Neuroscience Letters 441 (2008) 110–114

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

$P2X_3$ expression is not altered by lingual nerve injury

James E. Biggs[a,c,*], Julian M. Yates[a], Alison R. Loescher[a], Nick M. Clayton[b], Peter P. Robinson[a], Fiona M. Boissonade[a]

[a] Department of Oral & Maxillofacial Surgery, School of Clinical Dentistry, University of Sheffield, UK
[b] Neurology and GI CEDD, GlaxoSmithKline, UK
[c] Department of Pharmacology, University of Alberta, 9.75 Medical Sciences Building, Edmonton, Alberta, Canada T6G 2H7

ARTICLE INFO

Article history:
Received 31 March 2008
Received in revised form 20 May 2008
Accepted 24 May 2008

Keywords:
Lingual nerve
Trigeminal ganglion
$P2X_3$
Neuropathic pain
Dysaesthesia

ABSTRACT

We have investigated a possible role for the ATP receptor subunit $P2X_3$, in the development of neuropathic pain following injury to a peripheral branch of the trigeminal nerve. In nine anaesthetised adult ferrets the left lingual nerve was sectioned and recovery permitted for 3 days, 3 weeks or 3 months (3 ferrets per group). A retrograde tracer, fluorogold, was applied to the nerve to allow identification of cell bodies in the trigeminal ganglion with axons in the injured nerve. Indirect immunofluorescence for $P2X_3$ and image analysis was used to quantify the percentage area of staining at the site of injury. Additionally, the proportion of fluorogold-positive cells that expressed $P2X_3$ was determined and compared with expression in non-fluorogold containing cells in another part of the ganglion. Comparisons were made with results from control animals that only received the tracer injection. After lingual nerve injury there was no significant change in $P2X_3$ expression at the site of nerve injury or within cell bodies linked to either injured (lingual) or uninjured (ophthalmic) axons, at any of the time periods investigated. Overall, this study suggests that $P2X_3$ expression at these sites is not involved in the development of neuropathic pain following lingual nerve injury.

Injury to peripheral branches of the trigeminal nerve can result in the development of neuropathic pain. The lingual nerve, a branch of the mandibular division of the trigeminal nerve, is susceptible to iatrogenic damage during various surgical procedures, including the removal of impacted lower third molar teeth. In a small but significant number of patients this leads to permanent sensory disturbances such as dysaesthesia [25].

An ion channel that is potentially involved in the development of neuropathic pain is the ATP receptor subtype, $P2X_3$. Previous investigations have shown that after sciatic nerve axotomy and spinal nerve ligation (SNL) levels of $P2X_3$ are downregulated in injured DRG neurons [9,20,27]. Interestingly, levels of $P2X_3$ are increased in injured DRG and TG neurons after chronic constriction injury (CCI) of the sciatic [23] and inferior alveolar nerves, respectively [13]. $P2X_3$ also accumulates at the injury site after CCI of the inferior alveolar nerve (IAN) [13]. Differences in $P2X_3$ expression have also been observed in uninjured sensory ganglion neurons after nerve injury. $P2X_3$ mRNA is upregulated in uninjured DRG and trigeminal ganglion neurons after peroneal and infraorbital nerve injury, respectively [27], however, this is not observed in uninjured DRG neurons after L5 SNL [14,20]. Of particular interest is the fact that the time course of expression of $P2X_3$ following CCI of the IAN fits the time course of spontaneous activity produced after IAN injury [7,8,13]. Additionally, there are reports demonstrating that injured spontaneously active units respond to ATP administration [11,29]. Overall this suggests that $P2X_3$ is involved in the development and maintenance of ectopic activity.

In this study we determined whether section of the lingual nerve in the ferret causes changes in the expression of $P2X_3$, both at the injury site and in cells linked to the damaged axons in the trigeminal ganglion. In order to discover whether injury to the lingual nerve causes changes in the expression of $P2X_3$ in uninjured trigeminal ganglion neurons, changes in $P2X_3$ expression were also examined in cells linked to uninjured axons.

Twelve adult female ferrets (>9 months old) weighing between 0.6 and 1.0 kg were used in this study. All procedures were carried out under UK Home Office license regulations and approval (UK Animals [Scientific Procedures] Act, 1986). Ferrets were initially anaesthetised with a mixture of ketamine (Ketaset Injection, Fort Dodge Animal Health Ltd, Southampton, UK) (24 mg/kg im) and xylazine (Rompun 2%, Bayer plc, Newbury, UK) (1.4 mg/kg im). In 9 animals an incision was made in the submandibular region, the

* Corresponding author at: Department of Pharmacology, University of Alberta, 9.75 Medical Sciences Building, Edmonton, Alberta, Canada. Tel.: +1 780 492 2643; fax: +1 780 492 4325.
E-mail address: jbiggs@pmcol.ualberta.ca (J.E. Biggs).

0304-3940/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2008.05.118

underlying mylohyoid muscle was separated to reveal the left lingual nerve lying on the pharyngeal constrictor muscle. The lingual nerve was carefully separated from the surrounding connective tissue and sectioned with micro-scissors, and the cut ends of the nerve left in alignment. A single 6/0 black silk suture (Pearsalls Sutures) was placed in the soft tissue 3–4 mm lateral to the injury site to enable identification of the injury site after recovery. The wound was closed in layers and the skin closed with 4/0 catgut sutures (Bayer, Bury St. Edmonds, UK). A single dose of ampicillin antibiotic (Amfipen 15%, Intervet, Milton Keynes, UK) (22.5 mg/kg) was administered subcutaneously immediately after surgery. The animals were allowed to recover for a period of 3 days, 3 weeks or 3 months (3 ferrets per group). Three days prior to the end of the recovery period, fluorogold (Fluorochrome Inc, Eaglewood, Colorado USA), a retrograde tracer, was applied to the lingual nerve to identify the cell bodies within the trigeminal ganglion with axons in the lingual nerve. For the 3-day recovery group the fluorogold injection was performed during the nerve injury procedure. For the 3-week and 3-month recovery groups, the incision was reopened and the lingual nerve re-exposed. A small incision was then made in the epineurium of the lingual nerve with a razor chip, at a position central to the injury site. A microdialysis needle (0.1 mm o.d.; World Precision Instruments, UK) was inserted into the incision and 6 μl of 2% fluorogold solution in sterile distilled water was slowly injected into the lingual nerve. The three remaining ferrets served as controls and received fluorogold injection without the lingual nerve injury.

At the end of the recovery period, the animals were deeply anaesthetised with sodium pentobarbitone (Sagatal, Rhone Merieux, Harlow, UK) (42 mg/kg, i.p.). All animals were perfused via the left ventricle with 1 l of phosphate buffered saline (PBS) followed by 1 l of 4% paraformaldehyde. Following perfusion the left and right lingual nerves and trigeminal ganglia were harvested. In order to orientate the lingual nerves a 4/0 black silk suture (Pearsalls Sutures) was tied around the central end with the knot positioned on the lateral surface. All tissue was post-fixed in 4% paraformaldehyde for 4 h at 4 °C before being immersed in 30% sucrose solution overnight at 4 °C. Frozen sequential longitudinal sections (14 μm) embedded in O.C.T. medium (Tissue-Tek®, UK) were cut with a cryostat and sections thaw mounted on glass microscope slides coated with poly-D-lysine. Lingual nerve tissue was collected as 4 sets while trigeminal ganglion tissue was collected as 8 sets, such that each section was 56 and 112 μm from its adjacent section for lingual and trigeminal tissue, respectively. One set of sections from each nerve and ganglion were preincubated with 10% normal donkey serum (NDS; Jackson ImmunoResearch Inc, West Grove PA, USA) diluted in PBS + 0.2% triton (PBST; Fisons Scientific Equipment, Loughborough, UK) for 1 h in a moisture chamber at room temperature. The sections were then incubated with a primary antibody raised in rabbit against human $P2X_3$ (1:1000, Neuromics Antibodies, Edina MN, USA) diluted in PBST containing 5% NDS in a moisture chamber overnight at 4 °C. The next day the sections were incubated with the fluorescent secondary antibody (donkey anti-rabbit IgG conjugated to indocarbocyanine [Cy3] 1:600 diluted in PBST containing 1.5% NDS, Jackson ImmunoResearch Inc, West Grove PA, USA) in the dark at room temperature for 90 min. The prepared sections were then mounted in Vectashield mounting medium (Vector Laboratories, Peterborough UK) and coverslipped. Immunohistochemical controls were performed by preabsorption of the primary antibody with 66 μg/ml of its blocking peptide (Neuromics Antibodies, Edina MN, USA) over a 24-h period at 4 °C.

Images of ferret lingual nerve tissue were captured and the percentage area of staining determined across the whole section of nerve immediately central to the site of injury (or an equivalent area in the control nerves). An area of interest (AOI) was drawn around the nerve fibres excluding any labelling within the epineurium of the nerve using the image processing software. A threshold was then applied to the image to define the range of intensities to be included in the measurements. The upper level of the threshold was always set to the maximum (255) while the lower limit was subjectively set so that the highlighted area matched as closely as possible the positive labelling viewed down the microscope. The mean percentage area of staining (PAS) for three alternate sections (112 μm between sections) was then determined, representing the lateral, central and medial components of each nerve. A minimum area of 7×10^5 μm^2 was analysed for each of the nerves. For the trigeminal ganglion, all (minimum of 200) fluorogold-positive cells per ganglion were counted, from five sections within each set of tissue (a minimum of 112 μm between sections) and the proportion of fluorogold cells that also contained $P2X_3$ determined. The proportion of $P2X_3$ positive cells was also determined in the "uninjured" ophthalmic division of the trigeminal ganglion. A total of 200 fluorogold-negative cells (40 per section included in analysis) were counted from an area representative of the whole ophthalmic division. Every effort was made to ensure that the counts were not biased to include either more or less $P2X_3$ positive cells. Analysis was performed blind to the experimenter.

To confirm reproducibility of the analysis, three trigeminal ganglia and three lingual nerves were re-examined by the same operator. Recounts were blind and differed from the original analysis by less than 5%.

Statistical analysis was performed using GraphPad InStat v3.06 (GraphPad Software, San Diego, California, USA). The Kruskal–Wallis test with Dunn post-tests was used to compare PAS between control nerves and nerves at different recovery periods for both the ipsilateral and contralateral sides. The Kruskal–Wallis test was also used to compare the proportion of double-labelled lingual nerve cell bodies in injured and control animals at each recovery period. Additionally, the Mann–Whitney *U* test was used to compare the proportion of $P2X_3$ positive cell bodies in the injured and uninjured regions of the trigeminal ganglion at each time point. Differences were considered significant at $p < 0.05$.

Immunoreactivity for $P2X_3$ was present in all nerves examined in this study (Fig. 1A and B). Preabsorption of the $P2X_3$ primary antibody with its blocking peptide abolished $P2X_3$ labelling, confirming the specificity of staining (Fig. 1C). $P2X_3$ staining was observed along the entire length of the nerve examined. The recorded percentage area staining of $P2X_3$ in each of the regions is illustrated in Fig. 2. No significant differences in the level of immunoreactivity for $P2X_3$ were observed in either ipsilateral or contralateral nerves between any of the recovery periods ($p > 0.05$).

In the trigeminal ganglion, retrograde labelling with 2% fluorogold was successful. All fluorogold-positive cells were confined to the mandibular division in the ipsilateral ganglia and none were observed in the contralateral ganglia. Immunoreactivity to $P2X_3$ was seen in all trigeminal ganglia studied and was present in all divisions of the ganglion. Double-labelled cells could be identified (Fig. 3A and B) and preabsorption of the primary antibody with its blocking peptide abolished $P2X_3$ labelling (Fig. 3C). Two hundred or more fluorogold-positive cells were identified in 5 sections for each of the ganglia. No significant differences in the number of fluorogold-positive cells were identified between any of the recovery groups. The mean percentage of $P2X_3$ positive cells for each of the experimental and control groups is illustrated in Fig. 4. There was a tendency towards a reduction in the proportion of $P2X_3$ positive cells 3 days (9.0 ± 1.9[S.E.M.]%) and 3 weeks (8.2 ± 1.3%) after injury, however, it was not significantly different from the proportion of $P2X_3$ positive cells in the uninjured control animals (15.3 ± 5.5%, $p > 0.05$). Three months after injury the number of double-labelled cells increased back to a level that was similar to