrast to the studies above, we found that nerve injury resulted in modest decreases in expression over the same 4-7 day period. When we looked at EAAT2 expression further by Western blot, these reductions were lost. This may be due to transporter redistribution in astrocytes, exposing changes in epitope availability in immunohistochemical studies. It is hard to reconcile the differences in EAAT expression observed between ours and previous studies. Others have reported increased expression of all three transporters up to 4 days after chronic constriction injury in rats (Sung et al., 2003) but decreased expression after 7 days (Sung et al., 2003; Wang et al. 2006). Explanations could be that of species differences, as our experiments were performed in mice and not rat, or models used (partial nerve ligation versus chronic constriction injury). For example, in mice mechanical allodynia and expression changes of nociceptive markers (e.g. NK1) are typically restricted to the ipsilateral hind paw (Malmberg and Basbaum 1998), whereas in rat, these changes are bilateral. It has been shown previously partial nerve ligation and chronic constriction models induce contrasting phenotypes of hot and cold hyperalgesia as well as mechanical allodynia (Bennett and Xie 1988; Seltzer et al. 1990). These adaptations were thought to create conditions whereby synaptic glutamate has the potential to both
persist within the synapse but also to diffuse out of the synapse to bind extra-synaptic 
receptors thereby enhancing primary afferent synaptic transmission and plasticity.

To test whether or not this occurs at functioning synapses, we made use of the 
broad-spectrum glutamate transport inhibitor TBOA to drive moderately increased 
glutamate spillover in the spinal dorsal horn. By inhibiting all three transporters with 100 
μM TBOA, a substantial late phase decay time constant for AMPARs was produced. 
Furthermore, the area under the curve of the AMPAR current (current density) increased 
from 249% in 30 μM TBOA to 384 % of control in 100 μM TBOA, suggesting a significant role 
for EAAT1 and EAAT2 in glutamate reuptake in the dorsal horn. Due to the rapid 
desensitization of AMPARs following agonist binding, the facilitation of the synaptic current 
under these conditions is likely due to diffusion of synaptically released glutamate to 
proximal (early time constant) extra-synaptic AMPARs and more remote extrasynaptic 
receptors and synapses (Beurrier et al. 2009).

If nerve-injury results in a basal increase in synaptic and perisynaptic glutamate 
concentrations, then eEPSCs from nerve-injured mice should display slower decay kinetics 
than naïve mice, similar to that observed for TBOA-induced spillover. In the absence of 
TBOA, decay time constants did not differ between naive and nerve injured mice suggesting 
AMPAR composition in the vicinity of synapses is not greatly altered and spillover, if present, 
is modest. Interestingly, nerve injury was associated with an increased early decay time
constant of AMPARs, suggesting increased peri-synaptic glutamate spillover or, alternatively, increased perisynaptic AMPAR density.

This increased early decay time constant after nerve injury in the presence of TBOA (30 µM) does not appear to be due simply to increased sensitivity to TBOA. Firstly, the late decay time constant was unaffected after nerve injury in this concentration of TBOA but was greatly enhanced in control tissue by a higher concentration of TBOA (100 µM). Secondly, the early decay time constant was completely unaffected by the higher concentration of TBOA (100 µM) in control spinal cord. These findings strongly suggest the increased early decay time constant observed in TBOA reflects either an increased density of extrasynaptic AMPARs that are silent in the absence of transporter inhibition or, alternatively impaired transporter (EAAT1 and/or EAAT2) activity in close proximity to synapses that enhances sensitivity to TBOA.

In a similar study in rats by Nie and Weng (2010), an EAAT2-specific blocker, dihydrokainate (DHK), significantly increased NMDAR EPSC amplitude, latency, duration and decay time in naïve rats, but failed to elicit any response in nerve injured rats, suggesting complete loss of EAAT2 function despite expression levels of around 40% compared to controls. This finding is hard to reconcile with our results. However it should be noted that these experiments were performed at 8-14 days post-injury in rats, whereas our electrophysiological recordings were made at day 4 post-injury in mice.
The possibility that perisynaptic spillover is increased can also be tested by examining the effect of TBOA on NMDARs after blockade of active subsynaptic receptors with MK-801. In this case there was no significant difference to the degree of extra-synaptic NMDAR activation at primary afferent synapses from nerve injured animals. This experiment therefore failed to confirm the interpretation that the increased early decay time constants induced by TBOA in nerve injured animals was due to increased perisynaptic glutamate spillover. There was, however, a non-significant trend for increased peak amplitude of the extrasynaptic NMDAR-mediated eEPSC which is consistent with a small increase in spillover. There are other potential explanations for the discrepancy between the AMPAR and NMDAR-mediated results. It is possible that the increased early decay time constant of the AMPAR-mediated eEPSC in the presence of TBOA reflects spillover to very proximal extrasynaptic AMPARs or there may be an increase in extrasynaptic AMPAR density following nerve injury rather than increased spillover per se. There is evidence for AMPAR subunit adaptations during inflammatory pain that could be associated with extrasynaptic insertion of AMPARs (Tao 2010) but whether or not similar adaptations develop in nerve injury-induced pain is unknown. It is also possible that spillover to extrasynaptic NMDARs in the presence of TBOA (100 µM) is mediated more by receptors more distal to release sites than the AMPARs contributing to the early decay phase enhanced by nerve injury. If so, measured NMDAR spillover may be similar to the late decay phase of the AMPAR-mediated eEPSC in the presence of TBOA that is unaffected by nerve injury.
In conclusion, the present study suggests that peripheral nerve injury at a time associated with neuropathic pain and astrocyte activation does not greatly influence the expression of astrocytic glutamate transporters in the superficial dorsal horn, nor does it produce substantial extrasynaptic spillover of glutamate from primary afferent synapses. However, in the close vicinity of synapses, glutamate transporter function may be prone to ineffective uptake under extreme circumstances and lead to enhanced receptor activation.

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**Figure 1.** Effect of nerve injury on the development of neuropathic pain. A) Hind paw incapacitance test revealing significant weight shift from left (injured) to right hind paw. Numbers in columns represent number of animals tested. B) PNL produces significant increase in the rate of spontaneous behaviours indicative of neuropathic pain. C) GFAP immunoreactivity in a spinal cord section from a nerve injured mouse (inj = injured/ipsilateral) and histogram revealing significant increase in GFAP densitometry at day 4 post-injury (N = 4 in each group).

**Figure 2.** Effect of nerve injury on expression of spinal glutamate transporters. Immunohistochemistry of glutamate transporters EAAT1 (A), EAAT2 (B) and EAAT3 (C) following nerve injury reveals significant reduction of EAAT1 and EAAT2 between 4 and 7 days (* = P < 0.05, inj = injured/ipsilateral side, N = 4 in each group).

**Figure 3.** Effect of nerve injury on the kinetics of AMPAR and NMDAR-mediated eEPSCs. Example synaptic current traces from eEPSCs mediated by AMPAR (A) and NMDAR (B) reveal no significant change to rise or decay kinetics observed after nerve injury.

**Figure 4.** Effect of glutamate transporter inhibition on eEPSCs. A) Representative AMPAR eEPSC showing effect of 30uM TBOA. Thin red lines depict fitted exponentials for early and
late components of decay. B) Effects of 30 µM and 100 µM TBOA on parameters of AMPAR eEPSCs in dorsal horn neurons from naive mice expressed as percentage of pre-TBOA baseline. C) Representative current traces of naive vs. nerve injured mice AMPAR eEPSCs in the presence of TBOA (30 µM) compared to baseline. D) The effect of 30 µM TBOA on eEPSC amplitude, decay kinetics and AUC in naive vs. nerve injured mice (percentage increase; * = P < 0.05, numbers within histograms represent number of cells; N).

Figure 5. Effect of TBOA on miniature EPSCs. A) Representative recording of mEPSC events before (CONTROL) and during application of TBOA. Enlarged is a single captured event from a baseline recording. B) Representative mEPSC traces from averaged events in the presence of TBOA. The histogram on the right reveals no deviation from baseline for decay, amplitude, or rate, are observed in the presence of TBOA (N = 4). C) Cumulative probability plots for current amplitude and rate in the presence of TBOA.

Figure 6. Effect of nerve injury on glutamate spillover to peri-synaptic NMDARs. A) Example NMDAR current traces from a from a single dorsal horn neuron at baseline (i), following washout of MK-801 (ii), and then after superfusion of TBOA (iii). Current traces are overlaid for comparison in (iv). B) Effect of nerve injury on kinetics of peri-synaptic NMDARs in presence of TBOA following sub-synaptic NMDAR blockade with MK-801. Numbers within histogram represent numbers of cells (N).

References
Bennett, G. J. and Y. K. Xie (1988). "A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man." 


A

B

C
AMPAR Naive Injured

Naive  Injured

25 ms

50 ms

NMDAR

A  AMPAR  B  NMDAR

Naive  Injured
A
CONTROL

100 μM TBOA

10 pA
1 s

B
CONTROL 100 μM TBOA

Cumulative Probability

Decay Amplitude Rate

% of baseline

C
Cumulative Probability

Current (pA) Rate (s⁻¹)
1. NMDAR eEPSC
2. MK-801 washout
3. TBOA superfusion
4. Overlay

A

1. NMDAR eEPSC  2. MK-801 washout

iii

3. TBOA superfusion  4. Overlay

B

% baseline

Peak Ampl.  TTP  AUC

Naive  Injured

6  5  6  5  6  5