Neuronal P2X<sub>7</sub> Receptors Are Targeted to Presynaptic Terminals in the Central and Peripheral Nervous Systems

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The ionotropic ATP receptor subunits P2X<sub>1</sub>–6 receptors play important roles in synaptic transmission, yet the P2X<sub>7</sub> receptor has been reported as absent from neurons in the normal adult brain. Here we use RT-PCR to demonstrate that transcripts for the P2X<sub>7</sub> receptor are present in extracts from the medulla oblongata, spinal cord, and nodose ganglion. Using in situ hybridization mRNA encoding, the P2X<sub>7</sub> receptor was detected in situ oblongata, spinal cord, and nodose ganglion. Using the P2X<sub>7</sub> receptor are present in extracts from the medulla oblongata and spinal cord. Localizing the P2X<sub>7</sub> receptor protein with immunohistochemistry and electron microscopy revealed that it is targeted to presynaptic terminals in the CNS. Anterograde labeling of vagal afferent terminals before immunohistochemistry confirmed the presence of the receptor in excitatory terminals. Pharmacological activation of the receptor in spinal cord slices by addition of 2′- and 3′-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (BzATP; 30 μM) resulted in glutamate mediated excitation of recorded neurons, blocked by P2X<sub>7</sub> receptor antagonists oxidized ATP (100 μM) and Brilliant Blue G (2 μM). At the neuromuscular junction (NMJ) immunohistochemistry revealed that the P2X<sub>7</sub> receptor was present in motor nerve terminals. Furthermore, motor nerve terminals loaded with the vital dye FM1-43 in isolated NMJ preparations destained after application of BzATP (30 μM). This BzATP evoked destaining is blocked by oxidized ATP (100 μM) and Brilliant Blue G (1 μM). This indicates that activation of the P2X<sub>7</sub> receptor promotes release of vesicular contents from presynaptic terminals. Such a widespread distribution and functional role suggests that the receptor may be involved in the fundamental regulation of synaptic transmission at the presynaptic site.

Key words: ATP; purine receptor; synaptic transmission; excitatory amino acid transmission; spinal cord; medulla oblongata; neuromuscular junction

In the nervous system, ATP acts as a fast neurotransmitter at excitatory purinergic synapses and activates ligand-gated cationic channels, the P2X receptors (North and Surprenant, 2000). The P2X receptor family consists of seven cloned subtypes, of which P2X<sub>1</sub>–6 have been localized to neurons in the CNS at both presynaptic and postsynaptic sites (Collo et al., 1996; Vulchanova et al., 1997; Le et al., 1998; Llewellyn-Smith and Burnstock, 1998; Loesch and Burnstock, 1998; Atkinson et al., 2000) and have been shown to mediate both postsynaptic responses (Bardoni et al., 1997; Edwards et al., 1997; Nieber et al., 1997; Pankratov et al., 1998) and presynaptic release of neurotransmitters (Gu and MacDermott, 1997; Khakh and Henderson, 1998; Boehm, 1999). In contrast there is no evidence to support the presence of the P2X<sub>7</sub> receptor (P2X<sub>R7</sub>) in neurons in normal adult brain.

The P2X<sub>7</sub> receptor subtype has been localized to widespread tissues in the periphery (Afework and Burnstock, 1999; Bardini et al., 2000; Lee et al., 2000; Pannicke et al., 2000) and was previously identified pharmacologically in cells with potential immunological functions as the P2Z receptor (Ralevic and Burnstock, 1998). In many cell types activation of the P2X<sub>7</sub> receptor has been previously identified pharmacologically in cells with potential immunological functions as the P2Z receptor (Ralevic and Burnstock, 1998). In many cell types activation of the P2X<sub>7</sub> receptor has been associated with cell lysis and death (Surprenant et al., 1996; Rassendren et al., 1997; Chow et al., 1997; Virgino et al., 1999).

In the CNS the P2X<sub>7</sub> receptor has been reported as absent from neurons but present only in ependymal cells and activated microglia (Collo et al., 1997). This suggests a role in pathophysiology for the P2X<sub>7</sub> receptor because ATP can be released in the CNS in response to cell injury (Dubayak and el Moattassim, 1993) or other conditions such as anoxia (Lutz and Kabler, 1997). Furthermore, activation in microglia of a P2 receptor that is likely to be P2X<sub>7</sub> results in the production of inflammatory cytokines, which have been associated with progression of neurodegenerative diseases (Hide et al., 2000). Thus, the expression of P2X<sub>7</sub> receptor by brain macrophages rather than neurons would be consistent with a role in brain repair after inflammation, infarction, or immune insult.

However, in this current work we reveal unexpected findings that indicate a fundamental role for the P2X<sub>7</sub> receptor in the process of neuronal synaptic transmission. We report that the receptor is expressed by neurons and is functionally targeted to excitatory presynaptic terminals that are widespread throughout the CNS, as well as at the neuromuscular junction in the peripheral nervous system. Furthermore, the receptor is functionally present because its activation promotes release of vesicular contents from presynaptic terminals in both central and peripheral nervous systems.

Parts of this work have been published in preliminary reports (Deuchars et al., 2000; Knutsen et al., 2000).

MATERIALS AND METHODS

Detection of mRNA encoding the P2X<sub>7</sub> receptor. Total cellular RNA was extracted from freshly dissected spinal cord, medulla oblongata, and nodose ganglia using TRI reagent (Sigma, Poole, UK) according to the
were cut on the vibrating microtome at 50 µm in a final volume of 20 µl. One microliter aliquots were used for RT-PCR analysis in a 20 µl reaction volume using 500 nM primers, 250 µM deoxyribonucleotide triphosphates, and 0.2 U µl taq polymerase (Promega) final concentration.

Cycling conditions were 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min followed by a final extension step of 72°C for 10 min. Primers used were P2X2 162a (agcaaaagaatcagccggctggtg) and hprt 231s (ctcgctgattacattaaagc) and hprt 576a (gaagtactcattatagtcaagg). PCR products were separated by electrophoresis through a 2% agarose gel.

In situ hybridization. Rats were anesthetized with intraperitoneal Sagatal (60 mg/ml; Rhone Merieux, Essex, UK) and perfused transcardially with sucrose containing artificial CSF. Sections were cryostat cut at 10 µm, mounted on slides that had been pretreated with 3-aminopropyltriethoxysilane (Sigma), and stored at −20°C before removal of tissue. Tissue was dissected from 150–200 gm Wistar rats. Two micrograms of RNA were reverse-transcribed using oligo-dT and mouse murine leukemia virus reverse transcriptase (Promega, Southampton, UK) in a final volume of 20 µl. One microliter aliquots were used for RT-PCR analysis in a 20 µl reaction volume using 500 nM primers, 250 µM deoxyribonucleotide triphosphates, and 0.2 U µl taq polymerase (Promega) final concentration.

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Western blot analysis. The primary antibody was raised in rabbit against residues 576–595 of rat P2X2 receptor with additional N-terminal cysteine (anti-P2X2 receptor; Alomone Labs, Jerusalem, Israel). Specificity was determined by Western blot analysis. Rat brainstem and spinal cord were separately isolated and crushed under liquid nitrogen. Tissue was isolated by sonication in homogenization buffer [500 mM, pH 7.2. Adult C57BL mice were killed by CO2 intoxication, and collected, the coverslip was removed, and the relevant area was cut out and placed in homogenization buffer 

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Anterograde tracing. Vagal afferent fibers were anterogradely labeled in 150–200 gm Wistar rats (n = 5) under halothane anesthesia (5% in O2) by the injection of 5–10 µl of 10% biotinylated dextran amine (BDA; Molecular Probes, Eugene, OR, USA) into the right nodose ganglia. After 7–10 d of recovery, the rats were anesthetized with Sagatal (60 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde–0.1–0.5% glutaraldehyde. Sections were cut on the vibrating microtome at 50 µm and freeze-thawed in liquid nitrogen. Anterogradely transported BDA was visualized by incubating sections in ABC solution (Vector Laboratories, Peterborough, UK) for 18–20 hr at 4°C before the diaminobenzidine (DAB) reaction.

Fluorescence light microscopy. Tissue sections (rat) were obtained as described above and incubated in rabbit anti-P2X2 receptor antibody as above at a concentration of 1:1000–1:5000 in PBS with 0.1% Triton X-100. After three washes for 10 min each in PBS, some sections were incubated in biotinylated secondary antibody to rabbit IgG (1:200 in PBS) for 5 hr at 4°C followed by incubation in streptavidin Alexa 488 (1:1000 in PBS; Molecular Probes) for 3 hr at room temperature. Other sections were transferred to PBS containing Cy3-conjugated secondary IgG (Jackson ImmunoResearch, Strathe, Luton, UK) at 1:1000 for 4–12 hr at room temperature. Sections were then washed three times in PBS, dried onto gelatin-coated slides at 4°C and mounted in Vectamount (Vector Laboratories) under a coverslip. Slides were viewed on a Nikon E600 microscope equipped with epifluorescence using the appropriate filter sets.

Whole mounts of transversus abdominis (mouse) were preblocked with PBS containing 1% BSA and stained en bloc. Poststain acetylcholine receptors were labeled with tetramethylrhodamine isothiocyanate-conjugated a-bungarotoxin (TRITC-a-BTX; 5 µg/ml; Molecular Probes). P2X2 receptors were labeled with primary antibody (1:1500 in 1% BSA in PBS), and visualized with fluorescein-conjugated donkey anti-rabbit secondary antibody (1:200 in 1% BSA in PBS; Scottish Antibodies, Edinburgh, UK). Sections of brain were mounted in 2.5% DABCO in glycerol.

Electron microscopy. Sections (50 µm) were cut on a vibrating microtome and cryoprotected by incubation in 10% sucrose in 0.1 M PB for 10 min followed by 20% sucrose in 0.1 M PB for 20 min and then freeze-thawed twice in liquid nitrogen to permeabilize the membranes. Sections were then incubated in rabbit anti-P2X2 receptor diluted 1:500–1:15,000 in PBS for 12–24 hr at 4°C. After three washes for 10 min each in PBS, some sections were placed into biotinylated secondary antibody to rabbit IgG diluted 1:200 in PBS (Vector Laboratories) for 5 hr at 4°C and then into Vectastain Elite ABC reagent (Vector Laboratories) for 18–20 hr at 4°C. Sections were then washed in Tris HCl buffer, pH 7.4, and incubated in DAB solution (5 mg in 10 ml of Tris HCl buffer, pH 7.4, with 0.01% H2O2) for 10 min. Control sections were incubated in PBS in place of primary antiserum for 12–24 hr at 4°C followed by secondary antibody and reacted with ABC–DAB as above. Other control sections were incubated for 12–24 hr at 4°C in primary antiserum for the P2X2 receptor (1:5000) that had been preabsorbed with peptide antigen for 1 hr before use (1 µg of peptide for 1 µg of antibody) and then incubated in secondary antibody and reacted with ABC–DAB as above. Sections were washed in 0.1 M PB for 10 min and post-fixed in 0.5% osmium tetroxide (in 0.1 M PB) for 45 min. After washing in 0.1 M PB the sections were then dehydrated through a series of ethanol followed by drying and embedding in Durcupan ACM resin (Fluka, Gillingham, UK). The sections were then immersed in Durcupan ACM resin (Fluka, Gillingham, UK) for 12–20 hr, mounted on glass slides under coverslips, and heated at 60°C for 48 hr to polymerize the resin.

Sections with anterogradely labeled vagal afferent fibers were transferred into primary antibody against the P2X2 receptor (1:10,000) for 18–20 hr at 4°C, washed in PBS, and then incubated in secondary antibodies to rabbit IgG conjugated to 1 nm gold particles (Amersham Pharmacia Biotech) diluted 1:100 in PBS, pH 7.4, containing 0.8% fish gelatin and 0.1% bovine serum albumin for 18–20 hr at 4°C. After thoroughly rinsing the sections (four times for 10 min each) in distilled deionized water, the gold particles were silver-enhanced for 5–10 min using an IntenSE silver enhancement kit (Amersham Life Sciences). Sections were then osmicated and processed for electron microscopy as described above.

When areas with suitable staining for electron microscopy were selected, the coverslip was removed, and the relevant area was cut out and glued to the flat surface of a blank resin block. After trimming of the block, serial ultrathin sections (70 nm) were cut using a Leica (Nussloch, Germany) UltraCut S ultramicrotome and collected on Formvar-coated 1 mm slot grids. The sections were then stained with lead citrate before viewing on a Phillips CM10 transmission electron microscope. Negatives were digitized using an Agfa Duoscan scanner and manipulated in Corel Draw 8.0 as below.

Image capture and manipulation. Slides were subsequently examined at the light microscope level using a Nikon E600 microscope equipped with epifluorescence and captured directly from the slide using an Acquis Image Capture System (Synoptics, Cambridge, UK). Images were ma-
nippedulate in CorelDraw 8 to adjust gamma, brightness, and contrast to the desired levels.

Electrophysiology. To test whether activation of the P2X,R could influence neuronal activity in the CNS, we performed whole-cell patch-clamp recordings from neurons in the intermediolateral cell column (IML) of the spinal cord. Wistar rats aged 10–15 d were anesthetized with urethane (2 gm/kg, i.p.). The upper to middle thoracic spinal cord was exposed, isolated, and placed in ice-cold sucrose–acSF containing (in mM) sucrose (217); NaHCO3 (26); KCl (3); MgSO4 (2); NaH2PO4 (2.5); CaCl2 (1); and glucose (10) equilibrated with 95% O2 and 5% CO2. The dura and pia mater were removed, and the spinal cord was immersed in warm agar and placed on ice for rapid setting. Thin transverse slices (250 μm) of the embedded spinal cord were cut on a vibriscope (Campden Instruments, Sileby, UK) and placed in the recording chamber or a holding chamber for later use. The sections were submerged in aCSF [composition (in mM): NaCl (124); NaHCO3 (26); KCl (3); MgSO4 (2); NaH2PO4 (2.5); CaCl2 (2); glucose (10)] and superfused at a rate of 3–5 ml/min. All experiments were performed at room temperature. Visualized patch-clamp recordings were performed using an upright microscope (model BX50WI; Olympus, Tokyo, Japan). The IML was located at 10× magnification, and the cells were visualized at 60× magnification for recording. Patch electrodes (tip diameter, 3 μm; resistance, 4–6 MΩ) were filled with K-gluc (130 mM) K(+), EGTA (10 mM); MgCl2 (2); CaCl2 (1); HEPES (10) Na2GTP (0.3), and Na2ATP (5) at pH 7.2–7.5. Neurobiotin (0.5%) was included in the patch solution and diffused into the neuron during recording. Neurons within and immediately adjacent to the IML were targeted, and whole-cell patch-clamp recordings were obtained using standard techniques. All recordings were performed in current-clamp mode using an Axopatch 1D (Axon Instruments, Foster City, CA).

To characterize the neurons electrophysiologically as sympathetic preganglionic neurons or interneurons, rectangular hyperpolarizing and depolarizing current pulses (1 sec duration, +100 to −130 pA) were applied to the neuron at a holding potential of ~−60 mV, and the changes in voltage were recorded. The shape of the action potential was also noted, as was the duration of the action potential and amplitude of the afterhyperpolarization. Neurons were then held at a resting membrane potential of ~−60 mV, and a small square wave depolarizing current pulse was applied (+10 to +30 pA) every 5–10 sec to monitor input resistance of the neuron. Drugs were applied to the bathing medium in the following final concentrations [agonist, 2−; and 3′,3′-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate 30 μM (BzATP, Sigma); antagonists, oxidized ATP, 100 μM (Sigma) and Brilliant Blue G 2 μM (Sigma)]. We used BzATP as the agonist because it is several-fold more effective than ATP at recombinant P2X7 receptors (North and Foster, 1995, 1996). Because the P2X7 receptor has been reported to open in the presence of drugs. After washout of the BzATP and AP-5, BzATP was reapplied on its own 1.5 hr after the first application.

Visualization of nerve terminal destaining at the neuromuscular junction. Eight- to ten-week-old C57BL/6 mice were killed by inhalation of CO2. Preparation steps were identical to those described for the electrophysiology earlier and maintained in oxygenated aCSF in glass vials at both room temperature and 35°C. The fluorescent dyes YO-PRO-1 and carboxyfluorescein (40 μM; MW 376; Molecular Probes) or carboxyfluorescein (40 μM; MW 376; Molecular Probes) were added to the solutions previous or simultaneous to addition of BzATP (30 μM). In some cases, oxidized ATP (100 μM) was present before BzATP was added. In other cases the excitatory amino acid receptor antagonists NBQX (20 μM) and AP-5 (50 μM) were also added to the solution. As controls the fluorescent dyes were added in the absence of all drugs or in the presence of antagonists only. Slices were incubated in reaction solution for intervals between 10 and 60 min before being fixed in 4% paraformaldehyde for 60 min, resectioned at 50 μM using a vibrating microtome, and examined using filter sets appropriate to the fluorescent dye used. Similar experiments using YO-PRO-1 and carboxyfluorescein were also performed in a nerve–muscle preparation as described for the destaining experiments above.

RESULTS

mRNA encoding for the P2X7 receptor is present in neurons in the CNS

RT-PCR analysis revealed the presence of P2X7 transcripts in spinal cord and medulla RNA (Fig. 1, lanes 1, 2). No amplified products were detected when using water or RNA as template (lanes 3, 4, 5, 8). Veracity of the amplified products was confirmed by cloning the PCR product into pGEM T-Easy followed by DNA sequencing. As a positive control, the PCR products for the housekeeping gene hprt were also detected in both tissues (lanes 6, 7). Products resulting from a 1 kb molecular weight marker are indicated in lane M, and the bands correspond to the calculated size for all PCR products.
reaction. However, we performed in situ hybridization to detect P2X7 mRNA, and this revealed that expression was present in the cytoplasm of neurons throughout the medulla oblongata and spinal cord (Fig. 2). A positive reaction was observed only in sections hybridized with antisense and not in sections incubated with a sense probe or without a probe.

**P2X7 receptor immunoreactivity is targeted to presynaptic terminals in the CNS and at the neuromuscular junction**

Because in situ hybridization revealed neuronal expression of the P2X7 receptor, we examined the cellular localization using a commercially available antibody directed toward an intracellular portion of the receptor. Evidence that the primary antibody was specific to the P2X7 receptor was obtained from Western blotting, in which blotting of rat brainstem resulted in a major band at ~69 kDa (Fig. 3). This corresponds to the expected molecular weight for P2X7 (Surprenant et al., 1996). Furthermore, preabsorption of the antiserum with the peptide antigen abolished staining of the membrane. This specificity appeared to be retained in tissue sections because in control sections where the primary antibody had been omitted or preabsorbed with peptide antigen, there was no staining. Furthermore, the pattern of labeling was distinct to that obtained with antibodies to the P2X subunits P2X2 (Atkinson et al., 2000), P2X1, and P2X4 (unpublished data). Immunoreactivity for the P2X7 receptor was observed in punctate structures throughout the medulla oblongata and the spinal cord with both fluorescence and transmitted light microscopy (Fig. 4). The immunoreactivity was visible in all parts of the medulla and spinal cord examined, and there was no obvious preferential localization to particular nuclei (Fig. 4). Immunoreactive structures appeared to outline the somata and dendrites of neurons (Fig. 4). Because it was not possible at the light microscopic level to determine whether these structures were glial or neuronal processes, we examined the tissue at the ultrastructural level. This electron microscopy revealed that P2X7 receptor immunoreactivity was concentrated in neuronal synaptic terminals presynaptic to other neuronal structures in both the medulla oblongata and spinal cord (Fig. 5A,B). Synapses were characterized by the presence of clear vesicles clustered toward the presynaptic active zone, rigid apposition of presynaptic and postsynaptic membranes, and an asymmetric type (Gray’s type I) thickening of the postsynaptic membrane that was often associated with subjunctional bodies (Fig. 5A,B). Despite examining >230 terminals in the medulla oblongata and spinal cord, we did not find any P2X7-immunoreactive terminals associated with obvious symmetric (Gray’s type II) synapses. In addition, the P2X7 receptor was not colocalized with GABA, GAD, or the GABA vesicle transporter (vGAT) when tissue was double-stained for both antigens (data not shown). In accordance with the receptors being incorporated into the membrane, reaction product was often observed adjacent to the membrane (Fig. 5A,B,D,E). In addition, reaction product was present in the cytoplasm of the terminals (Fig. 5A–E), concordant with trafficking of the receptor to its final site or internalization after

**Figure 2. In situ hybridization reveals that messenger RNA coding for the P2X7 receptor is present in neurons throughout the spinal cord and medulla oblongata.**

**A.** Spinal cord section indicating positive signal after hybridization with a DIG-labeled antisense probe specific to the P2X7 receptor, visualized with alkaline phosphatase. The signal is present throughout the gray matter and is easily seen in the large motoneurons of the ventral horn (VH) as well as in dorsal horn (DH) neurons. The white matter (WM) is sparsely labeled. **B.** Spinal cord section indicating lack of signal when tissue was incubated with a sense probe to the P2X7 receptor. **C.** Medulla oblongata section indicating the widespread positive signal obtained after hybridization with the DIG-labeled antisense probe specific to the P2X7 receptor. Positive signal is visible in labeled neurons throughout the medulla. **D.** Medulla oblongata section indicating lack of signal when tissue was incubated with a sense probe to the P2X7 receptor. **E.** Larger magnification of the dorsal vagal complex of the medulla oblongata, indicating that hybridization reaction product can be observed in neuronal structures (arrows). **F.** Larger magnification of the ventral horn of the spinal cord indicating that hybridization reaction product can be observed in the cytoplasm of large ventral horn motoneurons (arrows). **G.** Neurons (arrow) in the dorsal horn of the spinal cord also contain hybridization reaction product in their cytoplasm.

**Figure 3. Western blotting indicates the tissue specificity of the antibody to P2X7 receptors.** Western blotting of rat brainstem resulted in staining of a major band running at ~69 kDa when detected with the anti-P2X7 receptor antibody. This is the predicted molecular weight of the P2X7 receptor. Preabsorption of the antiserum with the cognate peptide abolished staining of the membrane.
agonist stimulation as observed in neurons transfected with GFP-tagged P2X$_2$R (Li et al., 2000) or GFP-tagged P2X$_2$-R (Khakh et al., 2001).

Because synapses that exhibit asymmetric type morphology are considered to be excitatory in nature, we first selected terminals that we could be certain were excitatory by labeling anterogradely the central projections of vagal afferent fibers with the anterograde tracer biotinylated dextran amine. Using a procedure we have shown to be effective in localizing the P2X$_2$ receptor to vagal afferent terminals (Atkinson et al., 2000), the tracer was detected with DAB to yield an electron-dense amorphous reaction product, whereas P2X$_2$ receptor immunoreactivity was visualized by silver intensifying a gold-conjugated secondary antibody. Electron microscopic examination revealed silver deposits indicating P2X$_2$ receptor immunoreactivity in DAB-labeled myelinated fibers and terminals of central projections of vagal afferent neurons.
(n = 10) (Fig. 5D,E), indicating that the receptor is present in excitatory terminals in the CNS. Consistent with expression of the P2X7 receptor in vagal sensory nerve terminals, we detected P2X7 receptor transcripts in nodose ganglia extracts (Fig. 5F).

We did not find any P2X7 receptor immunoreactivity associated with the plasma membrane of neuronal somata or dendrites, suggesting that it is not functionally targeted to the postsynaptic membrane. However, because in situ hybridization indicated the presence of the P2X7 receptor in ventral horn motoneurons, we tested the neuromuscular junction for the presence of P2X7 receptor immunoreactivity (Fig. 4C). Immunofluorescence microscopy indicated that punctate P2X7 receptor immunoreactivity was localized to the neuromuscular junctions in neonatal and adult skeletal muscle (Fig. 4C). This immunoreactivity was completely blocked by preincubation with the control P2X7 antigen, Electron microscopy confirmed that the P2X7 immunoreactivity was located exclusively presynaptically and apparently restricted to the presynaptic face of labeled terminals (Fig. 5C). Notably, we found no immunoreactivity in motor nerve axons or terminal Schwann cells, but it was present in myelinating Schwann cells (data not shown). Identical preparations labeled with P2X1, P2X2, and P2X4 antibodies (all from Alomone Labs) resulted in no labeling at the neuromuscular junction.

**Activation of the P2X7 receptor results in excitation of CNS neurons via release of glutamate**

Because anatomical methods indicated the presence of the P2X7 receptor in CNS, we performed electrophysiological studies to determine whether activation of the receptor could affect neuronal activity. BzATP (30 μM) was bath-applied to 18 sympathetic preganglionic neurons recorded in the whole-cell patch-clamp configuration in spinal cord slices. After a delay for the drug to reach the tissue, there was a large depolarization of the neurons that often reached the threshold for action potential generation (Fig. 6A). Depolarization was sometimes so pronounced that it resulted in spike accommodation, which was maintained during the presence of the agonist (Fig. 6A). Because of the reported long preincubations required for antagonists to exhibit their effects (Jiang et al., 2000; North and Surpreman, 2000), we tested for antagonism by preincubating slices for 30 min in the P2X7 receptor antagonists oxidized ATP (100 μM; n = 5) or Brilliant Blue G (2 μM; n = 6) and then adding BzATP. There was no response to BzATP in the presence of either antagonist. BzATP has been reported to act also on P2X1 and P2X3 receptors (Bianchi et al., 1999), and this can be reversibly inhibited by oxidized ATP (Evans et al., 1995). However, because oxidized ATP irreversibly inhibits the P2X7 receptor (North and Surpreman, 2000), we reapplied BzATP 90 min after the first application and washout of oxidized ATP and observed no excitatory response (n = 3). On occasion, a reduction in firing rate of the recorded neuron was observed when BzATP was applied in the presence of antagonists (Fig. 6B). This may be because of unknown actions of BzATP or possible breakdown products on other receptors such as P2YR. Nevertheless, these data indicate that the receptor activated by BzATP that contributes to the excitatory response in these experiments is the P2X7 receptor.

Because anatomical studies indicated that the receptor was present presynaptically in excitatory terminals, we tested the hypothesis that the observed excitation was attributable to the release of glutamate. We therefore pharmacologically antagonized ionotropic glutamate receptors by incubating slices in NBQX (20 μM) and AP-5 (50 μM) before application of BzATP.

**Figure 6. Activation of the P2X7 receptor in CNS slices elicits depolarizations in neurons attributable to release of glutamate. A. The P2X7 receptor agonist BzATP depolarizes neurons. In this example the cell was at a potential of −60 mV, and a depolarizing current of +20 pA. 1 sec duration was applied every 7 sec, which on occasion caused the neuron to reach the threshold for firing (shown on a faster time base below). Application of BzATP (30 μM) depolarized the neuron, resulting in discharge of action potentials and eventual depolarizing block. Recovery after washout is shown in the inset. B. The effects of BzATP were antagonized by appropriate antagonists. When preincubated in the P2X7 receptor antagonist Brilliant Blue G (2 μM) for 30 min, BzATP failed to depolarize neurons. This neuron was also held at −60 mV, and depolarizing current pulses of +30 pA were applied C. BzATP-evoked depolarizations were blocked by excitatory amino acid receptor antagonists. Superfusion of the non-NMDA receptor antagonist NBQX (20 μM) and the NMDA receptor antagonist AP-5 (50 μM) prevented BzATP (30 μM)-evoked depolarization even after prolonged application (C1). After 90 min washout, application of BzATP caused a large depolarization and increase in firing rate that again resulted in depolarizing block. This neuron was held at −60 mV with current pulses of +15 pA applied every 7 sec.**
(Fig. 6C). When these antagonists were present, BzATP did not produce a response in the recorded neuron \( (n = 3) \) (Fig. 6Ci). However, when the antagonists were washed out and slices were allowed to recover from BzATP application for >90 min, a further application of BzATP elicited a characteristic response \( (n = 2) \) (Fig. 6Cii). These results indicate that BzATP causes release of glutamate, which excites recorded neurons.

**Activation of the P2X\(_7\) receptor promotes transmitter release at the neuromuscular junction**

Taken together, our electrophysiological results are consistent with the anatomy and suggest an enhancement of release of transmitter through vesicular release. However, activation of the P2X\(_7\) receptor has been suggested to interfere with glutamate uptake processes by transporters in Muller cells of the retina (Pannicke et al., 2000), and such reduced glutamate uptake could contribute to excitation in neurons, as observed in our experiments. We therefore tested whether activation of the P2X\(_7\) receptor can result in vesicular release by direct visualization of vesicle destaining at the neuromuscular junction (Figs. 7, 8). We performed experiments in which motor nerve terminals were loaded with vital styryl dyes, which results in labeling of actively recycling vesicles. Destaining of nerve terminals is taken as evidence for vesicle exocytosis, which reflects release of neurotransmitter (Cochilla et al., 1999). In control preparations only a minimal reduction in fluorescence was observed over the 30 min visualization period (89 ± 4%; \( n = 2 \)), which is attributed to photobleaching and spontaneous release of neurotransmitter (Figs. 7, 8). In the presence of BzATP (30 \( \mu \)M), nerve terminals destained with a sigmoidal time course to 48 ± 2% \( (n = 6) \) of their original value over the same time period (Figs. 7, 8). This is significantly different from the control preparation \( (p < 0.005; \text{Student's} t\text{ test}) \). When terminals were preincubated in the P2X\(_7\) receptor blockers, oxidized ATP (100 \( \mu \)M) or Brilliant Blue G (1 \( \mu \)M) terminal brightness was only reduced to 85 ± 3% \( (n = 4) \) and 85 ± 4% \( (n = 5) \), respectively (Fig. 8). These data are significantly different from the BzATP-treated preparations \( (p < 0.05; \text{Student's} t\text{ test}) \) and indicate that destaining, and hence agonist-induced neurotransmitter release had been prevented. Notably, we were able to retain terminals with styryl dyes subsequent to BzATP-driven destaining, suggesting that the nerve terminals had not become damaged by the treatment.

**The P2X\(_7\) receptor does not undergo large pore formation in our experimental conditions**

Several studies demonstrate that the activation of P2X\(_7\) receptors results in the opening of a nonselective large pore permeable to molecules up to 900 Da (Steinberg and Silverstein, 1989; Suprenant et al., 1996). We therefore sought to determine whether our observed responses were caused by opening of the selectively permeable channel or the nonselective large pore. However, we were unable to demonstrate loading of terminals with YO-PRO-1 in either the neuromuscular junction or in CNS slices. We did observe YO-PRO-1 uptake by neuronal cell bodies in CNS slices, but these were also present in control tissue without agonist present and were assumed to be attributable to cell injury during tissue preparation. Because YO-PRO-1 is fluorescent only when it binds to nucleic acids, there may be insufficient nucleic acids in the terminals to make it visible. We therefore repeated the same experiments using 6-carboxyfluorescein (MW 376; 40 \( \mu \)M). We again did not find any staining in terminals, and the low numbers of stained neurons were similar to those observed in control experiments. We therefore suggest that activation of the P2X\(_7\) receptor present in presynaptic terminals does not cause large pore formation under these experimental conditions in which the large pore might be blocked by the relatively high levels of divalent cations present (Virginio et al., 1997; Michel et al., 1999).

**DISCUSSION**

Ionotropic ATP receptors P2X\(_{1-6}\) participate in fast excitatory neurotransmission in the CNS, but to date the P2X\(_7\) receptor (P2X\(_7\)-R) has been excluded from an involvement in synaptic transmission. Here we show that the P2X\(_7\)-R has a remarkably widespread distribution throughout the brainstem and spinal cord where it is targeted to excitatory presynaptic terminals of neu-
The P2X7R is functionally targeted to excitatory presynaptic terminals. These data indicate that the native receptor exists in homomeric form. Furthermore, the P2X7R does not form heteromers with other P2X receptors in expression systems (Torres et al., 1999), and so it is likely that the native receptor exists in homomeric form.

The P2X7-R is considerably more prevalent at presynaptic terminals than other P2XR in the CNS. Indeed, although other P2XR are present presynaptically and postsynaptically at restricted sites in the CNS (Collo et al., 1996; Vulchanova et al., 1997; Le et al., 1998; Llewellyn-Smith and Burnstock, 1998; Loesch and Burnstock, 1998; Atkinson et al., 2000), we can only detect the P2X7 receptor in presynaptic terminals. In addition, our preliminary experiments and those of Armstrong and MacVicar (2000) indicate that the P2X7-R is also present in excitatory presynaptic terminals in forebrain regions such as the hippocampus. Furthermore, this is the first P2XR to be conclusively identified as involved in presynaptic release because of the presence of selective agonists and antagonists for this receptor. Furthermore, the P2X7-R does not form heteromers with other P2X receptors in expression systems (Torres et al., 1999), and so it is likely that the native receptor exists in homomeric form.

The widespread distribution of the P2X7-R receptor suggests a fundamental role for the P2X7-R at synapses. One possible function could be as an autoreceptor, mediating positive feedback from terminals because ATP can be coreleased with other neurotransmitters (Edwards et al., 1997). At the NMJ, ATP is present in vesicles in motor nerve terminals with acetylcholine and is released into the synaptic cleft during nerve stimulation, where it can reach an estimated concentration (at least transiently) of 30–300 μM (Silinsky et al., 1990; Smith, 1991; Ribeiro et al., 1996), well within the EC50 value of 100 μM for ATP at the rat P2X7-R (North and Surprenant, 2000). However, BzATP has been shown to act at recombinant P2X7-R with greater potency than P2X7-R, and at P2X7-R with similar potency (Bianchi et al., 1999). Nevertheless, neuronal P2X7-R or P2X7-R have not been reported in neurons in the region of the spinal cord where we conducted our electrophysiological recordings or at the NMJ. Furthermore, the BzATP responses were irreversibly blocked by oxidized ATP. Although oxidized ATP acts on P2X7 and P2X2 receptors, the actions on these receptors are reversible (Evans et al., 1995), whereas the action of oxidized ATP at the P2X7-R is irreversible (North and Surprenant, 2000). We were unable to repeat our responses after washout of oxidized ATP, consistent with an action on P2X7-R. In addition, Brilliant Blue G, recently reported as a selective P2X7-R antagonist (Jiang et al., 2000), also blocked the response to BzATP. Therefore it is likely that we have reported the results of activation of the P2X7-R.

**Implications for synaptic transmission**

Although P2X receptors have been reported to enhance or directly mediate transmitter release from presynaptic terminals (Gu and MacDermott, 1997; Khakh and Henderson, 1998; Boehm, 1999), the lack of specific agonists and antagonists has hindered identification of the particular subtypes responsible. Our findings indicate that the P2X7-R plays a similar role at the presynaptic terminal. However, this is the first P2XR to be conclusively identified as involved in presynaptic release because of the presence of selective agonists and antagonists for this receptor. Furthermore, the P2X7-R does not form heteromers with other P2X receptors in expression systems (Torres et al., 1999), and so it is likely that the native receptor exists in homomeric form. 

The P2X7-R is considerably more prevalent at presynaptic terminals than other P2XR in the CNS. Indeed, although other P2XR are present presynaptically and postsynaptically at restricted sites in the CNS (Collo et al., 1996; Vulchanova et al., 1997; Le et al., 1998; Llewellyn-Smith and Burnstock, 1998; Loesch and Burnstock, 1998; Atkinson et al., 2000), we can only detect the P2X7 receptor in presynaptic terminals. In addition, our preliminary experiments and those of Armstrong and MacVicar (2000) indicate that the P2X7-R is also present in excitatory presynaptic terminals in forebrain regions such as the hippocampus. Furthermore, this is the first P2 receptor to be identified at the neuromuscular junction, although ATP and its metabolites ADP and adenosine have long been known to affect synaptic transmission at the NMJ (Fu and Poo, 1991). Recently, ATP was shown to modulate neurotransmitter release from motor nerve terminals, but this was suggested to occur via an interaction with presynaptic nicotinic receptors (Salgado et al., 2000). In this light it is interesting to note that P2X7 and a nicotinic receptor inhibit one another when coactivated (Khakh et al., 2000), and because we could not detect P2X7 at the NMJ there is the possibility that P2X7 may interact in a similar manner with presynaptic nicotinic receptors at this site.

The widespread distribution of the P2X7-R receptor to excitatory synaptic terminals suggests a fundamental role for the P2X7-R at synapses. One possible function could be as an autoreceptor, mediating positive feedback from terminals because ATP can be coreleased with other neurotransmitters (Edwards et al., 1997). At the NMJ, ATP is present in vesicles in motor nerve terminals with acetylcholine and is released into the synaptic cleft during nerve stimulation, where it can reach an estimated concentration (at least transiently) of 30–300 μM (Silinsky et al., 1990; Smith, 1991; Ribeiro et al., 1996), well within the EC50 value of 100 μM for ATP at the rat P2X7-R (North and Surprenant, 2000). However, BzATP has been shown to act at recombinant P2X7-R with greater potency than P2X7-R, and at P2X7-R with similar potency (Bianchi et al., 1999). Nevertheless, neuronal P2X7-R or P2X7-R have not been reported in neurons in the region of the spinal cord where we conducted our electrophysiological recordings or at the NMJ. Furthermore, the BzATP responses were irreversibly blocked by oxidized ATP. Although oxidized ATP acts on P2X7 and P2X2 receptors, the actions on these receptors are reversible (Evans et al., 1995), whereas the action of oxidized ATP at the P2X7-R is irreversible (North and Surprenant, 2000). We were unable to repeat our responses after washout of oxidized ATP, consistent with an action on P2X7-R. In addition, Brilliant Blue G, recently reported as a selective P2X7-R antagonist (Jiang et al., 2000), also blocked the response to BzATP. Therefore it is likely that we have reported the results of activation of the P2X7-R.

**Identification of the presynaptic P2X receptor as the P2X7 receptor**

Because the P2X7-R has previously been reported as absent from normal adult rat brain (Collo et al., 1997), we combined several approaches to ensure that we were indeed studying the P2X7R. First, RT-PCR revealed the presence of messenger RNA for the receptor in CNS tissue. However, because a positive PCR reaction could be caused by glial cell expression, we performed in situ hybridization to determine the identity of the cells expressing the receptor. This procedure revealed widespread neuronal expression of the receptor in the medulla oblongata and spinal cord. Second, immunohistochemical procedures revealed specific immunoreactivity at the NMJ and in the medulla oblongata and spinal cord. The antibody used is generated against residues 576–595 of rat P2X7-R, a sequence not shared by any other known protein, as indicated by a BLAST database search. In addition, the C terminus of the rat P2X7-R is ~200 amino acids longer than other known P2X-R and is not present in other known P2X-R (Surprenant et al., 1996). Furthermore, Western blotting resulted in a band at the appropriate molecular weight that was abolished by preadsorption of the antibody with the antigenic peptide. These data suggest that the antibody specifically recognizes the rat P2X7-R. Third, our electrophysiological and destaining experiments are consistent with the activated receptor being the P2X7-R. We used BzATP as the agonist because it is several-fold more effective than ATP at recombinant P2X7-R (North and Surprenant, 2000). However, BzATP has been shown to act at...
2000). The prevalence of the P2X₇R and the possibility that synaptically released ATP can reach sufficient levels to activate the receptor suggest that the P2X₇R plays a key role in synaptic transmission.

A role for the P2X₇ receptor in neuroopathological conditions?

Another possible role for the neuronal P2X₇-R is that it is activated in response to injury. In keeping with this, activation of the P2X₇-R evokes glutamate release, and such release is the basis of excitotoxicity (Doble, 1999). ATP is released by neurons that are damaged or under certain neuropathological conditions such as ischemia and axonopathy (Dubyak and el Moattassim, 1993; Juranyi et al., 1999). Indeed, because ionic gradients collapse under anoxia and ischemia, resulting in increases in extracellular potassium levels but decreases in sodium, chloride, and calcium concentrations (Morris and Trippenbach, 1993; Xie et al., 1995; Vorisek and Sykova, 1997), P2X₇ receptor function might be expected to be enhanced (Virginio et al., 1997). ATP release may therefore coincide with an environment favoring activation of the P2X₇-R. It is evident that such conditions can activate the P2X₇-R because the P2X₇-R in the brain is upregulated in tissue surrounding a necrotic region (Collo et al., 1997), and microglial cell lines contain the receptor (Ferrari et al., 1997).

After injury and associated ATP release, the neuronal P2X₇-R may undergo large pore formation, as observed when the P2X₇ receptor is exposed to agonists for prolonged periods (Steinberg and Silverstein, 1989; Ballerini et al., 1996; Surprenant et al., 1996; Chessell et al., 1997; Ferrari et al., 1997; Rassendren et al., 1997). Such large pore formation by the P2X₇-R has been associated with a cytolytic effect (Virginio et al., 1999). We could find no evidence for large pore formation in presynaptic terminals, but there are several reasons why this might have been the case. Within the CNS slices it is possible that the high molecular weight fluorescent dyes do not gain access to the terminals, and therefore uptake cannot be observed. However, this limitation is not applicable to the neuromuscular junction where accessibility is indicated by the uptake of styril dyes into presynaptic terminals. Another possibility is that the amount of dye entering the terminals does not provide sufficient signal for visualization. We cannot discount this possibility but note that it is possible to visualize styril dyes in terminals. Nevertheless, the lack of large pore formation is consistent with findings when the rat P2X₇ receptor is expressed in oocytes (Petrout et al., 1997) and for the P2X₇ receptor in Muller glial cells of the human retina (Pannicke et al., 2000). Possibly the explanation lies in the relatively high concentrations of extracellular calcium and magnesium ions in our experiments because the large pore is more likely to open in low concentrations of divalent cations (Surprenant et al., 1996).

In conclusion, we show here that the P2X₇-R is expressed by neurons, is targeted to presynaptic terminals that are excitative, and that activation of this receptor in the CNS and PNS elicits transmitter release, which is likely to be glutamate in the CNS. Considering the well-established roles for glutamate in excitation and the activation of the P2X₇ receptor in response to injury, the P2X₇ receptor may represent a new therapeutic target to reduce cell death in times of stress.

REFERENCES

Deuchars J, Atkinson, L, Batten TFC, Deuchars SA Evidence that the P2X7 receptor is targeted to presynaptic terminals in the brainstem and spinal cord of rats. J Physiol (Lond) 520P, 167P. 2000.
Knutson PM, Deuchars, J, Parson SH (2000) Immunocytochemical evidence that the P2X7 receptor is present in mammalian motor nerve terminals. J Physiol (Lond) 526:60.


