Cellular/Molecular

Reanalysis of $P2X_7$ Receptor Expression in Rodent Brain

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P2X receptors are cationic-selective ion channels gated by extracellular ATP. There are seven subunits ($P2X_{1-7}$), the first six of which are expressed throughout the peripheral and central nervous systems. $P2X_7$ receptors are rapidly upregulated and activated as a result of inflammatory stimuli in immune cells, where they act not only as cationic channels but uniquely couple with rapid release of proinflammatory cytokines, cytoskeletal rearrangements, and apoptosis or necrotic cell death. The $P2X_7$ receptor has been termed the cytolytic non-neuronal P2X receptor because it had not been detected in neurons until recently when it has been immunolocalized to several brain regions, particularly the hippocampus, and has been suggested to be involved in presynaptic modulation of transmitter release. Because its expression in brain neurons may have substantial functional implications, we have performed detailed immunocytochemical, immunoblot, and immunoprecipitation studies on brain and non-neuronal tissue using all currently available antibodies. We first examined rats, but staining patterns were inconsistent among antibodies; we therefore studied mice for which there are two $P2X_7$ knock-out mice constructs available, one expressing the LacZ transgene. We found that $P2X_7$ receptor protein is strongly and reliably detected in the submandibular gland and lung of wild-type mice but not in either of the $P2X_7{}^{-/-}$ mice. However, we failed to find evidence for $P2X_7$ receptor protein in hippocampal neurons or their input–output projections. Either the $P2X_7$ protein in the hippocampus is below the limits of detection by the currently available methods or it is not present.

Key words: **purinergic receptors; hippocampal neurons; immunocytochemistry; ligand-gated ion channels; transgenic animals; presynaptic inhibition**

Introduction

$P2X_7$ receptors are unique ion channels; they have been studied extensively in monocytes and macrophage, including brain microglia. In these cells, their activation not only opens cationic channels and larger dye-permeable pores but also engages diverse signal cascades associated with inflammatory states. These include initiation of a rapid release and processing of the proinflammatory cytokines interleukin-1β and caspase-1, induction of rapid membrane blebbing and cytoskeletal rearrangements, activation of proapoptotic transcription factors and, depending on the cell background and duration of stimulus, apoptotic or necrotic cell death (Di Virgilio et al., 2001; Mackenzie et al., 2001; Suh et al., 2001; Le Feuvre et al., 2002, 2003; Guerra et al., 2003; Rothwell, 2003; Verhoef et al., 2003; Pfeiffer et al., 2004). These receptors have excited much interest in immune cell biology and drug development research as a promising target to treat inflammatory diseases, particularly arthritis, and the after-effects associated with cerebral ischemic attacks (Alcaraz et al., 2003; Baxter et al., 2003; Guerra et al., 2003; Rothwell, 2003). Much of this therapeutic interest stems from the notion that $P2X_7$ receptors are relatively specifically expressed in cells of the immune system, where they are rapidly upregulated and activated after initial inflammatory stimuli.

Initial studies showed mRNA for the $P2X_7$ receptor in many immune, endothelial, and epithelial cells but not in peripheral or central neurons; in the brain, significant mRNA expression was observed only in ependymal cells lining the ventricles and in activated microglia from ischemic rat and mouse brain (Collo et al., 1997). More recently, protein expression studies revealed similar non-neuronal localizations, but these have also shown immunostaining in many brain neurons, at both presynaptic and postsynaptic densities (Deuchars et al., 2001; Armstrong et al., 2002; Atkinson et al., 2002, 2004; Lundy et al., 2002; Sperlagh et al., 2002; Ishii et al., 2003; Cavaliere et al., 2004). However, immunostaining patterns in neurons have been inconsistent (Deuchars et al., 2001; Armstrong et al., 2002; Atkinson et al., 2002; Sperlagh et al., 2002; Ishii et al., 2003; Lundy et al., 2003; Atkinson et al., 2004). Although the panopoly of consequences of activation of this ion channel may well be functionally relevant in the immune system, it is difficult to see a role for $P2X_7$ receptors on neurons. Given the limitations to available antibodies (Abs), as well as the difficulty in interpreting functional studies without adequate antagonists, this prompted the need to reexamine the expression of the receptor on mammalian neurons.

In the present study, we sought to determine the protein expression pattern of the $P2X_7$ receptor in adult rodent brain and to compare this with results from parallel experiments on submandibular gland and lung. We examined most areas of the brain but focused our studies on the hippocampus because this is the region where most of the current evidence for the functional ex-

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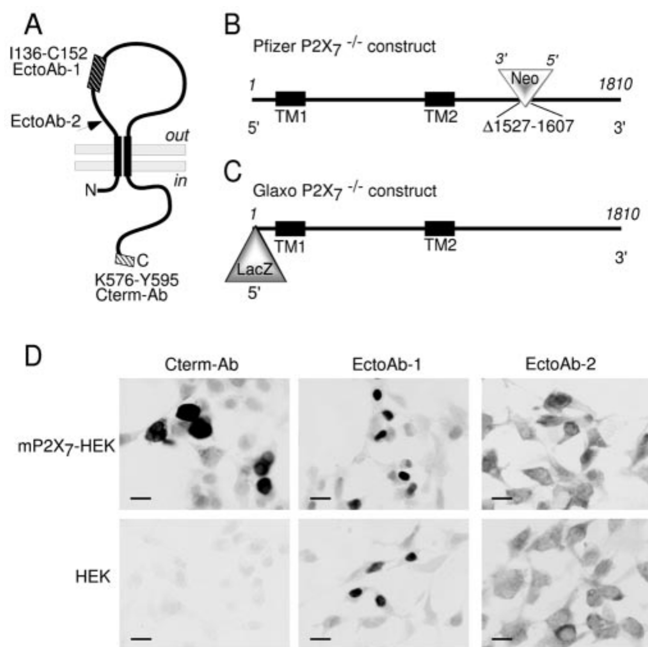

Figure 1. $P2X_7$ Abs and $P2X_7$ receptor knock-out mice constructs. *A*, $P2X_7$ receptor subunit topology and the corresponding positions against which the various Abs have been raised. The construct of the Pfizer $P2X_7^{-/-}$ (*B*) has nucleotides 1527–1607 deleted and a neomycin cassette inserted 5′ to 3′, whereas the construct of the Glaxo$P2X_7^{-/-}$ mice (*C*) had a *LacZ* gene inserted after ATG at the 5′ end. *D* shows typical immunostaining for each of the three Abs in HEK293 cells transiently transfected with m$P2X_7$ cDNA (top micrographs) or untransfected cells (bottom micrographs). The Cterm-Ab was highly selective in HEK cells; the ectoAb-1 nonspecifically stained nuclei, but the cytoplasmic/plasma membrane staining was only seen in transfected cells, and the ectoAb-2 staining did not show any obvious selectivity. In separate experiments with enhanced green fluorescent protein (GFP) cotransfection, only GFP-positive cells were immunopositive with the Cterm-Ab, thus further supporting its selectivity.

pression of the $P2X_7$ receptor has been obtained (Deuchars et al., 2001; Armstrong et al., 2002; Lundy et al., 2002; Sperlagh et al., 2002; Atkinson et al., 2004). We performed immunocytochemistry, Western blotting, and immunoprecipitation experiments using three separate Abs directed at different epitopes of the $P2X_7$ protein and on two sets of $P2X_7$ receptor knock-out mice generated using different strategies. One of these incorporated a LacZ insertion into the *$P2X_7$* gene, which allowed determination of β-galactosidase protein expression of the transgene. We can find no evidence for protein expression of $P2X_7$ receptors in hippocampal neurons or their input–output projections.

Materials and Methods

Animals used in the present study were male C57BL/6 mice (40–50 d old), $P2X_7$ knock-out mice derived from Pfizer (Groton, CT) and their corresponding wild-type B6D2 (C57B/L6 × DBA/2 F1) and $P2X_7$ knock-out mice derived from GlaxoSmithKline (Harlow, UK) with their C57B/L6 controls. Male Wistar rats (250 gm) were used in initial experiments. All experiments were performed under a United Kingdom Home Office License and in accordance with the regulations of the United Kingdom Animal (Scientific Procedures) Act (1986). Generation of the transgenic mice has been described (Sikora et al., 1999; Solle et al., 2001). Briefly, the Glaxo mice had a *lacZ* gene inserted at the beginning of the exon 1 region of the *$P2X_7$* gene, whereas Pfizer mice were constructed by a deletion in the region containing aa 506–532, followed by an insertion of a neomycin cassette in a 3′ to 5′ direction (Fig. 1). Using DNA isolate from ear tag, mice derived from Pfizer were genotyped as described by Le Feuvre et al. (2002), and mice from Glaxo were genotyped for lacZ insertion in the exon 1 region of $P2X_7$ (I. Chessell, personal communication). All mice used were genotyped immediately before use.

Immunocytochemistry. Adult male mice and rats were anesthetized with intraperitoneal Sagatal (60 mg/kg) and were perfused transcardially with 10 ml of saline containing heparin at room temperature, followed by cold fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. All experiments were performed under a United Kingdom Home Office License and in accordance with the regulations of the United Kingdom Animal (Scientific Procedures) Act (1986). Whole brains were removed and postfixed in the same solution for 2 hr at 4°C. Tissues were then transferred to 30% sucrose in 0.1 M phosphate buffer at 4°C for 2 d. Serial 40 μm sections were cut in cold TBS, pH 7.4, on a Vibratome (Leica, Milton Keynes, UK) and collected in multiwells containing TBS and processed for P2X receptor immunocytochemistry. Lung and submandibular glands were removed and embedded in optimal cutting temperature compound (Bright Instruments, Huntingdon, UK), frozen on dry ice, sectioned at 10 μm, and collected on SuperFrost Plus slides (VWR Scientific, West Chester, PA). In some experiments, brains were removed from nonperfused animals and treated similarly to lung and submandibular glands for comparative studies. The tissues were postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, before being processed for P2X receptor immunocytochemistry.

The immunocytochemistry procedure used in the present study was modified and adapted from those described previously (Skynner et al., 1999). We used two commercially available Abs [Cterm-Ab (epitope 567-595) and ectoAb-1 (epitope 136-152); Alomone, Jerusalem, Israel) and a polyclonal ecto-domain Ab (ectoAb-2; a gift from J. Barden, University of Sydney, Sydney, Australia) (Fig. 1). Free-floating sections and sections on slides were incubated in TBS solution containing 1% H_2O_2 and 40% methanol for 5 min, followed by three washes in TBS. Sections were then incubated for 1 hr at room temperature in preblocking TBS solution (bTBS) comprising of 5% NGS, 0.3% BSA, and 0.3% Triton X-100. Both permeabilized and nonpermeabilized (detergent-free bTBS) conditions were examined with both ecto-domain Abs. The free-floating sections were then incubated in either Cterm-Ab (0.30 μg/ml), ectoAb-1 (0.15 μg/ml), or ectoAb-2 (1:2000 dilution) for 24–48 hr at 4°C. After the primary Ab incubation, sections were rinsed (three washes for 15 min each) in TBS and exposed to goat biotinylated anti-rabbit secondary Ab complex (1:200; Vector Laboratories, Peterborough, UK) for 90 min before a 15 min incubation in HRP streptavidin (Vector Laboratories) at room temperature. The sections were washed again in TBS and finally reacted with the nickel–DAB chromagen using glucose oxidase. Controls consisted of the omission of the primary Abs from the immunostaining protocol and the use of $P2X_7$ Abs preadsorbed with the corresponding peptide (for P2X from Alomone at 5 μg/ml final concentration as instructed by the manufacturer). The free-floating sections were then mounted onto gelatinized glass slides and air dried. All slides were dehydrated in series of ethanol (70, 80, 90, 95, and 100%) and cleared in xylene. Sections were mounted in DPX (BDH Chemicals, Poole, UK) and examined with a BX40 microscope (Olympus, Lake Success, NY). Images were taken with a digital SPOT camera and software (Diagnostic Instruments, Sterling Heights, MI).

Western blotting and immunoprecipitation. HEK293 cells were transiently transfected with mouse $P2X_7$ receptors using Lipofectamine 2000 (Invitrogen, San Diego, CA) according to standard protocols. Animals were killed by excess anesthesia (isoflurane), and their tissues were removed quickly. Whole brain, lung, and submandibular glands were collected from wild-type $P2X_7^{+/+}$, Pfizer wild-type $P2X_7^{+/+}$, Pfizer $P2X_7^{-/-}$, and Glaxo$P2X_7^{-/-}$ mice and placed in cold PBS containing protease inhibitor (Complete; Roche Molecular Biochemicals, Stockholm, Sweden). The Abs used were the same as those for immunohistochemistry. Goat anti-rabbit HRP-conjugated secondary Ab was obtained from DAKO Cytomation. Mouse tissues were homogenized as follows: tissues were cut into small (1 mm^2) pieces and incubated in 3 ml of ice-cold hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, and one Complete tablet per 25 ml) for 10 min. The tissues were homogenized by 20 strokes of a 3 ml Dounce homogenizer, and the NaCl concentration was adjusted to 155 mM. Homogenate was centrifuged at 1000 × *g* for 5 min to pellet intact cells and other debris. Membranes were recovered from the supernatant by centrifugation at 100,000 × *g* for 40 min. The resulting membrane pellet was resuspended in 500 μl of solu-

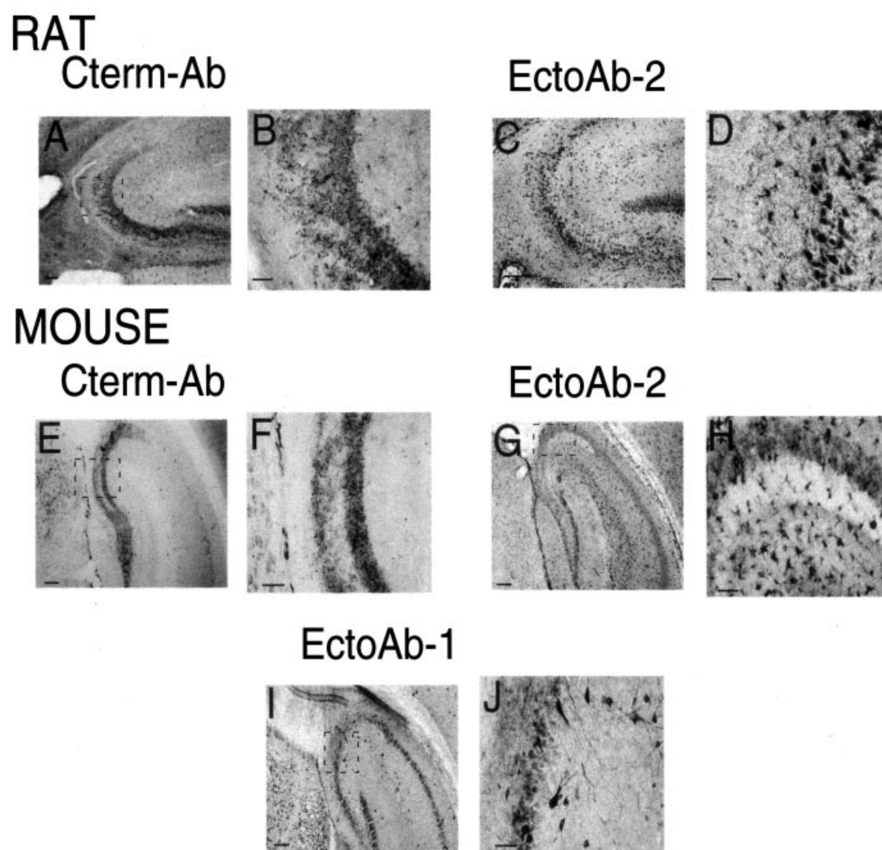

Figure 2. Discrete staining patterns obtained with different $P2X_7$ Abs in wild-type rat and mouse hippocampus. Sagittal views of adult rat and mouse hippocampi are shown at both 10× and 40× magnification for each Ab used and detected with Ni-DAB as described in Materials and Methods. Cterm-Ab revealed punctate staining indicative of mossy terminal input from the dentate gyrus in both rat and mouse (*A*, *B*, *E*, *F*), whereas ectoAb-2 primarily stained astrocytes scattered throughout the hippocampus (*C*, *D*, *G*, *H*). The ectoAb-2 also showed immunopositive neurons in the CA3 region of rat but not mouse (*D*, *H*). The ectoAb-1 stained large "interneuronal-like" cells within the CA3 region (*I*, *J*). Scale bars: *A*, *C*, *E*, *G*, *I*, 100 μm; *B*, *D*, *F*, *H*, *J*, 50 μm.

bilization buffer [20 mM dodecyl-D-maltoside (DM) in PBS plus protease inhibitors] and incubated on a rotating wheel for 16 hr at 4°C. After centrifugation at 13,000 rpm for 10 min in a refrigerated microfuge, the protein content of the solubilized membranes (supernatant) was determined using a protein assay kit (Bio-Rad, Hercules, CA). Samples of solubilized membranes were mixed with an equal volume of SDS loading buffer and boiled for 2 min to denature. Equal amounts of protein from the solubilized membranes were separated by SDS-PAGE on 8% gels and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Total cell lysates were used as positive controls for immunoblotting; these were obtained by lysing 2×10^6 cells in 50 μl of solubilization buffer and denaturing in an equal volume of SDS loading buffer, and 4 μl of this solution was loaded onto gels. The amount of protein loaded from the mouse tissues is indicated in the figure legends. PVDF membranes were rinsed in TBS (10 mM Tris and 250 mM NaCl, pH 7.5) and then blocked with 5% milk in TTBS (TBS plus 0.2% Tween 20) for 1 hr at room temperature. Primary Abs were applied at the appropriate concentration in blocking solution and incubated overnight at 4°C. Membranes were washed three times with TTBS, and a secondary Ab was added in blocking solution for 1 hr at room temperature. Membranes were washed four times in TTBS, and Western Lightning substrate (NEN Life Science, Boston, MA) was applied for 1 min. The blots were then exposed to Kodak Bio-max Light film, and the films were developed using an Optimax developing machine (IGP, Chelmsford, UK). The primary Abs and their dilutions were: Cterm-Ab (1:5000), ectoAb-1 (1:1000), and ectoAb-2 (1:5000). Secondary Ab was used at a 1:2000 dilution.

Immunoprecipitation. Membrane proteins from mouse tissues were solubilized using the same technique as for Western blotting, except that 2% (w/v) Triton X-100 was used instead of DM. Samples of solubilized membrane proteins were made up to 500 μl in PBS containing 2% (w/v) Triton X-100 plus protease inhibitors (IP buffer). Gamma-bind G Sepharose beads (25 μl; Amersham) were added to clear the membrane solubilizates, and the mixtures were tumbled for 3 hr at 4°C. After centrifugation to pellet the beads, supernatants were transferred to fresh tubes, 1 μl of C-terminal Ab (Alomone) was added, and the mixtures were tumbled for 2 hr at 4°C. Gamma-bind G Sepharose beads (25 μl) were added, and the mixtures were incubated overnight at 4°C to allow binding of immune complexes to the beads. After incubation, beads were recovered by centrifugation and washed six times in 0.5 ml of IP buffer. Washed beads were heated in SDS-PAGE sample buffer at 100°C for 5 min to denature and elute bound proteins, and the resultant mixtures were separated by SDS-PAGE and subjected to Western blotting as described above, using the Cterm-Ab. These blots then resulted in strong staining of the Ig heavy chain at ~50 kDa, but this did not interfere with detection of the 75 kDa $P2X_7$ receptor protein band (see Fig. 7*B*).

X-gal staining. Cryostat sections of submandibular glands (10 μm) and brain tissues (12 μm) were cut and collected on SuperFrost Plus slides (VWR). The tissues were postfixed in cold (4°C) PBS containing 0.2% glutaraldehyde, 5 mM EGTA, and 2 mM $MgCl_2$, pH.7.2, for 5 min, washed for 1 min in H_2O, and left to air dry. The sections were then washed in rinse buffer containing 1 mM $MgCl_2$ and 0.1% Triton X-100 in PBS at room temperature. The sections were then immersed in X-gal solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 4 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactosidase made up in rinse buffer) for 4–24 hr at 37°C to reveal transgene-expressing cells in the brain. Sections were counterstained with Neutral Red to highlight the cellular localization of the blue X-gal staining. All sections were mounted with glycerin and viewed on a Axiovert C25 microscope (Zeiss, Welwyn Garden City, UK). Images were taken with an Axiocam camera and Image Associates software (Zeiss).

Results

Immunohistochemistry

Wild-type rat and mouse brain

We used three Abs directed toward separate epitopes of the $P2X_7$ receptor: Cterm-Ab, EctoAb-1, and ectoAb-2 (Fig. 1*A*). These Abs have been used in previously published reports examining $P2X_7$ receptor localization and function in neuronal and non-neuronal tissues (Worthington et al., 1999a,b; Deuchars et al., 2001; Kim et al., 2001; Sluyter et al., 2001; Armstrong et al., 2002; Atkinson et al., 2002, 2004; Le Feuve et al., 2002; Lundy et al., 2002; Sperlagh et al., 2002; Ishii et al., 2003). We found that Cterm-Ab, but not ectoAb-1 or ectoAb-2, were highly selective for $P2X_7$ receptor protein when expressed in HEK293 cells and compared with untransfected (Fig. 1*D*) and $P2X_{1-6}$ receptors individually transfected into these cells (data not shown). Initial studies performed on rat brain with each Ab showed different patterns of $P2X_7$ immunoreactivity ($P2X_7$-IR) depending on the Ab used and the protocols used (see Materials and Methods). In

agreement with previous studies using the Cterm-Ab (Armstrong et al., 2002; Atkinson et al., 2004), we could find $P2X_7$-IR in microglia-like cells and punctate staining in the CA3 region of the hippocampus that was compatible with mossy fiber terminals (Fig. 2*A*,*B*), although other staining patterns, such as neuronal cell body immunoreactivity could also be observed by slightly altering the fixation, incubation protocols, or both. In contrast, ectoAb-2 produced strong staining in astrocytes and neuronal somata in CA3 but no evidence of mossy fiber staining (Fig. 2*C*,*D*). Several different fixation and staining protocols were tried, which again resulted in different patterns of $P2X_7$-IR throughout the hippocampus and other regions of the brain; however, under no condition did we observe a similar pattern of staining by the Cterm and ectoAb Abs. Thus, it was not possible to determine which staining pattern, if any, could be considered a reliable indication of $P2X_7$ receptor protein expression in rat brain.

Therefore, we turned our attention to the mouse model in which $P2X_7$ knock-out mice are available to assess which pattern of immunoreactivity might adequately represent $P2X_7$ receptor protein expression. Staining patterns in wild-type mouse brain were very similar to those obtained in rat brain; these initial results provided us with good evidence that results obtained in mouse brain may also be considered representative for rat central neurons, in which the majority of functional studies have been performed. Strong mossy fiber staining was observed with the Cterm-Ab similar to that observed in rat hippocampus (Fig. 2*E*,*F*). EctoAb-2 stained mainly astrocytic cells but no neuronal cell bodies or terminals in the CA3 region (Fig. 2*G*,*H*), whereas the ectoAb-1 stained large interneuronal-like cells scattered within the pyramidal layer and dentate gyrus with some weak staining in neurons within the pyramidal layers (Fig. 2*I*,*J*).

$P2X_7^{-/-}$ mice

Results from immunolocalization with the three Abs in the knock-out and their respective control mice are shown in Figures 3 (Pfizer $P2X_7^{-/-}$) and 4 (Glaxo$P2X_7^{-/-}$). The overall intensity of staining with the Cterm-Ab was reduced in the Pfizer $P2X_7^{-/-}$ mouse brain compared with control (Fig. 3*A*,*D*), and no microglial-like cells were observed (Fig. 3*D*), but the punctate immunoreactivity in the mossy fibers of the dentate gyrus and projections to the CA3 region were still observed (Fig. 3*D*). There were no apparent differences in immunoreactivity between the control and Pfizer $P2X_7^{-/-}$ mouse brain using either of the other two Abs (Fig. 3*B–F*). We observed no alterations in the staining patterns or reduction in intensity between control and Glaxo$P2X_7^{-/-}$ mice with any of the Abs (Fig. 4), although a consistent increase in the intensity of immunoreactivity by all three Abs was observed in the Glaxo$P2X_7^{-/-}$ mouse brain (Fig. 4*E–H*).

Submandibular gland

In striking contrast to the lack of specificity for $P2X_7$ receptor immunoreactivity by the different $P2X_7$ Abs in the brain, similar immunostaining of submandibular glands were observed with both the Cterm-Ab and ectoAb-1 in wild-type mice, and this staining disappeared in the knock-out mice. Figure 5 shows typical $P2X_7$ immunoreactivity in 10-μm-thick coronal sections of submandibular glands from Pfizer $P2X_7^{-/-}$ and Glaxo$P2X_7^{-/-}$ mice and their respective controls. Immunoreactivity was present as diffuse staining throughout duct cells of wild-type glands with both Abs; the only difference was that the Cterm-Ab also stained nuclei of both duct and acinar cells, but this nuclear staining was not altered in the knock-out tissues (Fig. 5*A*), indicating that the nuclear staining is most likely a nonspecific reaction to this particular Ab. A previous study has also observed immunolocalization in nuclei of hippocampal neurons using this Ab (Atkinson et al., 2002).

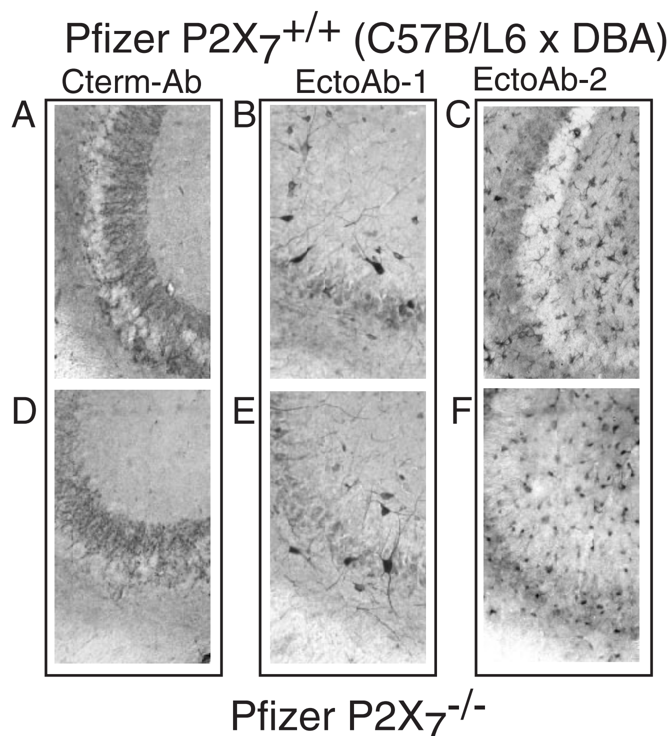

Figure 3. $P2X_7$ Ab immunoreactivity is unaltered in Pfizer $P2X_7^{-/-}$ knock-out mice. The sagittal view of the staining pattern using the Ni-DAB protocol of the three $P2X_7$ receptor Abs in hippocampal sections in wild-type (C57B/L6 × DBA) (*A–C*) and in Pfizer $P2X_7^{-/-}$ mice (*D–F*) is shown. No clear or consistent differences in staining were observed with any of the three Abs using this or other staining protocols. Scale bar, 40 μm.

Western blots

Previous studies on brain and peripheral tissues have used these Abs to examine $P2X_7$ receptor protein expression by Western blot analysis and have seen strong bands at the appropriate size (73–78 kDa) (Worthington et al., 1999a,b; Deuchars et al., 2001; Kim et al., 2001; Sluyter et al., 2001; Armstrong et al., 2002; Atkinson et al., 2002, 2004; Le Feuve et al., 2002; Sperlagh et al., 2002). Thus, it seemed reasonable to assume that although immunohistochemical localizations in the brain are unlikely to be reliable with these Abs, they may still provide an adequate means to determine and compare $P2X_7$ protein levels in central and peripheral tissues. We sought to verify this assumption by performing Western blots from the brain, lung, and submandibular glands of wild-type and knock-out mice, as well as from HEK293 cells transfected with mouse $P2X_7$ cDNA (m$P2X_7$/HEK). In all experiments, a band running at 75 kDa was detected in m$P2X_7$/HEK, which corresponds to the expected molecular weight for $P2X_7$ (Kim et al., 2001). Typical results obtained from knock-out mice tissue and their respective controls are shown in Figure 6. All of the Abs detected a band at 75 kDa in all tissues from wild-type mice, with the Cterm-Ab showing the highest level of discrimination (i.e., there were fewer bands of inappropriate size observed with this Ab) (Fig. 6). This 75 kDa band was completely absent from the lung and submandibular glands obtained from both sets of $P2X_7^{-/-}$ tissues when probed with either the

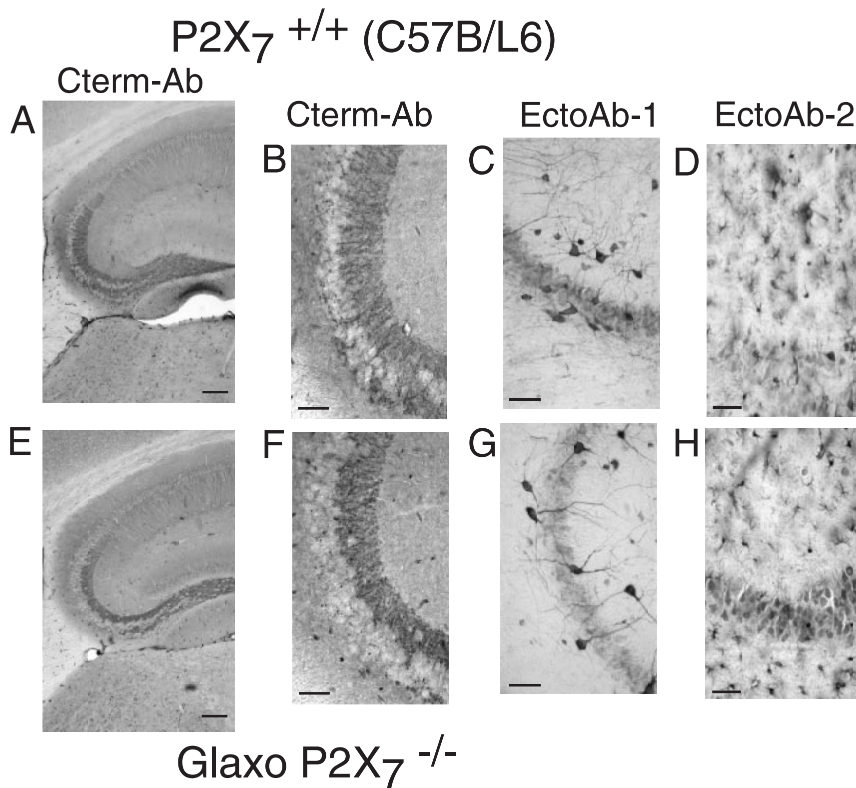

Figure 4. $P2X_7$ Ab immunoreactivity is unaltered in Glaxo$P2X_7^{-/-}$ mice. Sagittal views of the staining patterns of the three Abs in hippocampal sections from wild-type C56B/L6 (*A–D*) and Glaxo$P2X_7^{-/-}$ mice (*E–H*) are shown. The only consistent differences observed were an increase intensity of punctate staining in the mossy fiber region from knock-out mice using the Cterm-Ab (compare *B* with *F*) and the appearance of immunopositive neurons in the CA3 region with the ectoAb-2 in the knock-out mice that was absent in the wild-type mice (compare *D* with *H*). Scale bars: *A*, *E*, 100 μm; *B–D*, *F–H*, 40 μm.

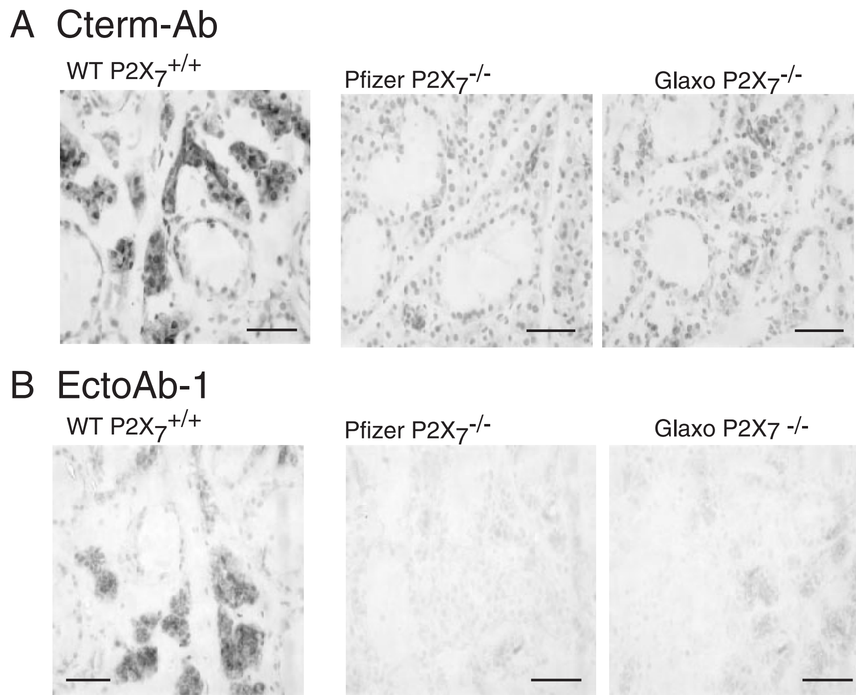

Figure 5. $P2X_7$ Ab immunoreactivity is absent from the submandibular glands of $P2X_7$ knock-out mice. Cterm-Ab shows strong Ni-DAB-positive duct cells with weaker staining apparent in acinar cells in wild-type mice but not in Pfizer or Glaxo knock-out mice (*A*), although the nuclear staining pattern is unaltered and represents nonspecific staining. The ectoAb-1 shows a very similar pattern of immunoreactivity in duct and acinar cells in wild-type submandibular glands; virtually all immunoreactivity disappeared in knock-out submandibular glands (*B*). Scale bars, 40 μm.

Cterm-Ab or the ectoAb-1, but there was no difference in the 75 kDa bands detected from brains of the same knock-out animals (Fig. 6*A*,*B*). There were no differences in the 75 kDa (or other) bands observed in any tissues from wild-type or knock-out mice using the ectoAb-2, and additionally it produced very high background staining at dilutions (1:5000) required to detect the 75 kDa band (Fig. 6*C*).

Immunoprecipitation

Our results show that the Cterm-Ab can clearly detect $P2X_7$ protein in the submandibular gland and lung on Western blots but not in the brain. If the $P2X_7$ protein is significantly present in the brain, it is likely masked by the presence of brain-specific proteins of a similar size that are also detected by this Ab (Fig. 7*A*). We therefore pursued the use of this Ab in immunoprecipitation experiments in an attempt to eliminate the contaminating protein(s). Control experiments on the submandibular gland, which contained no discernible contaminating protein at 75 kDa (based on its total disappearance in knock-out tissue) (Figs. 6, 7*A*), indicated that there was little loss of $P2X_7$ receptor protein after the immunoprecipitation protocol (20 μg for both Western blot only and immunoprecipitation/Western blot) (Fig. 7*B*, lane 1 vs lane 3). When the same amount of brain membrane protein (20 μg) was loaded after immunoprecipitation of a 100 μg total membrane fraction, no 75 kDa band was observed from either wild-type or knock-out mice (Fig. 7*B*, lanes 7 and 8). Moreover, even running 40 times as much brain protein (4 mg) through the immunoprecipitation and loading all of the recovered protein onto the Western blot still failed to detect any 75 kDa band in the wild-type or knock-out brain (Fig. 7*B*, lanes 9 and 10, *C*). These results suggest that the immunoprecipitation did remove all or most of the contaminating protein from the brain and that levels of $P2X_7$ protein in the brain, if present, are at least 200 times lower than in the submandibular gland.

Transgene expression of β-galactosidase

An indirect assay for $P2X_7$ protein expression and cellular localization was provided by the Glaxo$P2X_7^{-/-}$ mice in which the *LacZ* gene had been inserted into the *$P2X_7$* gene. X-gal staining for β-galactosidase, the protein encoded by the *LacZ* gene, revealed positive cells in the ependyma (Fig. 8*A*,*B*) but not in pyramidal cell layers or the dentate gyrus of the hippocampus (Fig.

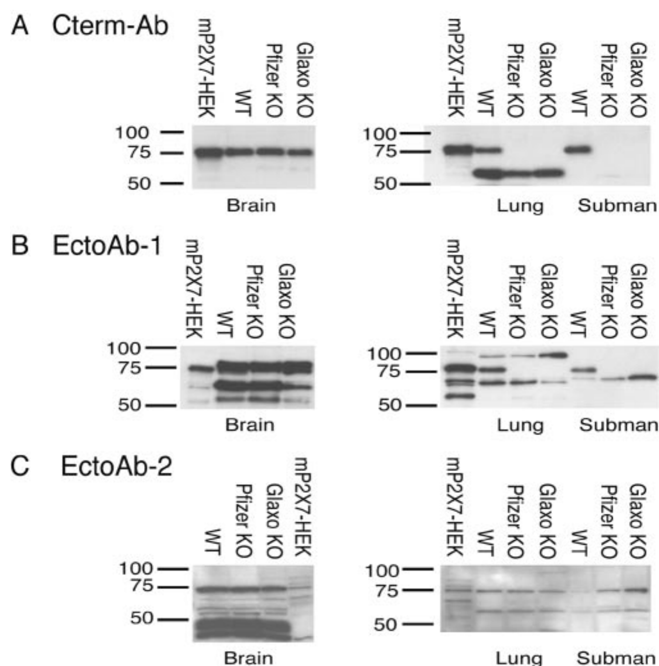

Figure 6. Western blots with $P2X_7$ Ab in brain and peripheral tissues from wild-type and knock-out mice reveals non-$P2X_7$ protein running at the same size as $P2X_7$ in the brain but not peripheral tissue. Membrane extracts of wild-type (WT) and knock-out (KO) mouse brain, lung, and submandibular gland (Subman) were subjected to SDS-PAGE on 8% (w/v) gels, followed by Western blotting as described in Materials and Methods. Extracts of HEK cells expressing mouse $P2X_7$ were used as positive controls in each blot. For each Ab (*A–C*), left gels show results from the brain and right gels are from the lung and submandibular gland. The 75 kDa bands observed in the lung and submandibular glands using the Cterm-Ab (*A*) and ectoAb-1 (*B*) Abs were absent from both knock-out mice, but there were no differences in any bands detected by these Abs in brain tissue. The ectoAb-2 detected several size bands in all tissues examined, including a 75 kDa band, but none were altered in any tissue from knock-out mice (*C*), rendering this Ab unsuitable for detection of the $P2X_7$ receptor protein. *A*, For Cterm-Ab (1:5000 dilution), equal loadings of 0.5 μg of brain, 16 μg of lung, and 16 μg of submandibular gland membrane protein were used. *B*, For ectoAb-1 (1:1000 dilution), equal loadings were 25, 16, and 16 μg for brain, lung, and submandibular gland, respectively. *C*, For ectoAb-2 (1:5000 dilution), loadings were 25, 16, and 16 μg, respectively.

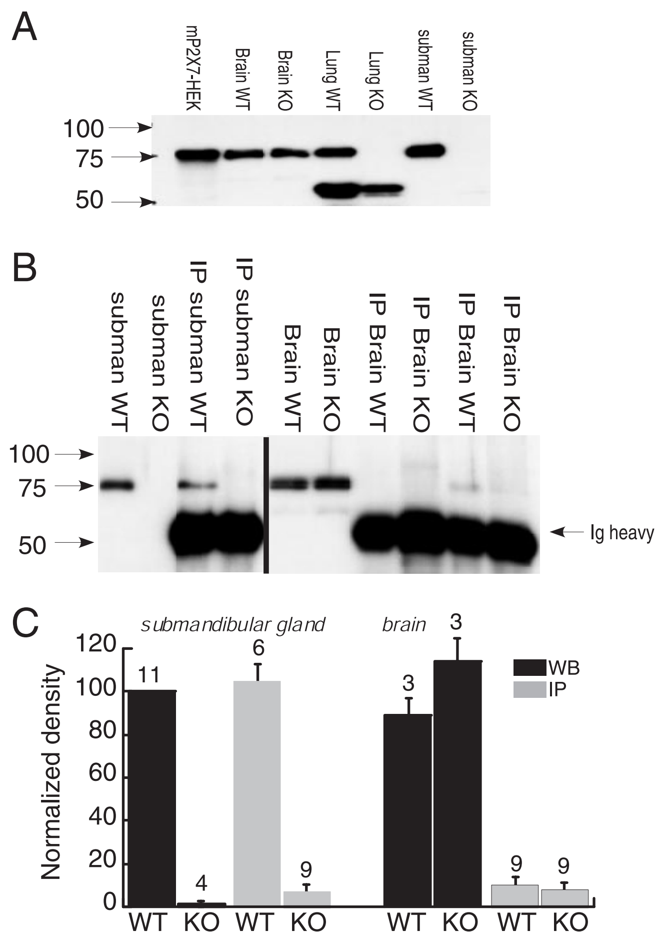

Figure 7. Immunoprecipitation with Cterm-Ab removes contaminating 75 kDa protein from brain but does not unmask $P2X_7$ receptor protein. *A*, Typical results from a single Western blot reveals the absence of the 75 kDa band in the lung and submandibular glands from knock-out mice but no change in staining in the brain from the same animals. *B*, Typical results from Western blots (lanes 1, 2, 5, 6) and after immunoprecipitation (lanes 3, 4, 7–10); a composite of two separate experiments is shown, as indicated by the vertical bar. The first four lanes are an experiment comparing equal amounts of submandibular gland membrane protein (20 μg) from wild-type and knock-out mice; no significant difference in the amount of 75 kDa protein detected was observed after immunoprecipitation from the submandibular gland of wild-type mice (lanes 1, 3), and this band disappeared from knock-outs (lanes 2, 4). Lanes 5–10 show a separate, but similar, experiment on brain tissue; here, 2 μg of brain membrane protein was loaded for the Western blot only (lanes 5, 6), a 20 μg equivalent (lanes 7, 8) or 4000 μg after immunoprecipitation (lanes 9, 10). This protocol virtually eliminated the 75 kDa band in both wild-type and knock-out brain (lanes 7–10). *C*, Denisitometry analysis of all results from experiments shown in *A* and *B*; the numbers above each bar represent the number of experiments. KO, Knock-out; WT, wild type; IP, immunoprecipitation; subman, submandibular gland.

8*C*,*D*). X-gal staining was prominent in both acinar and duct cells of submandibular gland sections from these mice (data not shown).

Discussion

The traditional hallmark of reliability and accuracy in immunocytochemical localization of proteins is a finding of similar cellular localization using multiple Abs directed at different epitopes of the protein of interest. This hallmark has been well fulfilled in the brain for localization of $P2X_4$ receptors and in autonomic–sensory–spinal systems for $P2X_1$, $P2X_2$, and $P2X_3$ receptors (Vulchanova et al., 1997; Le et al., 1998; Kanjhan et al., 1999; Chizh and Illes, 2000; Dunn et al., 2001; Rubio and Soto, 2001). In contrast, our inability to detect similar patterns of expression in the hippocampus and other brain areas of adult rats using the available $P2X_7$ Abs, as well as similar inconsistencies in the recent literature (Worthington et al., 1999a,b; Deuchars et al., 2001; Kim et al., 2001; Sluyter et al., 2001; Armstrong et al., 2002; Atkinson et al., 2002, 2004; Lundy et al., 2002; Sperlagh et al., 2002; Ishii et al., 2003), led us to undertake the present study in the mouse, in which two knock-out models are available. Except for a nonspecific nuclear staining, the two commercially available Abs were, in fact, highly selective for $P2X_7$ receptors expressed in HEK cells and showed identical staining patterns in the submandibular gland and peripheral macrophage; moreover, all immunocytochemical staining disappeared (except for the nuclear staining with Cterm-Ab) in submandibular glands from both the Glaxo$P2X_7{}^{-/-}$ and the Pfizer $P2X_7{}^{-/-}$ mice. The two Alomone Abs, but not ectoAb-2, also detected the $P2X_7$ receptor protein in the lung and submandibular gland (Figs. 6, 7) as well as in isolated microglia and macrophage because no 75 kDa band could be detected from any of these peripheral tissues in the knock-out mice (Sikora et al., 1999; Labasi et al., 2002; Le Feuvre et al., 2002). Finally, LacZ transgene expression of β-galactosidase was present in these peripheral tissues from Glaxo$P2X_7{}^{-/-}$ mice. Taken together, our results do provide very convincing evidence for $P2X_7$ protein expression and detection (by two of the three $P2X_7$ recep-

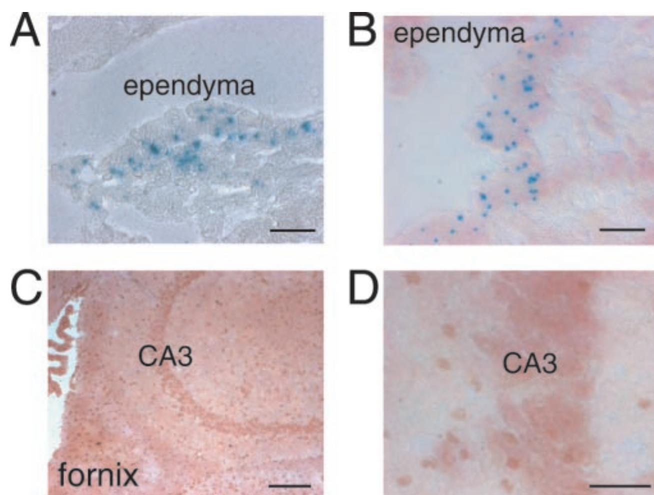

Figure 8. X-gal staining for expression of LacZ transgene shows positive cells only in ependymal cells surrounding the ventricle but no neuronal localization in hippocampus. *A*, *B*, Intense blue X-gal staining of intracytoplasmic organelles in the ependyma cell layer; *A* shows a section without neutral red counterstain. *C* and *D* show sagittal sections of the hippocampus; only the neutral red staining is seen in the pyramidal cell layer and dentate gyrus at either at 10× (*C*) or 60× (*D*) magnification. Scale bars: *C*, 100 μm; *A*, *B*, *D*, 40 μm.

tor Abs) in these peripheral immune and epithelial cells as well as equally convincing evidence for the absence of this protein in both $P2X_7^{-/-}$ mice lines.

In direct contrast to these results, none of the Abs could identify $P2X_7$ receptor expression in neurons, either by immunocytochemical localizations or by Western blots. Not only did none of the Abs give a similar pattern of staining to one another, but there was also little or no difference in either of the knock-out mice by immunocytochemistry or by Western blot with any of the Abs. Clearly, these Abs also recognize other proteins in brain that are not present in non-neuronal tissues that we and others have examined (Sikora et al., 1999; Suh et al., 2001; Labasi et al., 2002; Le Feuvre et al., 2002, 2003; Guerra et al., 2003; Rothwell, 2003), in particular one (or more) that runs at the same 75 kDa size as does the $P2X_7$ protein. We attempted to eliminate the contaminating protein(s) and unmask brain $P2X_7$ protein by immunoprecipitation before immunoblot; no 75 kDa band was observed using this protocol, except for a weak signal when 40- to 100-fold greater amounts of total brain membrane protein were used, and even this remaining signal was not altered in the knock-out mice. Moreover, no transgene expression of β-galactosidase could be detected in the hippocampal pyramidal cell layer or dentate gyrus, although it was readily apparent in ependymal cells lining the ventricle (Fig. 8). The only reasonable explanation for all of these results is that $P2X_7$ receptor protein is either not expressed in neurons of the normal adult rodent brain, or that levels are too low to be detected by any method currently available. This explanation would be in keeping with previous results that detected expression of $P2X_7$ receptor only in microglia surrounding an area of inflammation evoked by prior occlusion of the middle cerebral artery (Collo et al., 1997). In any event, a clear conclusion can be reached concerning the use of these $P2X_7$ receptor Abs in the brain: they are neither selective nor reliable. However, it is possible that the immunoprecipitation protocol we developed may be adequate under conditions in which $P2X_7$ protein levels are upregulated, as is known to occur in isolated microglial cells in response to inflammatory stimuli (Di Virgilio et al., 2001; Rothwell, 2003).

Recent functional studies, in combination with immunolocalization using the same Abs we used in this study, have concluded that presynaptic $P2X_7$ receptors in the hippocampus may modulate synaptic transmission at these sites (Armstrong et al., 2002; Lundy et al., 2002; Sperlagh et al., 2002; Atkinson et al., 2004; Cavaliere et al., 2004). However, extracellular ATP activates not only ionotropic P2X receptors but also metabotropic P2Y receptors and, because it is readily broken down to adenosine, adenosine receptors. In particular, there is extensive evidence for inhibition of both glutamatergic excitatory and GABA inhibitory synaptic transmission at mossy fiber terminals in hippocampal CA3 by presynaptically localized adenosine receptors (Malva et al., 2003; Kato et al., 2004). We found that 2′-3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP) produces a slow-onset inhibition of both evoked glutamatergic EPSCs and TTX-insensitive spontaneous GABAergic miniature IPSCs in CA3 of adult rat and mouse in the absence, but not in the presence, of the adenosine receptor blocker 8-cyclopentyl-1,3-dipropylxanthine (J. Sim, E. Tanaka, and F. Kato, unpublished observations). Thus, it is possible that the BzATP-induced presynaptic inhibition of mossy fiber excitatory input to CA3 neurons observed by Armstrong et al. (2002) may have been attributable to ATP hydrolysis to adenosine. Moreover, P2Y receptors and other P2X receptors ($P2X_2$, $P2X_4$, $P2X_6$) have been shown to act presynaptically in both the CA1 and CA3 regions of the hippocampus (North and Barnard, 1997; Brown et al., 2000; Norenberg and Illes, 2000; Khakh, 2001; Mori et al., 2001; Robertson et al., 2001; North, 2002; Malva et al., 2003); any of these receptors may also be targeted by breakdown of extracellular ATP. Certainly, recent functional studies on brain slices from wild-type and $P2X_2^{-/-}$ knock-out mice provide strong support for $P2X_2$ receptors playing a role in presynaptic modulation of transmitter release at hippocampal interneurons (Khakh et al., 2003).

Our study has failed to provide any evidence for neuronal localization of $P2X_7$ receptors in neurons of the rodent brain, but synthesis and membrane localization of these receptors in immune cells are rapidly upregulated in response to inflammatory stimuli (North, 2002; Guerra et al., 2003; Rothwell, 2003). In view of the apoptotic signaling cascades uniquely induced by activation of $P2X_7$ receptors in immune cells, it remains an important neuroscience issue to determine whether this ATP-gated ion channel may be similarly turned on in brain neurons after ischemic damage or other inflammatory diseases of the brain.

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