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Investigation into the role of $P2X_3/P2X_{2/3}$ receptors in neuropathic pain following chronic constriction injury in the rat: an electrophysiological study

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1 Two $P2X_3/P2X_{2/3}$ receptor antagonists with different potencies were profiled electrophysiologically in a rat model of nerve injury.

2 A-317491 has poor CNS penetrance (blood : brain, $1 : < 0.05$), and was therefore administered intravenously in chronic constriction injury (CCI)- and sham-operated rats to study the involvement of $P2X_3$ subunit-containing receptors in the periphery in neuropathic pain. A-317491 and Compound A were administered topically to the spinal cord to investigate the central contribution.

3 There were no significant inhibitory effects of A-317491 intravenous (i.v.) seen in sham-operated animals compared to vehicle controls. In CCI-operated animals, there were significant inhibitory effects of $3\,mg\,kg^{-1}$ A-317491 i.v. on C fibre-evoked responses, and with $10\,mg\,kg^{-1}$ A-317491 i.v. on Aδ and C fibre-evoked responses. No significant effects of A-317491 were observed after topical application to the spinal cord. In contrast, when Compound A was administered spinally in CCI animals, there was a decrease in Aδ and C fibre-evoked responses, and wind up.

4 These changes indicate that A-317491 has a selective effect on neuronal responses in CCI animals compared to sham, demonstrating an increased involvement of $P2X_3/P2X_{2/3}$ receptors in sensory signalling following nerve injury. In addition, the more potent antagonist Compound A was effective spinally, unmasking a potential central role of $P2X_3/P2X_{2/3}$ receptors at this site post nerve injury. These data support a role for $P2X_3/P2X_{2/3}$ antagonists in the modulation of neuropathic pain.
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Abbreviations: ATP, adenosine-5′-triphosphate; BBB, blood–brain barrier; CCI, chronic constriction injury; i.v., intravenous; VFF, von Frey filament; WDR, wide-dynamic range

Introduction

The current unmet need for the safe and efficacious treatment of neuropathic pain has led to rigorous and persistent investigations into its underlying pathophysiology using a variety of animal models of peripheral nerve injury to uncover a novel target for its treatment.

Chronic neuropathic pain can result from either peripheral or central neuronal injury. Changes that occur in the activity of somatosensory neurons such as increased excitability and sensitisation can alter the way pain signals are transmitted, resulting in hyperalgesia, where there is an increased response to a painful stimulus, and allodynia, whereby a previously innocuous stimulus is perceived as being noxious.

In the well-established chronic constriction injury (CCI) model of neuropathic pain (Bennett & Xie, 1988), four loose ligatures are tied around the sciatic nerve in the area proximal to the trifurcation point to induce a neuropathic hypersensitivity which can be maintained for more than 60 days. This model is a valuable experimental tool, as it demonstrates that after experiencing a painful neuropathy, animals exhibit behavioural changes in the perception of sensations, such as hyperalgesia and allodynia, which mirror the changes that are observed in human pain states (Bennett & Xie, 1988; Attal *et al*., 1990).

The physiological changes resulting from a neuropathic insult can be investigated using *in vivo* dorsal horn electrophysiology. Chapman *et al*. (1998) have previously described modifications in neuronal activity observed between sham animals and those having undergone ligation of spinal nerves L5–L6. These include an increase in the frequency of spontaneous action potential firing in neuropathic animals compared to sham-operated, and also an increase in the proportion of neurons exhibiting spontaneous activity at the level of the spinal cord. In addition, changes following CCI surgery have also involved recording activity from primary afferent bundles of the sciatic nerve following electrical and mechanical stimulation of the hind paw (Gabay & Tal, 2004). This study reported marked fibre loss 5–9 days post CCI surgery, with a degree of recovery observed at the later time point of 12–15 days following the insult. The functional changes that occur in response to nerve injury can be easily detected and described using sensitive recording techniques

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such as electrophysiology. Therefore, we utilised this technique to investigate the role of the adenosine-5′-triphosphate (ATP)-gated $P2X_3$ receptor, which is believed to be involved in pain following nerve injury.

The rationale for targeting the $P2X_3$ subunit-containing receptor for the treatment of neuropathic pain is well established in the form of various histological, pharmacological and behavioural studies. Receptors containing the $P2X_3$ subunit, expressed either as homomeric $P2X_3$ or heteromeric $P2X_{2/3}$, are highly expressed on peripheral and central terminals of primary sensory afferent fibres (Chen *et al*., 1995), areas which are known to be involved in nociceptive processing. The role of ATP in pain pathways is well documented (Burnstock, 1996; Burnstock & Wood, 1996). Activation of $P2X_3$ receptors by ATP has an excitatory effect on nociceptive neurons, causing glutamate release from presynaptic nerve endings (Gu & MacDermott, 1997). $P2X_3$ knockout mice exhibit a decreased response in pain behaviour tests (Cockayne *et al*., 2000) following ATP or formalin injection into the paw. Previous immunocytochemical studies have shown that following a peripheral neuropathic injury such as chronic constriction of the sciatic nerve, there is upregulation of the $P2X_3$ receptor in primary sensory neurons in dorsal root ganglia and ipsilateral spinal cord (Novakovic *et al*., 1999).

The present study aimed to used an electrophysiological approach to investigate the effect of the $P2X_3/P2X_{2/3}$ receptor antagonists, A-317491 (5-({(3-Phenoxybenzyl)[(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino}carbonyl)-1,2,4-benzenetricarboxylic acid) and Compound A (5-{[5-iodo-2-(1-methylethyl)-4-(methyloxy)phenyl]oxy}-2,4-pyrimidinediamine) on dorsal horn neuronal responses to electrical and mechanical stimulation of the hind paw in neuropathic and sham-operated rats. A-317491 (Jarvis *et al*., 2002) potently blocks $P2X_3/P2X_{2/3}$ receptors, demonstrating over 100-fold selectivity for $P2X_3$ subunit-containing receptors in comparison with other members of the P2X receptor family, and has been shown to have analgesic effects in both neuropathic and inflammatory behavioural models (Jarvis *et al*., 2002; McGaraughty *et al*., 2003; Wu *et al*., 2004). *In vivo* assessment of A-317491 gave rise to a blood : brain ratio of $1 : < 0.05$, and demonstrated poor CNS penetrance of the compound. As A-317491 was deemed unable to cross the blood–brain barrier (BBB), this tool compound was employed to try to elucidate the relative contributions of peripheral *versus* central $P2X_3/P2X_{2/3}$ receptors. Another $P2X_3/P2X_{2/3}$ antagonist from a different chemical series was also evaluated, Compound A (patent ref. US 2005/0209260 A1). The selectivity of Compound A has not been fully profiled; however, a structural analogue of Compound A has, and has been found to have no off-site activity. A phenol has been methylated in a region of the molecule known to tolerate substitution without any changes in activity; the overall template remains unchanged and a similar profile would be expected. Compound A has a blood : brain ration of 3.1 : 3, and is thus regarded as CNS penetrant, therefore it was only applied topically to the exposed spinal cord to examine in more detail the central involvement of these receptors.

Dorsal horn convergent neurons, which are known to have a role in the transmission of somatosensory processing, were studied employing multielectrode recordings to understand the contribution of $P2X_3/P2X_{2/3}$ receptors on neuronal activity post nerve injury.

Methods

CCI surgery

CCI of the sciatic nerve was performed according to the methodology previously described by Bennett & Xie (1988). Male Random Hooded rats (150–220 g) were anaesthetised with isofluorane (5% for induction, 3% for maintenance) in oxygen. The sciatic nerve was exposed by blunt dissection, and four loose ligatures (chromic 4.0 cat gut) were tied around the sciatic nerve. The wound was closed and secured with suture clips. For sham-operated animals, the sciatic nerve was exposed in the same manner; however, no ligatures were tied around the nerve.

Following CCI surgery, the presence of mechanical (tactile) allodynia was evident as rats demonstrated a reduction in paw withdrawal threshold to manually applied von Frey hair monofilaments (Stoelting, Wood Dale, IL, U.S.A.; range 1.4–26.0 g).

Electrophysiological studies

Single unit extracellular recordings were made from dorsal horn neurons in the L4–L5 region of the rat spinal cord *in vivo* using the Plexon multielectrode array system (Plexon Inc., Dallas, TX, U.S.A.; Sokal *et al*., 2000). An important aspect of this technique is that it decreases the number of animals required per study, and it increases the information gained from each animal by differentiating responses from individual neurons on the basis of waveform analysis. Male Random Hooded rats (190–300 g) were anaesthetised with isofluorane (5% for induction, 3% for maintenance) in oxygen. The trachea, jugular vein and carotid artery were cannulated to aid ventilation, administer compounds (including anaesthetic) and continuously monitor blood pressure, respectively. Upon completion of cannulations, the anaesthetic was switched from isofluorane to saffan anaesthesia administered at a constant rate of $30\,mg\,kg^{-1}\,h^{-1}$ intravenous (i.v.), *via* a perfusion pump for the remainder of the experiment. Body temperature was maintained at 36–38°C, using a rectal probe/homeothermic-heated blanket system. The rat was immobilised in a stereotaxic frame using ear bars and vertebral clamps, and the L4–L5 section of the spinal cord was exposed by dorsal laminectomy and careful removal of the overlying dura and pia.

Eight tungsten electrodes were simultaneously lowered into the dorsal horn in 10 μm steps using a motorised micromanipulator (Scientifica). Up to four different waveforms could be identified on each individual electrode. Each waveform was discriminated offline using Plexon software. Neuronal signals were amplified (5 K), filtered (bandwidth 500 Hz–5 kHz) and passed to an analogue-to-digital converter (CED1401 plus) for discrimination of single unit events before processing on a PC using Plexon software. Neurons were initially identified by response to both mechanical stimulation of the hind paw (touch, pinch and mechanical punctuate using von Frey filaments (VFFs)) to ensure that cells identified responded to both high- and low-threshold inputs and therefore were classed as wide-dynamic range (WDR). Transcutaneous electrical stimulation of the hind paw receptive field, using needle electrodes implanted into the centre of the cutaneous receptive field approximately 5 mm apart

(2 ms pulse width, 0.5 Hz), was used to stimulate the neurons thereafter. Thresholds for A and C fibre responses were determined and subsequent electrical stimuli were delivered at three times the C fibre threshold. Different fibre types were distinguished and characterised according to threshold and latency post-electrical stimulation. The typical latencies post-electrical stimulation of the different primary afferent fibre types were taken as 0–20 ms for Aβ fibres, 20–90 ms for Aδ fibres and 90–300 ms for C fibres. In some WDR neurons, a progressive increase in the number of long latency responses (C fibre and postdischarge) was seen during the application of a train of suprathreshold stimuli; this is known as wind-up. Wind-up was calculated by multiplying the number of action potentials fired in the 90–800 ms period following the first electrical stimulation by 20, and subtracting this value from the total number of action potentials fired within this time band for all 20 stimulations (Dickenson & Sullivan, 1986). Spontaneous activity of dorsal horn neurons (number of action potentials fired per second) was measured every 15 min for 10 s throughout the entire experiment.

Stimulation protocol

The hind paw received a train of electrical stimuli every 15 min. One train consisted of 20 electrical stimuli, delivered at three times the C fibre threshold (usually 0.2–0.9 mA), 2 ms pulse width and 0.5 Hz. At 5 min following each train of electrical stimuli, mechanical stimuli were delivered to the hind paw. Four VFFs were applied with increasing force, with each filament presented for 10 s with a 20 s interval in between presentations. The force delivered from the VFFs ranged from 1 to 185 g. Reproducible responses to electrical and mechanical stimulations were established before administration of compound.

Data analysis and statistics

Data analysis was carried out offline using NeuroExplorer software. The neurons were separated on the basis of waveform shape, using postrecording cluster analysis, thus ensuring that any multiple signals were split into single units. Each single unit can then be followed in isolation for the duration of the experiments, so effects of stimulation or compounds are quite clear. Only data demonstrating clear single waveform shapes were included. Data are presented as a mean percentage of control response $\pm$ s.e.m. Owing to the complex design of the experiments, involving several repeated factors, a general linear mixed model approach was used followed by *post hoc* planned comparisons on the predicted means to ensure the most relevant statistical analysis. A *P*-value of less than 0.05 was regarded as significant.

Pharmacokinetics

HPLC/MS/MS for A-317491 and Compound A Blood samples (50 μl) were extracted using protein precipitation with acetonitrile: 10 mM ammonium acetate (80 : 20) containing a proprietary internal standard (250 μl, 0.2 μg ml^{-1}). An aliquot of the supernatant was analysed by reverse phase HPLC/MS/MS (Applied Biosystems API4000) using a heat-assisted electrospray interface in negative ion mode. Nominal MRM transitions for A-317491 and internal standard were 564–520 and 427–200, respectively. Samples (3 μl) were injected using a CTC Analytics HTS Pal autosampler (Presearch, Hitchin, U.K.) onto a Hypersil Aquastar 3.0 $\times$ 30 mm, 3 μm column (Thermo, Runcorn, Cheshire, U.K.) operated at 40°C and at an eluent flow rate of 1 ml min^{-1}. Analytes were eluted using a high-pressure linear gradient programme, by means of an HP1100 binary HPLC system (Agilent, Stockport, Cheshire, U.K.), using 1 mM ammonium acetate as solvent A and acetonitrile as solvent B. The gradient went from 1% solvent B to 99% at 1.2 min, remaining at 99% until 1.6 min before returning to the starting conditions. The cycle time was 2.5 min per sample. Samples were assayed in the range 0.088–88.0 μM, and the lower limit of quantification was 0.088 μM. Brain concentrations were corrected for residual blood volume using 15 μl g^{-1} of brain tissue as the vascular space (Brown *et al*., 1986).

As for A-317491, nominal MRM transitions for Compound A and internal standard were 401–110 and 429–228, respectively. Samples (1 μl) were injected using a CTC Analytics HTS Pal autosampler on to a Hypersil Aquastar column. Analytes were eluted using a high-pressure linear gradient programme. The gradient went from 1% solvent B to 80% at 1.2 min, remaining at 80% until 1.6 min before returning to the starting conditions. The cycle time was 2.5 min per sample. Samples were assayed in the range 0.0125–12.5 μM and the lower limit of quantification was 0.0125 μM. Brain concentrations were corrected for residual blood volume using 15 μl g^{-1} of brain tissue as the vascular space (Brown *et al*., 1986).

Equilibrium dialysis measurements The methodology employed in this study was a modification of that reported by Kalvass & Maurer (2002). Briefly, a 96-well equilibrium dialysis apparatus was used to determine the free fraction in the blood and brain for each drug (HT Dialysis LLC, Gales Ferry, CT, U.S.A.). Membranes (3 kDA cutoff) were conditioned in deionised water for 40 min, followed by conditioning in 80 : 20 deionised water : ethanol for 20 min, and then rinsed in deionised water before use. Rat blood and brain were obtained fresh on the day of the experiment. Blood was diluted 1 : 1 with PBS, whereas the brain tissue was homogenised with PBS to a final composition of 1 : 2 brain : PBS, by means of ultrasonication (Tomtec Autogiser, Receptor Technologies) in an ice bath. Diluted blood and brain homogenates were spiked with the test compound (1000 ng g^{-1}) and 100 μl aliquots ($n = 6$ replicate determinations) were loaded into the 96-well equilibrium dialysis plate. Dialysis *versus* PBS (100 μl) was carried out for 5 h in a temperature-controlled incubator at 37°C (Stuart Scientific, Watford, U.K.), using an orbital microplate shaker at 125 revolutions per minute (Stuart Scientific). At the end of the incubation period, aliquots of blood, brain homogenate or PBS were transferred to Matrix ScreenMate tubes (Matrix Technologies, Hudson, NH, U.S.A.), and the composition in each tube was balanced with control fluid such that the volume of PBS to blood or brain was the same. Sample extraction was performed by the addition of 200 μl of acetonitrile containing an internal standard. Samples were allowed to mix for 15 min and then centrifuged at 2465 $\times$ *g* in 96-well blocks for 20 min. The unbound fraction was determined as the ratio of the peak area in buffer to that in blood or brain, with correction for dilution factor according to Equation (1) (Kalvass & Maurer, 2002).

$$\mathrm{fu} = \frac{(1/D)}{(1/\mathrm{fu(apparent)} + 1/D)} \quad (1)$$

where, D = dilution factor in blood or brain homogenate and fu (apparent) is the measured free fraction of diluted blood or brain tissue.

As described by Kalvass & Maurer (2002), if the unbound fractions in blood and brain are equal at steady state, then the *in vivo* Br : Bl ratio can be estimated from free fractions in blood and brain tissue as follows,

$$\frac{\text{fu(blood)}}{\text{fu(brain)}} = \frac{C(\text{brain})}{C(\text{blood})} \quad (2)$$

where fu (blood) and fu (brain) denote the unbound fractions in blood and brain.

Analysis of test compounds in equilibrium dialysis All samples were analysed by means of HPLC/MS/MS on a PE-Sciex API-4000 tandem quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada), employing a Turbo V Ionspray operated at a source temperature of 700°C (80 p.s.i. of nitrogen). Samples (3–10 μl) were injected using a CTC Analytics HTS Pal autosampler (Presearch, Hitchin, U.K.) on to a Hypersil Aquastar 3.0 × 30 mm, 3 μm column (Thermo, Runcorn, Cheshire, U.K.) operated at 40°C and at an eluent flow rate of 1 ml min^{-1}. Analytes were eluted using a high-pressure linear gradient programme, by means of an HP1100 binary HPLC system (Agilent), using acetonitrile as solvent B. For HPLC/MS/MS analysis in positive ion mode, solvent A comprised 1 mM ammonium acetate containing 0.1% (v v^{-1}) formic acid, whereas in negative ion mode, solvent A comprised 1 mM ammonium acetate. The gradient was held at 5% solvent B for 2 min, before increasing to 90% at 1.2 min, remaining at 90% until 1.6 min before returning to the starting conditions. The cycle time was 2.5 min per sample. Relative peak areas between the PBS and tissue half-wells were used to determine the respective free fractions.

Drugs

The $P2X_3/P2X_{2/3}$ receptor antagonists A-317491 (5-({(3-Phenoxybenzyl)[(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino} carbonyl)-1,2,4-benzenetricarboxylic acid) and Compound A (5-{[5-iodo-2-(1-methylethyl)-4-(methyloxy)phenyl]oxy}-2,4-pyrimidinediamine) were synthesised at GlaxoSmithKline laboratories in Harlow, U.K. Solutions were freshly prepared daily (taking into account the weight of the animal) by dissolving in DMSO (1%), PEG 200 (66%) and saline. A-317491 (or vehicle) was administered i.v. *via* the jugular cannula in cumulative doses of 1, 3 and 10 mg kg^{-1}. The volume of i.v. injection was 0.5 ml, followed by a 0.5 ml saline flush. When administered topically to the spinal cord, A-317491 (10, 100 and 300 μg), Compound A (0.4, 1.2 and 4 μg) or vehicle was applied in a 100 μl volume. A-317491 and Compound A were administered cumulatively, and effects of each dose were observed for 45 min. Electrical and mechanical stimuli were applied every 15 min throughout every dosing period.

Results

Pharmocokinetics

In vitro assessment of the tissue binding of A-317491 indicated that the compound is able to penetrate brain tissue and spinal cord. A-317491 displays a high degree of binding to brain tissue with 97.2 ± 0.1% bound (i.e. 2.8 ± 0.1% free). Binding to blood is higher still (99.8 ± 0.1% bound, 0.2 ± 0.1% free). *In vivo*, there is poor CNS penetration following subcutaneous administration of A-317491 (30 mg kg^{-1}), where a blood : brain ratio 1 : < 0.05 was noted, albeit with very high blood concentrations (ca. 70 μM). In contrast, Compound A was found to be CNS penetrant following subcutaneous dosing, and the blood : brain ratio was 1 : 3.3. This is consistent with tissue binding of Compound A in blood and brain, where the compound displayed a higher propensity for brain tissue binding (99.0 ± 0.1% bound, 1.0 ± 0.1% free) relative to blood (96.8 ± 0.2% bound, 3.2 ± 0.2% free).

Behavioural responses

Following CCI surgery, rats demonstrated a reduction in paw withdrawal threshold to manually applied VFFs on the ipsilateral leg. Typically, a significant reduction in withdrawal threshold is evident as early as 6 days postligation in comparison to presurgery baseline levels (presurgery CCI: 26 ± 0 g *versus* 6 days CCI 6.1 ± 0.7 g). These reductions are maintained out to at least 40 days postsurgery (typically 5.9 ± 0.5 g; data not shown).

Responses to A-317491 administered intravenously in sham-operated animals

In a total of 13 sham-operated rats, six rats received A-317491 i.v. and seven rats were administered with vehicle i.v. Electrophysiological recordings were made at an average depth of 1093.6 ± 45.2 μm from the surface of the spinal cord in the A-317491 group, and 1095.7 ± 51.8 μm in the vehicle group. No significant changes in Aβ or Aδ fibre-evoked responses (42), wind-up (19), spontaneous activity (29) or mechanically evoked responses (15) were observed at any dose of A-317491 at any time point (number of neurons recorded from in parenthesis). There was a significant increase in the C fibre-evoked response 45 min following 1 and 3 mg kg^{-1} A-317491 (17.1 ± 5.9% increase, $P < 0.01$ and 36.8 ± 8.4% increase, $P < 0.01$, respectively; $n = 39$ neurons) compared to time-matched vehicle controls (Figure 1).

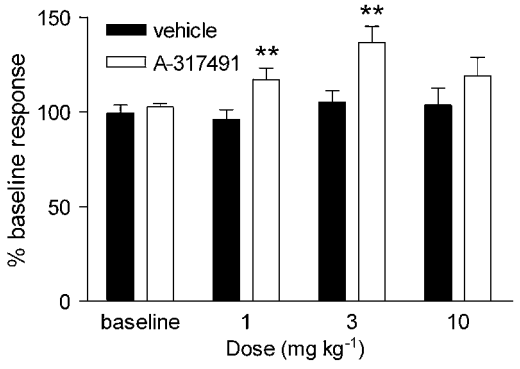

Figure 1 Effect of i.v. administration of A-317491 on C fibre-evoked response in sham-operated animals. Note the lack of inhibitory effects. **$P < 0.01$, when compared to time-matched vehicle controls.

Responses to A-317491 administered intravenously in CCI-operated animals

In a total of 12 separate CCI-operated rats, six rats received A-317491 i.v. and six rats were administered with vehicle i.v. Electrophysiological recordings were made at an average depth of $826.3 \pm 69.0\,\mu$m from the surface of the spinal cord in the A-317491 group, and $1129.3 \pm 38.9\,\mu$m in the vehicle group. There was no inhibitory effect observed on the Aβ fibre-evoked response at any dose of A-317491 at any time point ($n = 30$ neurons). At $3\,mg\,kg^{-1}$, A-317491 caused a significant inhibition of the C fibre-evoked response ($49.0 \pm 7.3\%$, $P = 0.0127$; $n = 20$ neurons; Figure 2b) when compared to vehicle controls 45 min post administration. The time-matched vehicle control had a mean electrically evoked C fibre response of 275.7 ± 35.1 action potentials per train of electrical stimuli, whereas the value for the group treated with $3\,mg\,kg^{-1}$ A-317491 was 170.6 ± 31.3 action potentials per stimulation train. At $10\,mg\,kg^{-1}$ A-317491, there was a significant inhibition of the Aδ fibre-evoked response ($15.0 \pm 7.2\%$, $P = 0.0169$; Figure 2a) and C fibre-evoked response ($69.4 \pm 5.2\%$, $P = 0.0086$; Figure 2b) compared to vehicle responses, 45 min after dosing. The mean electrically evoked C-fibre response of the group treated with $10\,mg\,kg^{-1}$ A-317491 was 112.2 ± 25.4 action potentials per train of electrical stimuli, whereas the value for the time-matched vehicle control was 236.6 ± 30.3 action potentials per stimulation train.

Spontaneous activity following $10\,mg\,kg^{-1}$ A-317491 had an average value of 3.9 ± 0.8 action potentials per second, whereas the vehicle control value was 5.6 ± 1.3 action potentials per second. There was a $58.1 \pm 9.8\%$ decrease in the wind-up response, but this was not deemed significant. No changes in mechanically evoked responses to VFFs were observed at any time point for any dose of A-317491 ($n = 15$ neurons).

When statistically compared to the pretreatment baseline response, only the C-fibre-evoked response came out as being significantly inhibited by A-317491 at both $3\,mg\,kg^{-1}$ ($P = 0.012$) and $10\,mg\,kg^{-1}$ ($P = 0.0004$). The baseline C fibre response in the A-317491-treated animals was 315.0 ± 44.2 action potentials per stimulation train; following administration of 3 and $10\,mg\,kg^{-1}$ A-317491, there were 170.6 ± 31.3 and 112.2 ± 25.4 action potentials fired per stimulation train, respectively.

A-317491 applied topically to spinal cord in CCI animals

In a total of 10 CCI-operated rats, five rats received A-317491 topically to the spinal cord and five rats were administered with vehicle by the same route. Electrophysiological recordings were made at an average depth of $1016.5 \pm 99.9\,\mu$m from the surface of the spinal cord in the A-317491 group, and $1216.7 \pm 22.9\,\mu$m in the vehicle group. No significant changes in Aβ, Aδ or C fibre-evoked responses ($n = 31$ neurons; Figure 3), wind-up, spontaneous activity or mechanically evoked responses were observed at any dose of A-317491 at any time point.

Compound A applied topically to spinal cord in CCI animals

In a total of 10 CCI-operated rats, five rats received Compound A topically to the spinal cord and five rats had vehicle administered *via* the same route. Electrophysiological recordings were made at an average depth of $968.0 \pm 90.7\,\mu$m from the surface of the spinal cord in the Compound A group, and $1216.7 \pm 22.9\,\mu$m in the vehicle group. There were significant effects observed on the Aβ fibre-evoked responses at all doses ($n = 39$ neurons); however, the maximum inhibition observed was $9.9 \pm 3.0\%$. There were no significant inhibitory effects on mechanical responses ($n = 18$ neurons) at any dose of Compound A at any time point. The Aδ fibre-evoked response was significantly inhibited at $0.4\,\mu$g ($11.7 \pm 5.4\%$ inhibition;

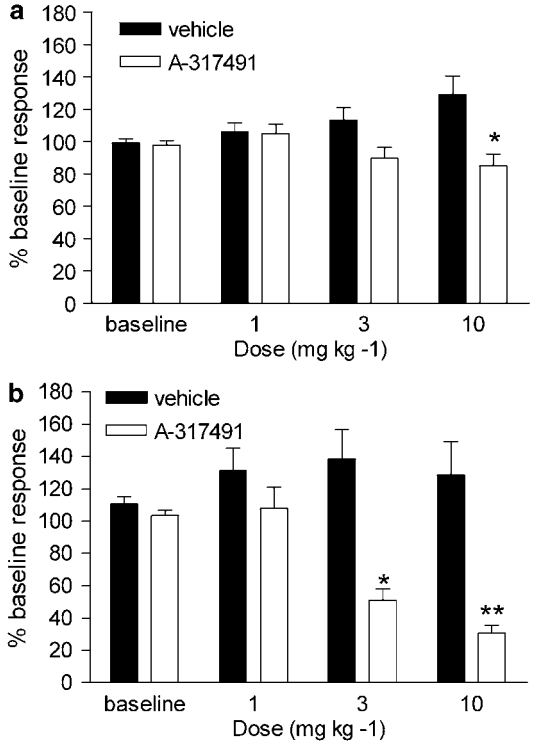

Figure 2 Effect of i.v. administration of A-317491 on Aδ fibre-evoked response (a) and C fibre-evoked response (b) in CCI-operated animals. $^*P < 0.05$, $^{**}P < 0.01$, when compared to time-matched vehicle controls.

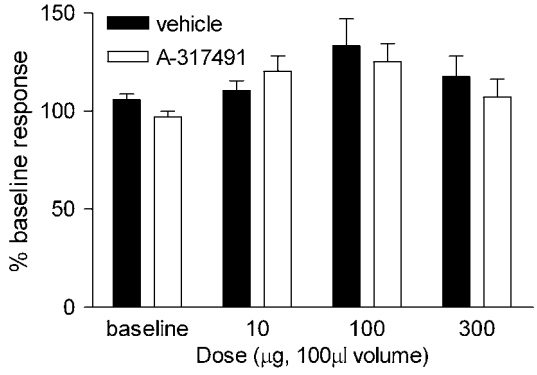

Figure 3 Example of administration of A-317491 topically to the spinal cord. C fibre-evoked response was not inhibited at 10, 100 or 300 μg A-317491.

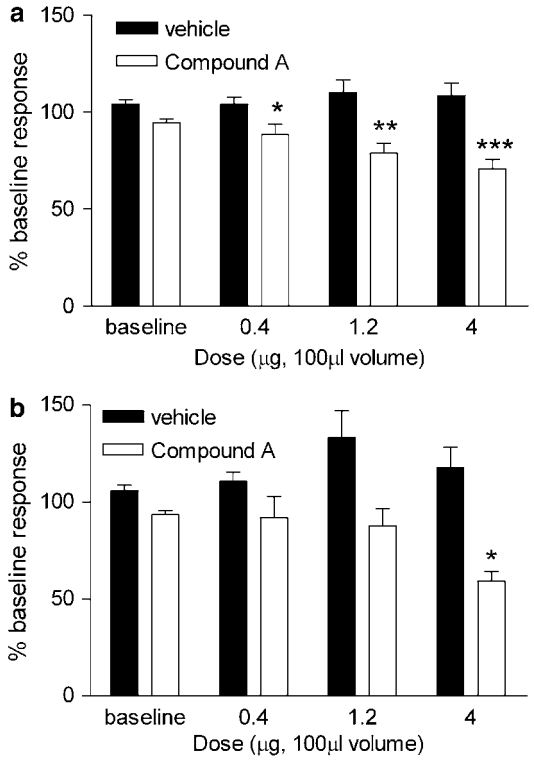

Figure 4 Effect of 0.4, 1.2 and 4 μg Compound A applied topically to the spinal cord on Aδ fibre-evoked response (a), and C fibre-evoked response (b) in CCI-operated animals. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ when compared to time-matched vehicle controls.

$P=0.0146$; $n=38$ neurons, Figure 4a) and 1.2 μg ($21.2\pm5.1\%$ inhibition; $P=0.0021$; $n=38$ neurons, Figure 4a), when compared to the time-matched vehicle controls. At 4 μg, there was a significant inhibition of Aδ ($29.3\pm4.7\%$ inhibition; $P=0.0003$; $n=38$ neurons, Figure 4a) and C fibre-evoked responses ($40.7\pm5.0\%$; $P=0.0463$; $n=36$ neurons, Figure 4b), and wind-up ($77.5\pm6.5\%$; $n=7$ neurons), compared to vehicle controls.

The baseline mean electrically evoked C fibre response was 488.1 ± 62.3 action potentials per train of electrical stimuli. For the group treated with 4 μg Compound A, this value was 262.4 ± 28.5 action potentials, whereas the number for the time-matched vehicle control was 460.2 ± 42.0 action potentials per stimulation train.

When statistically compared to the pretreatment baseline response, the C fibre-evoked response came out as being significantly inhibited by Compound A at 4 μg ($P=0.0018$), as did the Aδ fibre-evoked response ($P=0.01$) and spontaneous activity ($P=0.008$). Wind-up was significantly different to baseline at 0.4 μg ($P=0.02$) and 4 μg ($P=0.004$), and was only just not significant at 1.2 μg ($P=0.051$).

Discussion

The main focus of this investigation was to examine the effect of the $P2X_3/P2X_{2/3}$ antagonists, A-317491 and Compound A, on dorsal horn neuronal responses using an *in vivo* electrophysiology model.

As A-317491 does not cross the BBB, this compound was used to try to elucidate the relative contribution of $P2X_3/P2X_{2/3}$ receptors in the central and peripheral nervous system. To do this, A-317491 was administered systemically or topically to the exposed spinal cord in sham and CCI animals. The results from this study indicate a selective involvement of $P2X_3/P2X_{2/3}$ receptors following neuropathic insult, such that greater reductions in excitability were observed in the CCI-operated animals compared to sham animals following treatment with the $P2X_3/P2X_{2/3}$ receptor antagonist. When administered intravenously in sham-operated animals, A-317491 had no significant inhibitory effects on electrically or mechanically evoked dorsal horn responses or spontaneous activity, whereas in CCI-operated rats, i.v. administration of A-317491 caused a significant decrease in Aδ and C fibre-evoked responses, which are believed to be important in noxious processing and in altered somatosensory processing (Schouenborg & Sjolund, 1983; Dickenson & Sullivan, 1986; Davies & Lodge, 1987).

Clinically, pain resulting from nerve damage is refractory to many current pharmacological treatments (Dworkin, 2002; McQuay, 2002). There is a great unmet need for patients with neuropathic pain who do not have adequate pain control. In view of this, over the last decade animal models of nerve injury such as the Chung, Seltzer and CCI models have been employed to try to understand the changes that occur as a result of the insult, using histological, pharmacological and electrophysiological techniques (Bennett & Xie, 1988; Seltzer *et al*., 1990; Kim & Chung, 1992). In patients with nerve damage, there is a loss of and altered activity of primary afferents (Lang *et al*., 1995; Rowbotham *et al*., 1996). Nerve injury following CCI in rodents has been shown to cause extensive loss of myelinated afferent fibres (Basbaum *et al*., 1990), or a loss of myelinated and nonmyelinated axons distal to the neuroma (Gabay & Tal, 2004). In addition to fibre loss, there is also a change in the way primary afferents process somatosensory information. Post-nerve injury, a 'phenotypical switch' has been observed, where low threshold A fibres start to take on properties normally associated with C fibre afferents; for example, expression of substance P and CGRP (Neumann *et al*., 1996; Miki *et al*., 1998). As the CCI model displays some similarities to humans in altered somatosensory processing, an increase in inhibitory effects of the $P2X_{2/3}$ antagonists, as we have shown with A-317491, may also be effective in humans with nerve injury.

One hypothesis for the enhanced involvement of $P2X_3/P2X_{2/3}$ receptors in CCI animals compared to sham is an upregulation of $P2X_3/P2X_{2/3}$ receptors in pathways involved in somatosensory processing. This supports previous findings from neuropathic pain studies (Novakovic *et al*., 1999), which showed histologically that a peripheral neuropathic insult such as CCI can result in an upregulation of $P2X_3$ subunit-containing receptors in small and medium diameter primary afferent fibres in dorsal root ganglia and ipsilateral spinal cord. Given this precedence of upregulation of $P2X_3/P2X_{2/3}$ in neuropathy, we did not specifically investigate the expression patterns of this receptor in the current electrophysiological investigation.

As well as wishing to understand if $P2X_3/P2X_{2/3}$ receptors had an altered role in a nerve injury model, we also wanted to understand the site of action.

Receptors generated in dorsal root ganglia can be translocated to peripheral and central terminals of primary afferents (North, 2003). Therefore, $P2X_3/P2X_{2/3}$ receptors have the

potential to have a function in transmission *via* peripheral and central actions. In order to address this, we wanted to use a tool compound that could not cross the BBB, and administer it intravenously and centrally. McGaraughty *et al*. (2003) showed that A-317491 can penetrate the BBR. Contradictory to this is the data from Wu *et al*. (2004), who analysed plasma and brain samples from animals dosed with A-317491 (subcutaneously) in the Freund's Complete Adjuvant model of inflammatory pain. They found that although the antagonist had good systemic bioavailability, only low levels of A-317491 were detected in the brain. According to recent reports, the fact that A-317491 has higher levels of binding in the blood than the brain leads to a sequestration of the drug in the systemic circulation relative to the brain (Kalvass & Maurer, 2002). Owing to the contradictory evidence in the literature, we performed our own pharmacokinetic analysis. We found that A-317491 did not readily penetrate the CNS, and the blood to brain ration is $1:<0.05$. Therefore, we applied A-317491 to the spinal cord or gave it intravenously. Surprisingly, there was no decrease in electrically or mechanically evoked responses, or spontaneous activity, following topical administration of A-317491 to the spinal cord. A-317491 has been given *via* intrathecal administration in behavioural studies in a neuropathic model and has resulted in antinociception (McGaraughty *et al*., 2003). One major difference between the behavioural study and our study is that in the study by McGaraughty *et al*., the spinal cord remains intact and thus compounds administered into the intrathecal space can be transported *via* the cerebro-spinal fluid to the brain. Thus, one possible theory could be that the effects of A-317491 in this study were not at the level of the spinal cord. In order to address whether the lack of spinal effects caused by A-317491 was due to a compound effect or a class effect, we also tested Compound A, another $P2X_3/P2X_{2/3}$ antagonist (patent ref. US 2005/0209260 A1). A-317491 has a pIC_{50} of 6.5 at rat $P2X_{2/3}$ receptors, whereas Compound A has a pIC_{50} of 7.1 and is therefore deemed more potent at $P2X_{2/3}$ receptors. As this compound is CNS penetrant, we could not use it to determine the contribution of peripheral *versus* central receptor function. While Compound A has not been profiled against other receptors and ion channels in-house, a structurally similar compound has, and was shown to be selective for $P2X_3/P2X_{2/3}$ receptors (patent ref. US 2005/0209260 A1, example number 42). It is highly unlikely that such a small alteration in structure (methylating a phenol) would cause Compound A to be acting at any other receptor subtype, and thus it is assumed that it is acting *via* $P2X_3/P2X_{2/3}$ receptors. Compound A caused inhibition of the Aδ and C fibre-evoked responses, thus demonstrating a possible involvement of $P2X_3/P2X_{2/3}$ receptors at the level of the spinal cord.

We did not observe any inhibitory effects of A-317491 or Compound A on mechanically evoked responses by either route of administration. This is in contrast to Jarvis *et al*. (2002), who gave A-317491 subcutaneously in inflammatory and neuropathic models, and showed a significant reduction in mechanical hyperalgesia using VFFs. This is hard to reconcile with our data, but the lack of effects on mechanical responses in this model was at least confirmed by testing a second $P2X_3/P2X_{2/3}$ receptor antagonist from a different chemical series and obtaining the same result.

In conclusion, we have shown using electrophysiological methods that there is an increased involvement of $P2X_3$ subunit-containing receptors in neuropathic pain. We have demonstrated an enhanced antinociceptive effect of the $P2X_3/P2X_{2/3}$ antagonist A-317491 on dorsal horn neuronal responses *via* an action at peripheral $P2X_3/P2X_{2/3}$ receptors. In addition, we have shown a potential central involvement of $P2X_3/P2X_{2/3}$ receptors, by employing an antagonist from a different chemical series. These two antagonists have different pharmacokinetic properties and potencies that may account for the different effects seen with them. We have clearly demonstrated that inhibiting this class of receptor attenuates neuronal firing, known to underlie nociceptive transmission, in a model of neuropathic pain and thus may be a useful target to manage altered somatosensory processing in the clinic.

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