

A positive allosteric modulator of the adenosine A₁ receptor selectively inhibits primary afferent synaptic transmission in a neuropathic pain model.

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Running title: Adenosine A1R allosteric modulation in a chronic pain model.

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Page number: 31

Figure number: 6

Reference number: 63

Abstract word number: 248

Introduction word number: 742

Discussion word number: 1482

Abbreviations: A1R, adenosine A₁ Receptor; ACSF, artificial cerebrospinal fluid; ADA, adenosine deaminase; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP5, (2*R*)-amino-5-phosphonovaleric acid; ATP, adenosine triphosphate; CGRP, calcitonin gene-related peptide; CPA, N6-cyclopentyladenosine; DMSO, dimethyl sulfoxide; DPCPX, 8-Cyclopentyl-1,3-dipropylxanthine; DRG, dorsal root ganglion; EPSC, excitatory postsynaptic current; GPCR, G protein-coupled receptor; IB4, Isolectin IB4; IPSC, inhibitory postsynaptic current; NK-1R, Neurokinin-1 receptor; PAM, positive allosteric modulator; PBS, phosphate buffered saline; PNL, partial nerve ligation; PPR, paired pulse ration; PWT, paw withdrawal threshold; SCI, spinal cord compression injury; T62, 2-amino-3-(4-chlorobenzoyl)-5,6,7,8-tetrahydrobenzothiophen; VCP171, (2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl)(phenyl)methanone.

Abstract:

In the spinal cord and periphery, adenosine inhibits neuronal activity through activation of the adenosine A₁ receptor (A1R) resulting in antinociception and highlighting the potential of therapeutically targeting the receptor in the treatment of neuropathic pain. This study investigated the changes in adenosine tone and A1R signalling, together with the actions of a novel A1R positive allosteric modulator (PAM), VCP171, on excitatory and inhibitory neurotransmission at spinal cord superficial dorsal horn synapses in a rat partial nerve-injury model of neuropathic pain. In the absence of A1R agonists, superfusion of the A1R antagonist, DPCPX (1 μM), produced a significantly greater increase in electrically evoked AMPAR-mediated synaptic current (eEPSC) amplitude in both lamina I and II neurons from nerve-injured animals than in controls, suggesting that endogenous adenosine tone is increased in the dorsal horn. Inhibitory GABAergic and glycinergic synaptic currents were also significantly increased by DPCPX in controls but there was no difference following nerve-injury. The A1R agonist, N6-Cyclopentyladenosine (CPA), produced greater inhibition of eEPSC amplitude in lamina II but not lamina I of the spinal cord dorsal horn in nerve-injured versus controls, suggesting a functional increase in A1R sensitivity in lamina II neurons after nerve-injury. The A1R PAM, VCP171, produced a greater inhibition of eEPSC amplitude of nerve-injury versus control animals in both lamina I and lamina II neurons. Enhanced adenosine tone and A1R sensitivity at excitatory synapses in the dorsal horn after nerve-injury suggest that new generation PAMs of the A1R can be effective treatments for neuropathic pain.

Introduction

Despite the widespread prevalence of chronic pain, pharmacological management remains limited (Finnerup *et al.*, 2010). Current therapeutic options are inadequate for many patients due to insufficient pain relief, dose-limiting side effects and/or development of tolerance (Woolf, 2004). Among potential novel targets, adenosine signalling is important in pain modulation (Ribeiro *et al.* 2002; Sawynok and Liu, 2003; Zylka, 2011). Regulation of pain transmission by adenosine is mediated largely through activation of G protein-coupled receptors (GPCRs), particularly the adenosine A₁ receptor (A1R) subtype (Nakamura *et al.* 1997; Poon and Sawynok, 1998). A1R expression has been reported on the central terminals of primary afferent neurons of the spinal cord and cell bodies in the dorsal horn (Choca *et al.* 1987, 1988). Activation of the A1R produces presynaptic inhibition of primary afferent neurotransmission onto dorsal horn neurons where it decreases glutamate (Ackley *et al.*, 2003; Tian *et al.*, 2010; Choi *et al.*, 2011), substance P (SP) and calcitonin gene-related peptide (CGRP) release from primary afferents (Carruthers *et al.* 2001; Sjolund *et al.* 1997). Another mechanism implicated in A1R nociceptive effects include hyperpolarization of dorsal horn neurons by increasing K⁺ conductance (Sawynok and Liu, 2003). However, mechanisms underlying A1R actions at different dorsal horn sites, including inhibitory synapses, and mechanisms of adaptations to the adenosine signalling system in neuropathic pain are poorly understood (Sawynok and Lui, 2003; Zylka, 2011).

Orthosteric adenosine A1R agonists have antinociceptive effects following systemic or spinal administration (Sawynok, 1998; Gong *et al.* 2010; Lee and Yaksh, 1996; Lavand'homme and Eisenach, 1999), but poor selectivity across the four subtypes of adenosine GPCRs and side effect liability have limited their therapeutic development (Zylka, 2011). For instance, A1Rs are not restricted to the spinal cord and primary afferents, but are also expressed elsewhere in the CNS, heart and adipose tissue and can thus mediate side effects such as bradycardia and reduced arterial pressure (Yang *et al.* 2002). An approach to potentially improve selectivity and therapeutic efficacy in pathological states involving GPCRs has been through the targeting of topographically distinct allosteric sites, which can show greater sequence divergence between receptor subtypes

(Christopoulos, 2002; May et al., 2007; Christopoulos et al., 2014). Therapeutically effective positive allosteric modulators may also exhibit enhanced tissue-based selectivity if the concentration of the natural ligand is elevated in the pathologically affected tissue relative to other regions of the body (May et al., 2007). This mechanism is particularly pertinent to adenosine GPCR pharmacology, because increased extracellular adenosine concentrations (adenosine tone) have been widely reported in hypoxic neural tissue (Fredholm, 1997; Pedata *et al.* 2007) and other conditions, such as drug withdrawal syndromes (Hack and Christie, 2003). There is physiological evidence that adenosine tone can be detected at primary afferent synapses in the spinal cord dorsal horn (Ackley *et al.*, 2003; Tian et al., 2010) but whether or not adenosine tone is increased in neuropathic pain is unknown (Sawynok and Liu, 2003).

Allosteric enhancement of agonist binding and function at the A1R was reported as early as 1990 with the identification of 2-amino-3-benzoylthiophenes that could enhance agonist activity at lower concentrations, but which displayed antagonistic effects at higher concentrations (Bruns and Fergus, 1990; Bruns *et al.*, 1990). More potent positive allosteric modulators (PAMs) have since been described (van der Klein *et al.* 1999; Baraldi *et al.* 2006; Baraldi *et al.* 2007), with T62 and TRR469 able to promote a reduction in mechanical allodynia in rodent models (Li *et al.* 2002; Vincenzi et al., 2014). We recently described a novel 2-amino-3-benzoylthiophene PAM, VCP171, (2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl)(phenyl)methanone, that possessed higher affinity for the allosteric site on the A1R than other PAMs, including T62 and TRR469, and also had the potential to engender pathway biased signalling in the actions of orthosteric A1R agonists (Aurelio et al., 2009; Valant et al., 2010; Vincenzi et al., 2014). Given that a number of mechanisms underlying A1R signalling in neuropathic pain and the impact of PAMs on these processes remain poorly defined, the current study utilized VCP171 as a tool in patch-clamp recordings from adult rat spinal cord slices to examine adaptations to A1R function and adenosine tone at primary afferent synapses and inhibitory dorsal horn synapses in a nerve-injury model of neuropathic pain. We found evidence for enhanced A1R function and tone at primary afferent synapses but not inhibitory synapses. The actions of VCP171 on excitatory synaptic neurotransmission were enhanced in the neuropathic pain model,

suggesting that newer generation A1R PAMs may produce relief of neuropathic pain with a reduced side effect profile.

Materials and Methods

Neuropathic pain model. Neuropathic pain was induced in 5-6 week old male Sprague-Dawley rats (n = 58) by performing a partial nerve ligation (PNL) of the left sciatic nerve, as described previously (Seltzer *et al.* 1990). Briefly, rats were anaesthetized with isoflurane and the sciatic nerve proximal to its trifurcation was surgically exposed and a single suture was tied tightly around a third to a half of the nerve. Rats were assessed for mechanical allodynia two weeks post-PNL surgery using a von Frey assay and used for electrophysiology and behavioural experiments between 2.5-4 weeks post-surgery. Sham surgery rats with all other procedures except nerve ligation were used as controls (n = 58). Rats were housed in a temperature controlled environment 22 ± 2 °C with a 12 hour light/dark cycle. Animals were housed in groups of 3 or 4 and had free access to food and water. All experiments involving animals were approved by the University of Sydney Animal Ethics Committee. 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). Every precaution was taken to prevent animal suffering during these experiments.

Mechanical allodynia testing. Mechanical paw withdrawal threshold (PWT) was used to assess mechanical allodynia to confirm the development of neuropathic pain prior to electrophysiology experiments. To measure mechanical PWT, animals were acclimatized to clear Perspex containers with a steel mesh floor. Animals were acclimatized to the apparatus several times before the day of experiment, and for around 15 min prior to testing on the day of experiment. A series of von Frey filaments (0.41-15 g) were presented using an up-down paradigm to calculate 50% withdrawal threshold (Chaplan, 1994). Mechanical PWT was tested prior to surgery on day 0 and 14 days following surgery. A reduction in von Frey threshold from a pre-surgery baseline of (14.9 ± 0.4 g, n = 58) to below 4 g 14 days after surgery was used as a threshold criterion that neuropathic pain had developed. Fifty-six animals met this threshold with an overall threshold of 0.9 ± 0.1 g (n = 56), i.e. only 2 animals were not used for electrophysiology experiments.

Preparation of spinal cord slices. Adult male Sprague-Dawley rats (n = 18 naive rats, 5-6 postnatal weeks, n = 114 PNL rats and sham controls, 10-12 postnatal weeks) were anaesthetized with isoflurane, decapitated and the lumbar region of the spinal cord was removed. Parasagittal slices (340 μm thick) of spinal cord were cut on a vibratome (Leica VT 1200s) in oxygenated ice-cold sucrose-based artificial CSF (sACSF) that contained (mM): 100 sucrose, 63 NaCl, 2.5 KCl, 1.2 NaH_2PO_4 , 1.2 MgCl_2 , 25 glucose, and 25 NaHCO_3 . Slices were transferred to a submerged chamber containing NMDG-based recovery ACSF (rACSF) for 15 minutes at 34°C, equilibrated with 95% O_2 and 5% CO_2 and composed of (mM): 93 NMDG, 93 HCl, 2.5 KCl, 1.2 NaH_2PO_4 , 30 NaHCO_3 , 20 HEPES, 25 Glucose, 5 Na ascorbate, 2 thiourea, 3 Na pyruvate, 10 MgSO_4 and 0.5 CaCl_2 . Following the recovery incubation, slices were transferred to normal oxygenated ACSF where they were allowed to recover for 1 hour at 34° C and maintained at room temperature prior to transfer to the recording chamber. Normal ACSF had the following composition (mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 1.2 MgCl_2 , 2.5 CaCl_2 , 25 glucose, and 11 NaHCO_3 and was equilibrated with 95% O_2 and 5% CO_2 .

Electrophysiology. Slices were transferred to a recording chamber and superfused continuously at 2 ml/min with oxygenated normal ACSF which was maintained at 34°C with an inline heater and monitored by a thermister in the slice chamber. Dodt-contrast optics were used to identify laminae I and II neurons in the superficial dorsal horn. Large (capacitance ≥ 20 pF), putative NK-1R positive, lamina I cells were identified by position, size and fusiform shape with dendrites that were restricted to lamina I (Yu *et al.* 2005). Lamina I cells were filled with biocytin and identified post-hoc by immunostaining with anti-NK-1R antibody. Lamina II_i cells were identified by their position in the translucent substantia gelatinosa layer and were filled with biocytin so their position could be confirmed post-hoc relative to IB4 staining, which fluorescently stains small-diameter non-peptidergic afferent fibres in lamina II. A Cs^+ -based internal solution was used to record electrically evoked excitatory post-synaptic currents (eEPSCs) and contained (mM): 140 CsCl, 10 EGTA, 5 HEPES, 2 CaCl_2 , 2 MgATP, 0.3 NaGTP, 5 QX-314.Cl and 0.1% biocytin (osmolarity 285-295 mosmol l^{-1}). We were interested in investigating the effects of A1R activation on the primary afferent evoked ESPCs in laminae I and II and the IPSCs that affect nociceptive signals in lamina II. Therefore, we chose to use an electrode solution that prevented any postsynaptic effects so we could focus on nociceptive

transmission in the spinal cord. Patch electrodes had a resistance of between 3-5 M Ω . Synaptic currents were measured in whole-cell voltage-clamp (-70 mV) from lamina I or II cells. Bipolar tungsten electrodes placed in the dorsal roots (for excitatory currents) or inner laminae (nominally lamina III for inhibitory currents) were used to elicit eEPSCs and inhibitory post-synaptic currents (eIPSCs) using a stimulus strength sufficient to evoke reliable submaximal eEPSCs/IPSCs. For paired pulse experiments, evoked currents were elicited by two consecutive stimuli of identical strength separated by 70 ms. Paired pulse ratio (PPR) was calculated by dividing the second pulse by the first (PSC_2/PSC_1). All eEPSCs were recorded in the presence of picrotoxin (100 μ M), strychnine (0.5 μ M) and AP5 (100 μ M) and eIPSCs were recorded in CNQX (10 μ M), AP5 (100 μ M) and strychnine (0.5 μ M) or picrotoxin (100 μ M). At the conclusion of each experiment, CNQX (10 μ M), strychnine (0.5 μ M) or picrotoxin (100 μ M) were added to the perfusion to confirm that recorded currents were AMPA-mediated EPSCs, glycine-mediated IPSCs or GABA-mediated IPSCs, respectively. Drugs were superfused onto slices at a rate of 2ml/min in normal oxygenated ACSF at 34°C.

Immunohistochemistry. All neurons were filled with biocytin so their morphology and NK-1R expression, or lamina II position, could be confirmed *post hoc* through immunostaining. At the end of each electrophysiological recording, slices were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.1 for 2-4 hours and then washed in 0.1 M PBS. Slices were then incubated at 4°C overnight in 0.3% Triton X-100 in 0.1 M PBS. The following day slices were incubated at room temperature in 5% normal horse serum, 1% bovine serum albumin and 0.3% Triton X-100 in 0.1 M PBS for 1 hour. For immunohistochemical detection of NK-1R, slices were incubated in 1:1000 rabbit anti-NK1 receptor antibody (GeneTex) in 1% bovine serum albumin and 0.3% Triton X-100 in 0.1 M PBS for 6 hours at room temperature and 48-72 hours at 4°C. Slices were then incubated for 2 hours at room temperature then overnight at 4°C with goat anti-mouse Alexa Fluor-633 (Invitrogen) for visualization of the primary antibody and Streptavidin-FITC (Sigma) to visualize biocytin-filled cells. For identification of cells recorded in lamina II, slices were incubated in conjugated Isolectin B4 Alexa Fluor-488 (Invitrogen) and Cy5-Streptavidin (Sigma) for 2 hours at room temperature then overnight at 4°C. All antibodies were diluted in 1% BSA and 0.3% Triton X-100 in 0.1 M PBS. Following incubation with secondary antibodies, slices were washed in 0.1 M PBS and mounted onto slides,

briefly air dried and immersed in Fluoromount-G (ProSciTech). Images of immunolabelled neurons were obtained with a confocal microscope.

Drugs, reagents and solutions. VCP171 ((2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl)(phenyl)methanone) was synthesized as previously described (Aurelio *et al.* 2009) and a 10 mM stock solution was prepared in 100% DMSO. CPA (N6-cyclopentyladenosine) was purchased from Sigma Australia and a 10 mM stock solution was made in 100% DMSO. DPCPX (8-Cyclopentyl-1,3-dipropylxanthine) was purchased from Sigma Australia and 5 mM stock solutions were made in water. Picrotoxin, CNQX and strychnine were purchased from Sigma Australia. QX-314 was purchased from Alomone Labs, Israel. APV was purchased from Tocris Bioscience, UK. Adenosine deaminase was purchased from Worthington through Scimar Australia. All other chemicals were purchased from Sigma, Australia. Stock solutions of these compounds were diluted in ASCF for experiments and DMSO concentrations did not exceed 0.03%.

Data analysis. Pooled values are presented as mean \pm SEM, or drug effect normalized to the baseline. Data was analysed using Prism 6 (Version 6.03). Statistical tests between treatment groups were made using a two-tailed unpaired *t* test assuming unequal variance. Comparisons of two treatments in the same group were made using a two-tailed paired *t* test. When multiple comparisons were tested, ANOVA with Bonferroni *post hoc* test to correct for multiple comparisons were used, unless otherwise stated in figure legends. Normalized concentration-response data was pooled and fitted with a logistic function using Prism 6 software. Significance was set at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ levels.

Results.

A1R activation decreases excitatory synaptic transmission in nociceptive cells of the spinal cord dorsal horn. AMPAR-mediated eEPSCs in parasagittal spinal cord slices were evoked by stimulating the dorsal roots and measured in the lamina I cells by whole-cell voltage clamp in the presence of picrotoxin (100 μ M), strychnine (0.5 μ M) and AP5 (100 μ M). Putative NK1+ cells were chosen by their large size (capacitance \geq 20 pF) and position at the margins of the substantia gelatinosa in the slice. To confirm NK1R expression, cells were filled with biocytin via the recording electrode and immunostained with antibodies for NK1R and co-stained with a fluorescent streptavidin conjugate to detect biocytin at completion of the experiment (Fig. 2D). The success rate of recovering stained neurons that had been filled during recording was 42% and of these 100% co-stained for both NK-1R and biocytin. Both paired pulse and single stimulus recordings were performed and stimulus intensity was adjusted to produce eEPSCs of similar amplitude (<1 nA). Between 10-20 stable baseline eEPSC recordings were taken before superfusing any drugs. Primary afferent synaptic transmission onto putative NK1+ lamina I neurons was decreased by the A1R agonist, CPA (Fig. 1A, C). CPA (1 μ M) significantly decreased currents by $73 \pm 13\%$ ($P = 0.0005$, paired t-test, Fig. 1A, C) and increased paired pulse ratio by $51 \pm 16\%$ ($P = 0.02$, paired t-test, Fig. 1E).

In the absence of applied agonist, the highly selective A1R antagonist, DPCPX (Lohse et al., 1987), increased eEPSC amplitude, presumably because it prevented the actions of endogenous adenosine present in the dorsal horn (Fig. 1A, C). DPCPX increased eEPSCs by $19 \pm 6\%$ ($P = 0.012$, Fig. 1B, D) and decreased the paired pulse ratio by $23 \pm 6\%$ ($P = 0.019$), suggesting a presynaptic site of action. Slices were then superfused with CNQX (10 μ M) which completely (and reversibly) inhibited the current, confirming that the eEPSCs were AMPA-mediated.

Animals with neuropathic pain have an increase in adenosine tone in lamina 1 of the spinal cord. To determine if nerve injury-induced neuropathic pain affects adenosine tone AMPAR-mediated eEPSCs from Lamina I neurons from nerve-injured and sham surgery control animals were compared

before and after exposure to DPCPX (Fig. 2A). Superfusion of DPCPX increased AMPAR-mediated eEPSCs in slices from sham animals by $10 \pm 3\%$ ($n = 7$ cells, $P = 0.013$ versus pre-exposure baseline, paired t-test) but by $25 \pm 5\%$ in injured animals ($n = 9$ cells, $P = 0.002$ paired t-test). The DPCPX-induced increase in nerve-injured animals was significantly different from sham controls ($P = 0.03$, unpaired t-test, Fig. 2B), suggesting a possible increase in adenosine tone at A1Rs in neuropathic pain. DPCPX reduced the paired pulse ratio (PPR) in cells from both sham ($11 \pm 4\%$, $n = 6$ cells, $P = 0.03$) and injured ($16 \pm 6\%$, $n = 8$ cells, $P = 0.02$) animals but this did not differ significantly between groups.

To determine whether the enhanced A1R tone in the dorsal horn of neuropathic animals was due to an increase in adenosine or increased A1R sensitivity, concentration-response curves to the A1R agonist, CPA, were compared between slices from nerve-injured and sham animals. Baseline AMPAR-mediated eEPSCs from lamina I cells of sham ($n = 15$) and injured ($n = 22$) animals were recorded before application of 0.01-10 μM CPA. Responses were normalized to baseline controls. There was no significant difference between the two groups using a two-way ANOVA ($P = 0.8$, Fig. 2C), suggesting the enhanced tonic inhibition of eEPSCs by A1R activation in neuropathic pain was not due to increased A1R sensitivity. The $p\text{EC}_{50}$ for CPA from the fitted logistic function in the sham animal group was 7.8 ± 0.6 and for the nerve-injured group it was 7.6 ± 0.4 . The maximum inhibition of eEPSC was $58.5 \pm 0.6\%$ for sham animals and $41.2 \pm 7.7\%$ for injured animals. The PPR of AMPAR-mediated eEPSCs in lamina I increased with CPA (0.1 – 1 μM , pooled) in sham ($40 \pm 14\%$, $n = 14$ cells, $P = 0.049$) and injured ($115 \pm 22\%$, $n = 14$ cells, $P = 0.03$) groups.

The A1R PAM, VCP171, is more effective at decreasing excitatory synaptic currents in lamina I after nerve-injury. VCP171 might be expected to have a greater effect on primary afferent transmission when endogenous adenosine is elevated following the development of neuropathic pain. VCP171 (10 μM) reduced AMPAR-mediated eEPSCs in lamina I cells in sham ($13 \pm 2\%$, $n = 7$ cells, $P = 0.02$, paired t-test) and nerve-injured animals ($24 \pm 4\%$, $n = 8$ cells, $P = 0.004$, paired t-test) compared to pre-drug controls (Fig. 3A,B). The decrease in current following exposure to VCP171

was significantly greater in nerve-injured animals compared to sham controls ($P = 0.04$, unpaired t -test). The pEC_{50} for VCP171 (normalized to the eEPSC amplitude following application of DPCPX) on lamina I eEPSCs from the fitted logistic function of sham controls was 5.6 ± 0.2 and for injured animals it was 5.7 ± 0.4 . The maximum inhibition of eEPSC (normalized to DPCPX) was $21.3 \pm 2.4\%$ for sham animals and $35.3 \pm 5.1\%$ for injured animals. VCP171 increased PPR in cells from sham control animals ($12 \pm 3\%$, $n = 4$ cells, $P = 0.03$) and injured animals ($16 \pm 12\%$, $n = 4$ cells, $P = 0.3$). Inhibition by VCP171 was reversed with the A1R antagonist DPCPX ($1 \mu\text{M}$) in all cells tested (Fig. 3B). The inhibitory effect of VCP171 on lamina I AMPAR-mediated eEPSC amplitude was significant over a range of concentrations ($0.3 - 10 \mu\text{M}$) ($P < 0.0001$ between the two groups using a two-way ANOVA) (Fig. 3C).

To confirm that VCP171 acts as a PAM and has no orthosteric activity at the A1R, slices were exposed to adenosine deaminase (ADA, $20 \mu\text{g/ml}$) to degrade any extracellular adenosine before additionally superfusing VCP171 ($10 \mu\text{M}$, $n = 4$ cells). ADA increased the eEPSC, but was not elevated further when VCP171 was superfused in the presence of ADA, suggesting that VCP171 has no actions when endogenous adenosine is reduced with ADA (Fig. 3D). AMPAR-mediated eEPSCs returned to baseline levels approximately 15 minutes after superfusion of ADA ceased (Fig. 3D).

A1R sensitivity is enhanced in excitatory, but not inhibitory, lamina II synapses and adenosine tone is increased following nerve-injury. Both excitatory and inhibitory neurons in Lamina II of the dorsal horn play important roles in pain neurotransmission and neuropathic pain (Woolf, 2004). Primary afferent synaptic transmission was therefore studied in neurons in lamina II and location confirmed by co-staining with IB4. Lamina II cells were filled with biocytin and their location confirmed post-hoc relative to IB4 staining. The success rate of recovering stained neurons in lamina II that had been filled with biocytin during recording was 65% and 100% of those were in the vicinity of IB4 positive fibres (Fig. 5C). By contrast with lamina I neurons, the inhibitory effect of A1R activation by CPA was significantly more effective at reducing AMPAR-mediated eEPSCs in lamina II from nerve-injured animals ($n = 24$ cells) than sham controls ($n = 21$ cells) using a two-way ANOVA ($P < 0.0001$

for injured versus sham, Fig. 4A). This suggests an increase in A1R expression or functional coupling of the receptors to intracellular signalling pathways mediating presynaptic inhibition in nerve terminals in lamina II but not lamina I. The pEC_{50} from the fitted logistic function for CPA on lamina II eEPSCs of sham controls was 7.1 ± 0.4 and for injured animals it was 8.3 ± 0.2 . The maximum reduction in eEPSC was $48.5 \pm 9.0\%$ for the sham group and increased to $85.4 \pm 3.8\%$ for the injured group. This increased inhibitory effect of CPA was not observed at inhibitory synapses in lamina II, where both GABAergic and glycinergic synaptic currents from nerve-injured ($n = 20$ cells and $n = 16$ cells, respectively) and control animals ($n = 18$ cells and $n = 22$ cells, respectively) were reduced similarly in response to CPA (Fig. 4A). The pEC_{50} for CPA on GABAergic eIPSCs from lamina II of sham controls was 7.0 ± 0.6 and for injured animals it was 7.0 ± 0.8 . The pEC_{50} for CPA on glycinergic eIPSCs from lamina II of sham controls was 7.0 ± 0.5 and for injured animals it was 7.3 ± 0.6 . PPR of AMPAR-mediated eEPSCs in lamina II cells increased with CPA ($0.1 \mu\text{M}$), for both sham ($35 \pm 11\%$, $n = 5$ cells, $P = 0.04$) and injured ($111 \pm 35\%$, $n = 4$ cells, $P = 0.04$) groups, suggesting a pre-synaptic site of action. PPR of inhibitory synaptic currents also increased with CPA ($0.01 - 1 \mu\text{M}$, pooled), both for GABAergic currents from sham ($34 \pm 10\%$, $n = 8$ cells, $P = 0.05$) and injured animals ($44 \pm 16\%$, $n = 10$ cells, $P = 0.02$), and glycinergic currents from sham ($34 \pm 11\%$, $n = 8$ cells, $P = 0.02$) and injured animals ($28 \pm 10\%$, $n = 12$ cells, $P = 0.02$).

Similar to lamina I, DPCPX ($1 \mu\text{M}$) increased lamina II AMPAR-mediated eEPSCs by $14 \pm 2\%$ ($n = 7$ cells, $P = 0.001$) in sham controls and $31 \pm 5\%$ ($n = 7$ cells, $P = 0.0008$, paired t-test) in nerve-injured animals. PPR of AMPAR-mediated eEPSCs decreased with DPCPX treatment in sham ($8 \pm 1\%$, $n = 5$ cells, $P = 0.04$) and injured ($34 \pm 20\%$, $n = 7$ cells, $P = 0.1$, paired t-test) animals. Because there was increased presynaptic A1R sensitivity at lamina II synapses following nerve-injury, it is not possible to interpret whether or not increased adenosine tone contributed to the increased response to DPCPX. Adenosine tone was also identified at inhibitory synapses recorded from control lamina II neurons but this was not increased by nerve-injury (Fig. 4). The eIPSC in the presence of DPCPX was significantly increased compared to the baseline (pre-drug) controls for GABAergic-mediated currents from sham-surgery control ($14.8 \pm 5.9\%$, $n = 4$ cells, $P = 0.04$) and injured animals ($15.4 \pm 3.4\%$, $n = 5$ cells, $P = 0.03$), and glycinergic-mediated eIPSCs from sham ($26.5 \pm 10.2\%$, $n = 4$ cells, $P = 0.04$) and

injured animals ($20.2 \pm 2.3\%$, $n = 5$ cells, $P = 0.02$, paired t-test). The effects of DPCPX on GABAergic and glycinergic eIPSCs were similar in sham and nerve-injured groups and there was no significant difference between these inhibitory synapse types (Fig. 4B). The PPR of GABA-mediated eIPSCs decreased in response to DPCPX for sham ($24 \pm 15\%$, $n = 3$ cells, $P = 0.2$) and injured ($17 \pm 6\%$, $n = 5$ cells, $P = 0.04$, paired t-test) animals, and also for glycinergic-mediated eIPSCs from sham ($25 \pm 15\%$, $n = 3$ cells, $P = 0.2$) and injured ($44 \pm 15\%$, $n = 5$ cells, $P = 0.04$, paired t-test) animals.

The A1R PAM, VCP171, is more effective at decreasing excitatory synaptic currents in lamina II of animals with neuropathic pain. To determine whether VCP171 was more effective in lamina II following nerve-injury, as it is in lamina I, AMPAR-mediated eEPSCs in lamina II cells of sham and nerve-injured animals were compared before and during exposure to VCP171 (Fig. 5A,B). VCP171 was significantly more effective in lamina II neurons from nerve-injured animals than sham controls ($P = 0.0002$ for sham versus injured using a two-way ANOVA) (Fig. 5B). Inhibition by VCP171 was reversed with the A1R antagonist DPCPX ($1 \mu\text{M}$) in all cells tested. The $p\text{EC}_{50}$ from the fitted logistic function for VCP171 on lamina II eEPSCs of sham controls was 6.2 ± 0.4 and for injured animals it was 6.6 ± 0.2 . The maximum inhibition of eEPSC (normalized to the eEPSC amplitude following application of DPCPX) was $25.5 \pm 4.8\%$ for sham animals and $34.1 \pm 3.6\%$ for injured animals. The PPR of AMPAR-mediated eEPSCs in lamina II increased, but not significantly, with VCP171 treatment in sham ($14 \pm 10\%$, $n = 6$ cells, $P = 0.2$) and injured ($28 \pm 18\%$, $n = 6$ cells, $P = 0.3$) animals.

Discussion.

The current results establish that adaptations in adenosine tone and/or A1R sensitivity at different levels of the superficial dorsal horn in neuropathic pain increase the effect of a novel A1R PAM at primary afferent excitatory synapses. This provides further support for the pursuit of newer generation A1R PAMs with CNS activity as an attractive alternative to the use of A1R agonists that have off-target effects, as the PAMs may have higher selectivity for the affected tissues where endogenous adenosine levels are known to be elevated, or receptor sensitivity is increased.

A1R activation in primary afferent neurons decreases synaptic currents in lamina I. Adenosine has been reported to act at both pre- and postsynaptic sites (Sebastião and Ribeiro, 2009), but activity at the A1R is predominantly presynaptic (Patel *et al.* 2001). In this study the A1R agonist, CPA, significantly reduced AMPAR-mediated eEPSCs in lamina I evoked by dorsal root stimulation. The increased paired-pulse ratio suggests a presynaptic site of action. A1R expression in primary afferent neurons has previously been described by Lima *et al.* (2010), who immunostained rat DRG sections and found that A1R is selectively expressed by small- to medium-sized, presumably nociceptive neurons. Many of these neurons also expressed TRPV1, substance P, or IB4, whilst A1R was mostly absent in the large NF200-positive neurons that are presumed low threshold mechanosensors. Reeve and Dickenson (1995) administered CPA intrathecally and recorded responses in wide dynamic range dorsal horn cells *in vivo*. CPA dose-dependently inhibited C-fibre evoked responses, but did not significantly change A β -fibre responses, while A δ -fibre responses were facilitated. This suggests that the inhibitory effects reported here in the superficial laminae may be due to inhibition of C-fibre afferents. However, the relative proportions of C and A-fibres activated by dorsal root stimulation in the present experiments are unknown.

Adenosine tone is increased in lamina I of the dorsal horn in neuropathic animals. The current results suggest that extracellular adenosine levels are enhanced in lamina I following nerve-injury because the A1R antagonist DPCPX produced a larger increase in eEPSCs in neuropathic animals

without any change in presynaptic sensitivity to a directly acting A1R agonist, CPA. Furthermore, the A1R PAM, VCP171, produced greater inhibition of lamina I eEPSCs in neurons from injured than sham operated animals as would be expected if increased concentrations of adenosine were activating A1R orthosterically. Moreover, treatment with adenosine deaminase, which should attenuate adenosine tone, abrogated the actions of VCP171, suggesting that the modulator's effects are indeed due to potentiation of endogenous adenosine. However, adenosine concentrations in extracellular fluid were not directly quantified in neuropathic versus control lamina I of the dorsal horn so the magnitude of increased adenosine concentration is unknown.

The mechanism of enhancement of adenosine tone at excitatory lamina I synapses is uncertain. It is known that the extracellular availability of endogenous adenosine in the hippocampus is increased by neuronal excitation (Cunha et al., 1996; Mitchell *et al.* 1993; Grover and Teyler, 1993) and it has been suggested that the endogenous adenosine in the spinal cord adapts following neuropathic nerve-injury, possibly in response to elevated excitation, which may increase inhibitory tone through activation of A1Rs. Availability and release of adenosine is enhanced by injury and by depolarization, which would be expected to occur under conditions of enhanced excitatory primary afferent neurotransmission in neuropathic pain (Bantel et al., 2002). Liu *et al.* (2002) showed that adenosine release is increased in the rat hind paw following spinal cord injury, but, to our knowledge, there have been no studies to directly quantify extracellular adenosine in the dorsal horn in neuropathic pain models. There is significant evidence that neuropathic pain is initiated by the activation of astrocytes and microglia (Cao and Zhang, 2008; Wiesler-Frank et al., 2004), and activated glial cells release ATP which is converted to adenosine by ectoenzymes (Newman, 2003) suggesting potential mechanisms for increased adenosine tone in neuropathic pain.

A difference in the maximal inhibition produced by the A1R-agonist on eEPSC amplitude was also observed between young (~5 week old) naive rats (Fig 1C) and the sham surgery rats (~10-12 weeks old – approximately 3 weeks post surgery) (Fig 2C). It is possible that this is due to developmental changes in A1R expression or functional coupling during the transition from adolescence to adulthood. Postnatal A1R expression has been shown to decrease (Geiger et al., 1984) and increase (Metsola et al., 2014) in different tissues. Geiger et al. (1984) also reported that CPA binding to the

high affinity A1R sites increases in whole spinal cord during postnatal days 1-14, followed by a decrease and increases again in adulthood.

A1R activation in lamina II_i inhibits both excitatory and inhibitory synaptic currents in the dorsal horn. Yamaoka *et al.* (2013) reported that A1Rs are densely distributed in neuronal cell bodies in lamina II of the dorsal horn, and suggest this area is important in endogenous pain relief by adenosine. We found that the inhibitory effect of the A1R agonist, CPA, was similar for both eEPSCs and eIPSCs in lamina II, which suggests that A1Rs are located on both excitatory and inhibitory terminals that innervate this region. The increased paired-pulse ratio observed at lamina II synapses suggests the site of action is presynaptic in both cases. Moreover, the use of Cs-filled electrodes in this study would be expected to largely block post-synaptic GIRK conductances that may be activated by A1R in lamina II cells decreasing the likelihood that postsynaptic actions contributed to synaptic current inhibition. This suggests that, although nociceptive signals in this region are reduced by A1R activation, the inhibitory circuits in this region would also be reduced, which may enhance excitability. This differs from the lateral horn, where CPA has been shown to affect excitatory but not inhibitory postsynaptic potentials (Deuchars *et al.* 2001).

A1R functional coupling in Lamina II is enhanced in neuropathic pain. The A1R agonist, CPA, is more potent and effective at reducing AMPAR-mediated eEPSCs in lamina II following nerve-injury. By contrast, CPA was equally effective for lamina I eEPSCs and lamina II eIPSCs in control and nerve-injured animals. This finding suggests that there is an increase in A1R expression, or that the receptors are more effectively coupled to inhibition of glutamate release mechanisms following injury only in lamina II. Consistent with this, a recent study by Yamaoka *et al.* (2013) found an increase in A1R mRNA following spinal cord mild-compression injury (SCI). The increase in A1R in SCI was accompanied by a proliferation of microglia in the spinal cord. Different pain models affected A1R expression in different ways, because a plantar incision (PI) pain model decreased A1R expression in lamina II (Yamaoka *et al.* 2013). The lack of change in A1R sensitivity for Lamina I eEPSCs is

consistent with Yamaguchi *et al.* (2014), who reported that A1R expression did not change in dorsal root ganglia and peripheral nerves following nerve-injury using immunohistochemistry.

Lack of increase in adenosine tone at inhibitory synapses in Lamina II_i. The A1R antagonist, DPCPX, enhances both eEPSCs and eIPSCs in lamina II_i which suggests there is significant adenosine tone at both excitatory and inhibitory synapses in uninjured control animals. Following nerve-injury there was no change in eIPSCs in response to DPCPX, but there was an increase in eEPSCs, which could be due either to increased adenosine tone, or increased A1R sensitivity, as discussed above, or both. It is of interest that DPCPX produced no greater effect in eIPSCs of nerve-injured animals, for either GABAergic or glycinergic synapses, suggesting that there is no increase in adenosine tone at inhibitory synapses. It is therefore tempting to speculate that there is little or no increase in adenosine tone after nerve injury in lamina II, with the increased effect of DPCPX at excitatory synapses being due to increased A1R sensitivity. If this interpretation is correct, increased adenosine tone after nerve injury is largely restricted to lamina I and does not extend substantially to inner lamina II (Fig 6).

Nerve-injury increases the inhibitory activity of VCP171 on eEPSCs in primary afferent synapses. The results support the hypothesis that an increase in endogenous adenosine in the dorsal horn increases the activity of VCP171 in reducing glutamatergic currents in nociceptive circuits. The inhibitory effect of VCP171 on eEPSCs was significantly increased in neuropathic animals that have increased endogenous adenosine in the dorsal horn. This effect was due purely to allosteric modulation of A1R, with no orthosteric influence by testing VCP171 in the presence of adenosine deaminase, which breaks down any endogenous extracellular adenosine present in the dorsal horn (Geiger *et al.* 1986; Bantel *et al.* 2002). Although the effect that VCP171 has on reducing eEPSCs is modest compared to CPA, it may have greater therapeutic potential as it would be expected to be more effective in tissues where endogenous adenosine tone or A1R sensitivity is increased. It is therefore less likely to produce unwanted side-effects. This suggests that new A1R PAMs can, theoretically, be more effective as a therapeutic for neuropathic pain.

Authorship Contributions.

Participated in research design: Imlach and Christie.

Conducted experiments: Imlach and Bhola.

Performed data analysis: Imlach.

Wrote or contributed to the writing of the manuscript: Imlach, Bhola, May, Christopoulos and Christie.

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Footnotes:

This work was supported by a Sydney University Postdoctoral Fellowship [Grant 2012-02237]; the National Health and Medical Research Council of Australia [Grants 569927, 1041875], Australian Research Council [Grant DE130100117].

Figure Legends.

Figure 1. A1R activation decreases synaptic AMPAR mediated eEPSCs in nociceptive lamina I neurons. **(A, B)** Examples of AMPAR-mediated eEPSC traces and a time plot of peak amplitude (dark blue plot) and paired-pulse ratio (light blue plot) from the same lamina I neuron pre- and post-exposure to **(A)** CPA (1 μ M) and **(B)** DPCPX (1 μ M; separate experiment) in spinal cord slices from naive animals. Black bars indicate period of drug superfusion. **(C)** CPA significantly decreases evoked AMPA currents in lamina 1 neurons (n = 9 cells). **(D)** DPCPX significantly increases evoked AMPA currents in lamina I neurons (n = 9 cells). **(E)** Paired pulse ratios of eEPSCs were increased in lamina 1 following exposure to 1 μ M CPA (n = 7 cells) and **(F)** decreased following DPCPX exposure (n = 7 cells). Example traces show the relative change in the second eEPSC before (dark green trace) and during superfusion of drug (light green trace) when the first eEPSC amplitudes were normalised to the amplitude of the first eEPSC. PPR was calculated by dividing the second pulse by the first (PSC2/PSC1). Individual cells are colour coded for traces and plots (same set of neurons in A, C and E; another set of neurons in B, D and F). Asterisks show significant differences from the control for all cells: * - P < 0.05; *** - P < 0.001 (paired t-tests).

Figure 2. Adenosine tone in Lamina I increases post-nerve-injury. AMPAR-mediated eEPSCs pre- and post- treatment with the A1R antagonist DPCPX (1 μ M) in lamina I neurons. **(A)** Examples of time plots of peak eEPSC amplitude (normalized) in neurons from sham control (black) and injured (red) animals. The black bar indicates period of DPCPX superfusion. **(B)** Normalized eEPSCs from sham control (n = 8 cells) and injured animals (n = 9 cells) show a greater increase in eEPSC amplitude following injury. Circles show individual (normalized to pre-DPCPX) data points. **(C)** The A1R agonist CPA has similar inhibitory activity on eEPSCs in sham and injured animals. Cell numbers for each point are in parentheses. **(D)** NK-1R immunolabelling and biocytin staining of a lamina I neuron. Scale bar = 50 μ m. Asterisks show significant difference from control: *P < 0.05; ***P < 0.001. Significance was determined using a one-way ANOVA with Tukey's multiple comparisons test.

Figure 3. VCP171 is more effective at reducing eEPSC amplitudes in Lamina I of nerve-injured animals compared to sham controls. **(A)** AMPAR-mediated eEPSC traces and a time plot of peak

amplitude from a lamina I cell of an injured animal showing a decrease in eEPSC in response to 10 μ M VCP171, which is reversed in the presence of 1 μ M DPCPX. **(B)** VCP171 (10 μ M) was significantly more effective at reducing eEPSCs in injured animals compared to sham controls and effects were reversed by DPCPX (1 μ M). **(C)** Concentration-response curve shows that VCP171 is more effective at reducing eEPSCs in injured lamina 1 over a range of concentrations. Numbers in parentheses are cell numbers for each data point. **(D)** VCP171 does not reduce eEPSCs in lamina 1 in the presence of adenosine deaminase (ADA) ($n = 7$). Significance was determined using a one-way ANOVA with Dunns multiple comparisons test. Data is mean \pm SEM. Asterisks show significant difference from control: * $P < 0.05$; ** $P < 0.005$.

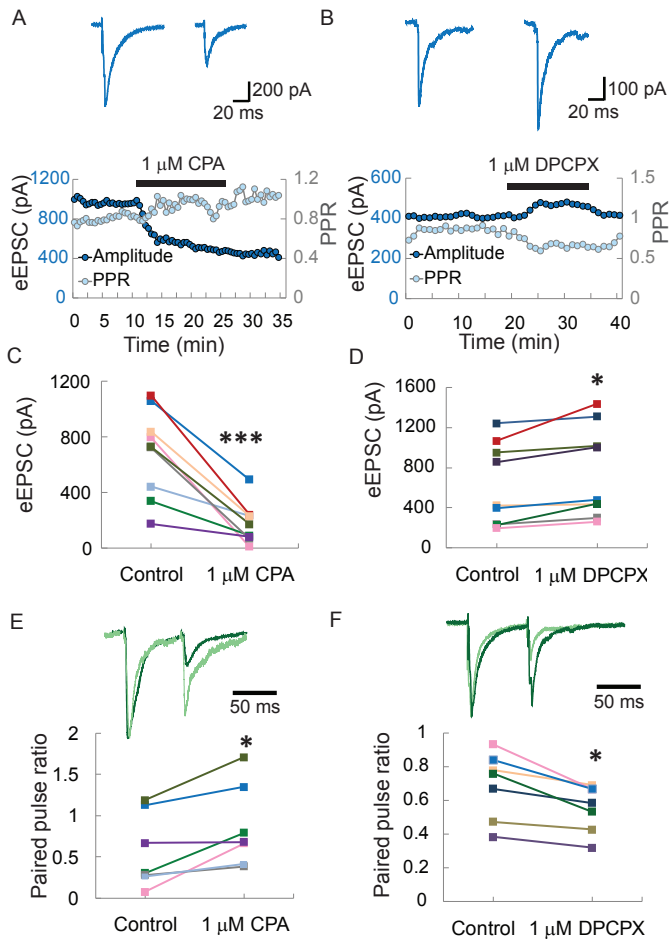
Figure 4. A1R functional coupling is enhanced in excitatory, but not inhibitory Lamina II synapses following nerve-injury and adenosine tone in Lamina II is increased in excitatory neurons following nerve-injury. **(A)** The A1R agonist CPA is more effective at inhibiting AMPAR-mediated eEPSCs in lamina II of injured animals compared to sham controls, but inhibition of GABA and Glycine eIPSCs are similar in neurons from injured and control animals. **(B)** DPCPX enhances eEPSC amplitude to a greater extent in neurons from injured than sham control animals. DPCPX increases eIPSC amplitude to a similar extent in neurons from sham control and injured animals. Data is mean \pm SEM. Numbers in parentheses indicate cell number for each data point. Asterisks show significant difference from control: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$. Significance between treatment groups was determined using unpaired two-tailed t-tests.

Figure 5. VCP171 is more effective at reducing eEPSC amplitudes in Lamina II of nerve-injured animals compared to controls. **(A)** Examples of AMPA eEPSC from a lamina II neuron of an injured animal showing a decrease in eEPSC in response to 10 μ M VCP171, which is reversed in the presence of 1 μ M DPCPX. **(B)** Concentration-response curves show that VCP171 is more effective at reducing eEPSCs in injured lamina II over a range of concentrations. Numbers in parentheses indicate cell numbers for each data point. **(C)** The location of lamina II cells was confirmed post-hoc by staining IB4 and labelling biocytin. Scale bar = 20 μ m. Asterisks show significant difference from

control: *P < 0.05. Significance between treatment groups was determined using unpaired two-tailed t-tests.

Figure 6. Changes in adenosine signalling occur in the spinal cord dorsal horn following the development of neuropathic pain. Synaptic responses were measured using whole-cell patch-clamp techniques with recording electrodes on neurons in either lamina I with electrical stimulation of primary afferents, or lamina II with electrical stimulation of neurons in the inner laminae. In lamina I there is an increase in adenosine tone which decreases excitatory nociceptive signals in putative NK-1R positive cells. In lamina II there is an increased response to adenosine signalling at excitatory glutamatergic (GLUT) synapses which causes a decrease in excitatory signals in these interneurons (purple circle). This may be due to an increase in A1R expression or functional coupling. There is no change in adenosine signalling at inhibitory synapses (GABA/GLY) in lamina II following neuropathic pain development.

Figure 1.



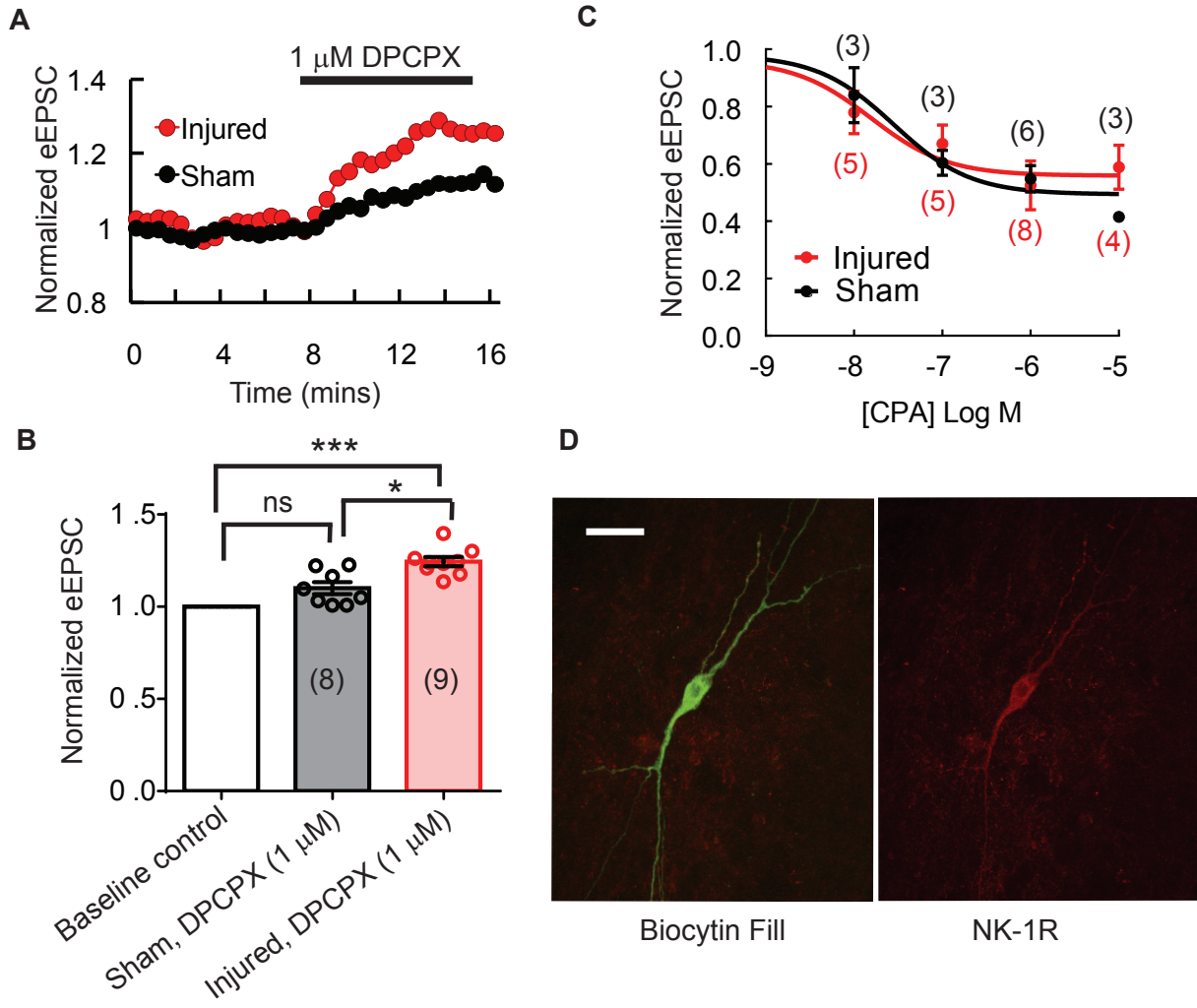


Figure 2

Figure 3.

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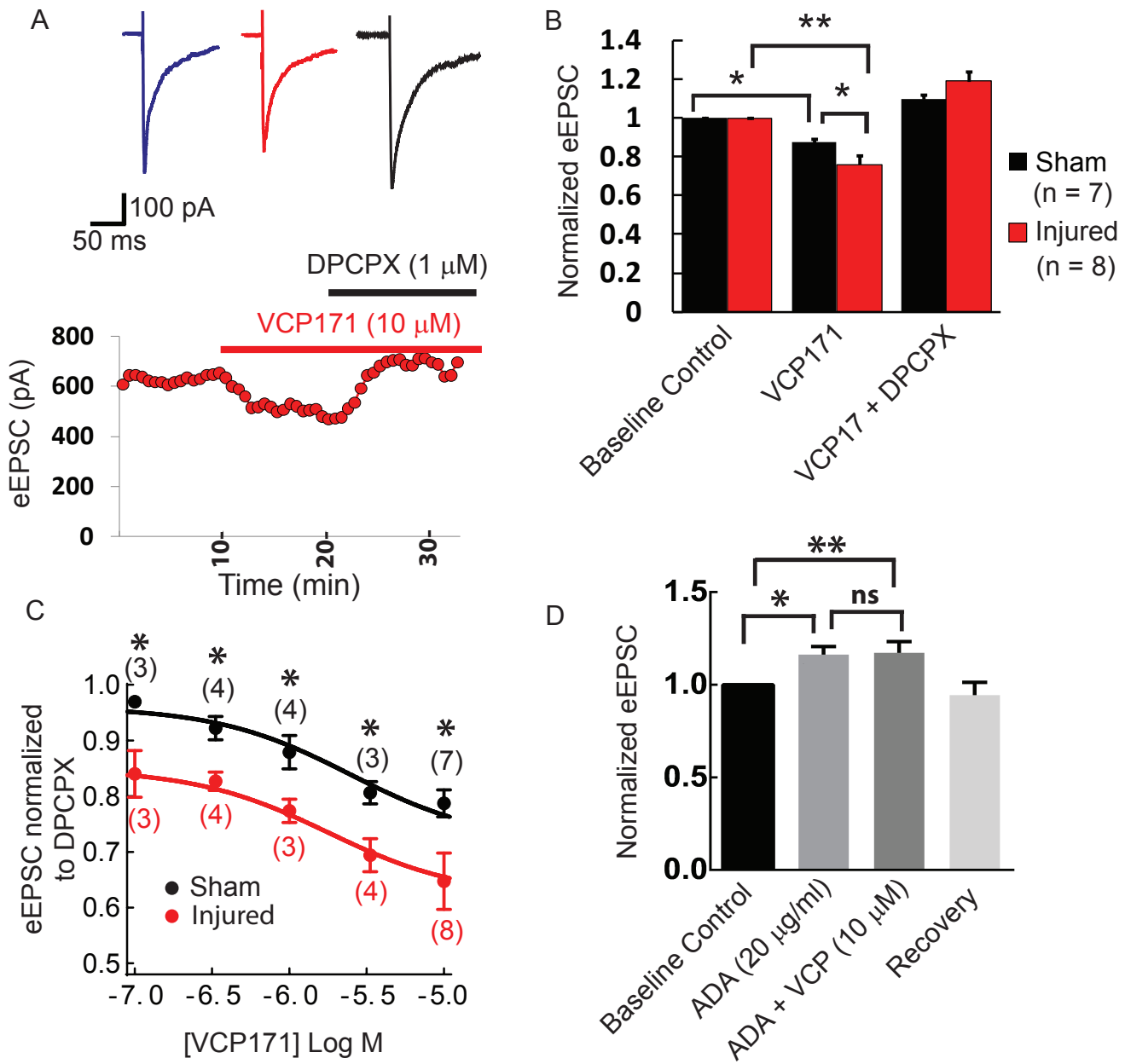


Figure 4.

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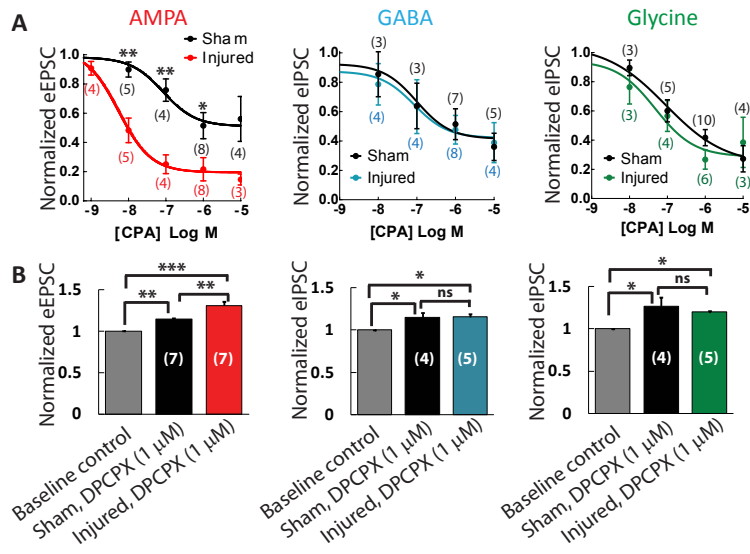


Figure 5

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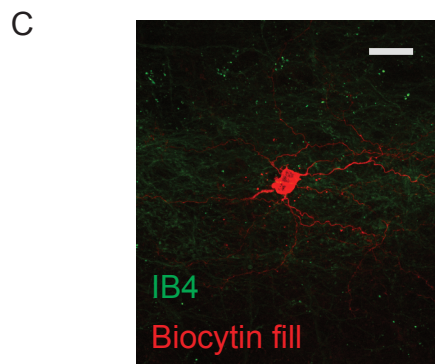
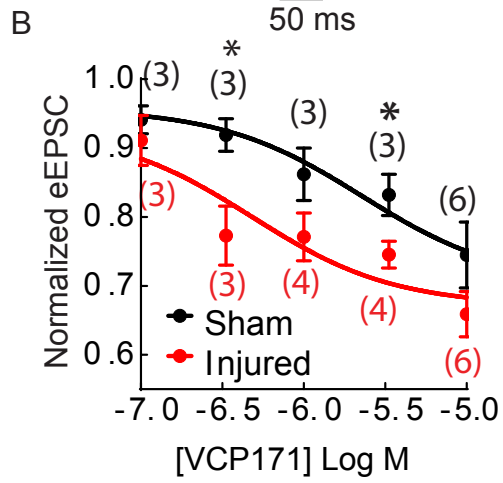
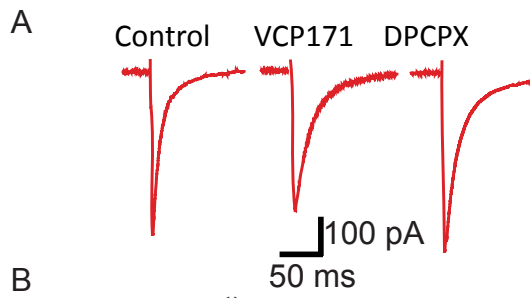


Figure 6.

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