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ACCELERATED COMMUNICATION

Localization of P_{2X} Purinoceptor Transcripts in the Rat Nervous System

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SUMMARY

We used transcript-specific oligonucleotides to examine the localization in the rat nervous system of the corresponding mRNAs for the two P_{2X} purinoceptor genes cloned recently from the rat vas deferens and PC12 cells. PC12 P_{2X} purinoceptor mRNA was labeled in the olfactory tubercle, striatum, hypothalamus, hippocampus, dentate gyrus, amygdala, cortex, and cerebellum, whereas the vas deferens P_{2X} purinoceptor-specific probes labeled the cerebellum and, at lower levels of expression, the striatum, hippocampus, and cortex. Both types of P_{2X} purinoceptor transcript were found on cell bodies in the nodose and superior cervical ganglia. The presence of these two purinoceptor transcripts in the brain was confirmed by polymerase chain reaction. Two partial cDNAs, identical to sections of the PC12 or vas deferens P_{2X} purinoceptor coding sequences, were amplified from neonatal brain and cerebellum poly(A)$^+$ RNA, respectively. These findings are in broad agreement with earlier Northern blot studies on the PC12 P_{2X} purinoceptor mRNA but differ from those for the vas deferens P_{2X} purinoceptor mRNA, which had not previously been detected in adult brain. This difference is attributed to the low levels seen in the adult compared with the neonate and to the greater sensitivity of the methods used in the present study. The neonate medial habenula had low levels of transcripts for the PC12 but none for the vas deferens P_{2X} purinoceptor. Because pharmacologically the recombinant PC12 P_{2X} purinoceptor differs from the functional purinoceptor in the medial habenula, these results suggest the existence of other, unidentified, P_{2X} purinoceptors in the rat nervous system.

Evidence has been accumulating suggesting that ATP is a neurotransmitter in both the central and peripheral nervous systems (1–3). Extracellular ATP acts at two fundamentally distinct purinoceptor groups: the first includes the P_{2Y}, P_{2T}, and P_{2U} purinoceptors, which are G protein-coupled (4), and the second includes P_{2X} purinoceptors, which are ligand-gated cation channel receptors, mediating fast excitatory neurotransmission (2). ATP-evoked excitatory responses have been observed in many types of peripheral and central neurons, including the locus ceruleus (5–7), nucleus tractus solitarius (8, 9), medial habenula (10), supraoptic nucleus (11), hippocampus (12, 13), reticulospinal vasomotor neurons (14), SCG (15, 16), coliac ganglion (17), dorsal root ganglion (18), NG (16), and vagus nerve (19). Recent studies also suggest the presence of ATP-mediated fast excitatory responses in rat microglial cells (20).

The discrete localization of P_{2X} purinoceptors in the nervous system has been hampered by the limitations of the main available ligand, [^{3}H]$\alpha\beta$-methylene ATP (21, 22). This compound, which is inactive at P_{2Y} purinoceptors but is a potent agonist at some P_{2X} purinoceptors (23), has been used for autoradiographic localization of P_{2X} purinoceptors in the rat central nervous system (24, 25). However, $\alpha\beta$-methylene ATP is neither an agonist nor an antagonist at the PC12 P_{2X} purinoceptor (26, 27) and therefore cannot be used to identify all types of P_{2X} purinoceptor. Furthermore, [^{3}H]$\alpha\beta$-methylene ATP has recently been shown to bind to 5′-nucleotidase in endothelial cells (28) and therefore has limited usefulness for mapping the distribution of P_{2X} purinoceptors.

Two cDNA sequences encoding distinct P_{2X} purinoceptors have been isolated independently from the rat vas deferens (29) and the PC12 pheochromocytoma cell line (27). The receptor proteins display 38% identity but no primary sequence homology with other ligand-gated cation channels, having only two transmembrane domains: a large extracellular loop and short intracellular amino and carboxyl termini

ABBREVIATIONS: SCG, superior cervical ganglion; NG, nodose ganglion; RT-PCR, reverse transcriptase-polymerase chain reaction; SSC, saline sodium citrate; bp, base-pair(s);

(23). Results of Northern blotting suggested that the vas deferens P_{2X} purinoceptor was expressed in the spinal cord and the SCG (29), whereas the PC12 P_{2X} purinoceptor was found in the brain, spinal cord, and SCG (27).

The aims of the present study were to examine the localization of the mRNA for the two recombinant P_{2X} purinoceptor types in the rat central and peripheral nervous systems using *in situ* hybridization and transcript-specific oligonucleotide probes and RT-PCR and to determine whether there is any correlation between the distribution of these mRNAs and the functional receptors involved in ATP-mediated fast excitatory responses in the nervous system.

Materials and Methods

***In situ* hybridization.** Transcript-specific antisense 45 base oligonucleotide probes were custom-synthesized (Cruachem Ltd., Glasgow, UK). They were complementary to bases 251–295 in the amino terminus (5′ CCGGGGAGTGTCATATTCAAAGAAGAAGGCTGACAGCTCATCTTG 3′, oligonucleotide 1), 833–877 in the extracellular loop (5′ CATGTAGGTGCCGTTCACCTCCTCTACCAGGTTGCGCCTGTTGAC 3′, oligonucleotide 2), and 1427–1471 in the carboxyl terminus (5′ CACACACTGCGTCAAGTCCGGATCTCAAGACTAAGGTCAGGAGGT 3′, oligonucleotide 3) for the cDNA encoding the vas deferens P_{2X} purinoceptor (29) and to bases 619–663 in the extracellular loop (5′ CTTGAGGTAGTCACTCTTCTGGCTTGCAATGTTGCCCTTTGAGAA 3′, oligonucleotide I) and 1399–1443 in the carboxyl terminus (5′ AAGTTGGGCCAAACCTTTGGGGTCCGTGGATGTGGAGTCCTGTTG 3′, oligonucleotide II) for the PC12 P_{2X} purinoceptor cDNA (27). The probes were 3′ end-labeled with [^{35}S]dATPαS (>1000 Ci/mmol; Amersham, Little Chalfont, UK) using terminal deoxynucleotidyl transferase as described by Young (30) and purified by separation on a Sephadex G-50 column. *In situ* hybridization was carried out essentially according to Senaris *et al.* (31). Briefly, 5-day-old neonate and adult male Sprague-Dawley rats (230–250 g) were killed, and the brains were removed and frozen immediately. NG and SCG were also removed from the adult rats and frozen. Then, 12-μm coronal brain sections and 12-μm sections from several ganglia embedded in Cryo-M-Bed (Bright, Huntingdon, UK), were cut, thaw-mounted on gelatin-coated slides, and frozen until use. Sections were fixed in 4% paraformaldehyde for 20 min; dehydrated sequentially through 70%, 80%, 90%, 95%, and 100% ethanol (3 min each); and dried. Sections were hybridized in 150 μl of hybridization buffer under Parafilm (American National Can, Greenwich, CT) coverslips overnight at 37° in a moist chamber. The hybridization buffer contained 50% formamide, 1× Denhardt's solution (0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll), 10% dextran sulfate, 0.25 mg/ml salmon testes DNA, 4× SSC (0.6 M sodium chloride, 0.06 M sodium citrate), 0.3% β-mercaptoethanol, and either ~3.6 × 10^5 dpm/slide of the pooled vas deferens P_{2X} purinoceptor oligonucleotides or ~3.6 × 10^5 dpm of either of the two PC12 P_{2X} purinoceptor oligonucleotides. The oligonucleotides directed against the vas deferens P_{2X} purinoceptor had to be pooled as the images obtained in preliminary studies with each one separately were too faint to be interpreted successfully. Control sections were hybridized in the presence of a 50-fold excess of the appropriate unlabeled oligonucleotides. Control hybridizations performed with sense oligonucleotides gave the same results. After hybridization, the sections were rinsed for 5 min at 21° in 1× SSC and then washed three times for 30 min at 55° in 1× SSC and for 60 min at 21° in 1× SSC. Finally, they were briefly (2 min) rinsed in sterile water, then in 70% ethanol with 300 mM ammonium acetate, and last in 100% ethanol. The sections were air dried; those from the brain were exposed to Hyperfilm β-Max (Amersham) for 6 weeks, whereas the ganglia sections were dipped in Ilford K5 autoradiographic emulsion (Ilford, Knutsford, UK), exposed for 6 weeks, and counterstained with hematoxylin and eosin.

RT-PCR. Total RNA was extracted from neonatal rat whole brain or cerebellum as described by Chomzynski and Sacchi (32). Oligo(dT)-primed cDNAs were synthesized from poly(A)$^+$-selected RNAs using Moloney-murine leukemia virus reverse transcriptase (1st-Strand cDNA Synthesis kit, Clontech, Palo Alto, CA). Control reactions in the absence of reverse transcriptase were also carried out. PCR reactions were performed on 2-μl aliquots of cDNAs made from either neonate whole brain or cerebellum poly(A)$^+$ RNA. The primers were either degenerate (27 mer each) directed against the most conserved region of the two known P_{2X} purinoceptor cDNAs sequences (27, 29) (sense, bases 448–474; antisense, bases 991-1017) or specific for the vas deferens P_{2X} purinoceptor cDNA (sense, oligonucleotide 2; antisense, oligonucleotide 3; see Materials and Methods for *in situ* hybridization). Amplifications were performed with Dynazyme DNA polymerase (2.5 units, Flowgen, Sittingbourne, UK) in the presence of 200 ng of each of the primers and 200 μM of each of the dNTPs. The conditions were as follows: 94°, 1 min; 55°, 30 sec; and 72°, 1 min; 35 cycles for the whole brain cDNA using the degenerate primers, and 94°, 1 min; 59°, 30 sec; and 72°, 1 min; 35 cycles for the cerebellar cDNA using the specific primers, and a final

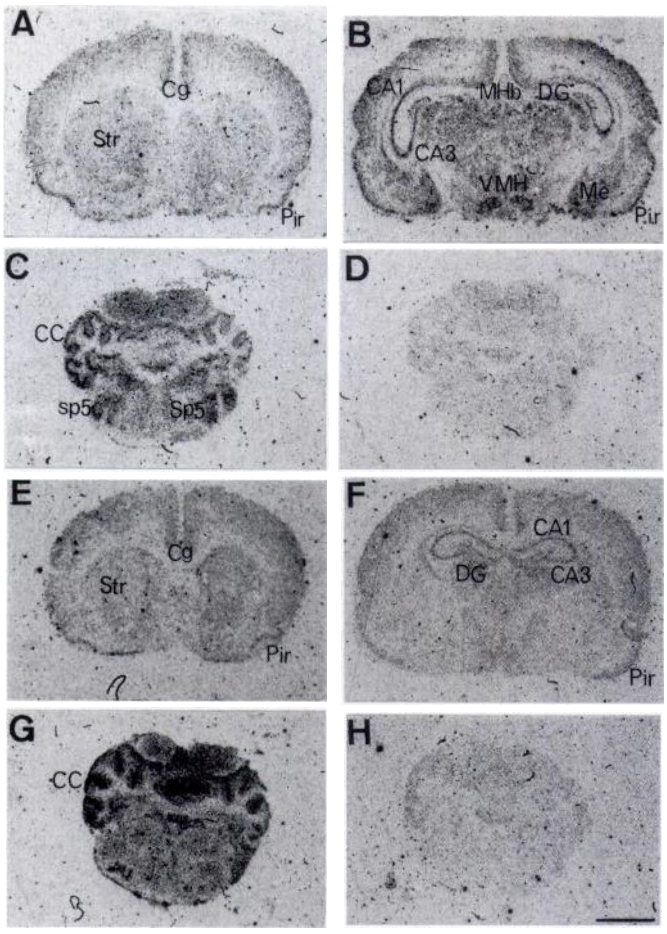

Fig. 1. Photomicrographs of autoradiograms of coronal sections from a 5-day-old neonate rat brain. A–D, mRNA was hybridized with an ^{35}S-labeled oligonucleotide (oligonucleotide II) specific for the PC12 P_{2X} purinoceptor cDNA. E–H, mRNA was hybridized with three ^{35}S-labeled oligonucleotides (oligonucleotides 1–3) for the rat vas deferens P_{2X} purinoceptor cDNA. Figures progress from rostral to caudal. Exposure time was 6 weeks. D and H, Control sections that were incubated with a 50-fold excess of the unlabeled probes. *CA1* and *CA3*, fields CA1 and CA3 of the hippocampus; *CC*, cerebellar cortex; *Cg*, cingulate cortex; *DG*, dentate gyrus; *Me*, medial amygdaloid nucleus; *MHb*, medial habenula; *Pir*, piriform cortex; *sp5*, spinal trigeminal tract; *Sp5*, spinal trigeminal nucleus; *Str*, striatum; *VMH*, ventromedial hypothalamic nucleus. *Scale bar* = 2 mm.

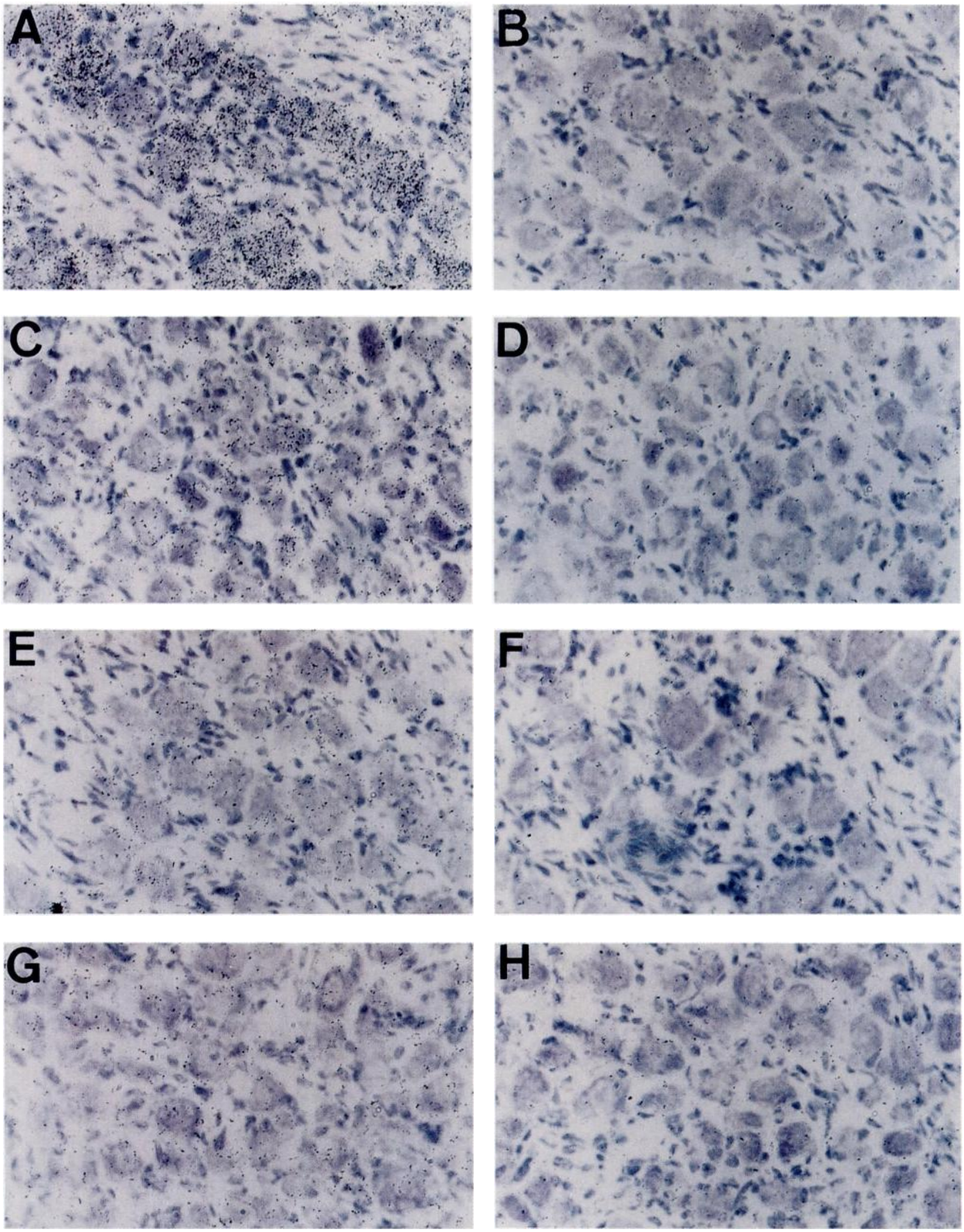

Fig. 2. Photomicrographs (×400) from emulsion autoradiographs of hematoxylin and eosin-stained sections of adult rat NG (A–B and E–F) and SCG (C–D and G–H). A–D, mRNA was hybridized with an ^{35}S-labeled oligonucleotide (oligonucleotide II) specific for the PC12 P_{2X} purinoceptor cDNA. E–H, mRNA was hybridized with three ^{35}S-labeled oligonucleotides (oligonucleotides 1–3) for the rat vas deferens P_{2X} purinoceptor cDNA. Sections were dipped in autoradiographic emulsion. Exposure time was 6 weeks. B, D, F, and H are control sections that were incubated with a 50-fold excess of the unlabeled probes.

extension at 72°, 5 min for both reactions. The resulting PCR products were cloned into the pCR II vector (Invitrogen, Leek, the Netherlands) according to the manufacturer's instructions and sequenced using Sequenase v2.0 enzyme (Amersham).

Results and Discussion

The present study constitutes the first regional distribution of transcripts for P_{2X} purinoceptors in the rat central and peripheral nervous systems and shows for the first time the presence of the vas deferens P_{2X} purinoceptor in the rat brain.

The distribution of vas deferens and PC12 transcripts in coronal brain sections from a 5-day-old rat is shown in Fig. 1. The labeling was eliminated in control sections incubated with the appropriate excess unlabeled oligonucleotides (Fig. 1, D and H). The strongest labeling with the three oligonucleotides specific for the vas deferens P_{2X} purinoceptor transcript was seen in the cerebellum (Fig. 1G). In addition, less intense labeling was observed in the striatum, CA1–3 regions of the hippocampus, dentate gyrus, and cortex (Fig. 1, E and F). Oligonucleotides specific for the PC12 P_{2X} purinoceptor transcript labeled the olfactory tubercle (not shown), striatum, ventromedial nucleus of the hypothalamus, CA1–3 regions of the hippocampus, dentate gyrus, medial amygdala, cortex, and spinal trigeminal tract and nucleus (Fig 1, A–C). The signal for this type of mRNA was also present in the cerebellum (Fig. 1C) but was less intense than that seen with the vas deferens-specific oligonucleotide probes. The medial habenula exhibited low levels of transcripts for the PC12 P_{2X} purinoceptor (Fig. 1B) but none for the vas deferens P_{2X} purinoceptor. In contrast, sections of adult rat brain, labeled and processed under conditions identical to those used for the neonatal brain, showed poorly detectable labeling in all areas (and are therefore not shown). This was true for the oligonucleotides derived from both the PC12 and the vas deferens P_{2X} purinoceptor cDNAs. Differences in labeling with the two sets of oligonucleotides specific for the two P_{2X} purinoceptor transcripts were also seen in adult rat NG and SCG (Fig. 2). The PC12 P_{2X} purinoceptor oligonucleotide probes showed very strong labeling of cell bodies in the NG, but less labeling was seen over the cell bodies in the SCG (Fig. 2, A and C). However, there was a similar, low level of mRNA signal for the vas deferens P_{2X} purinoceptor over cell bodies in both the NG and SCG (Fig. 2, E and G). The labeling was eliminated in control sections incubated with the appropriate excess unlabeled oligonucleotides (Fig. 2, B, D, F, and H).

In addition, we amplified a number of cDNAs by PCR using a template synthesized from rat neonatal whole brain poly(A)$^+$ RNA and degenerate primers directed against the most conserved regions of the two known recombinant P_{2X} purinoceptor cDNAs. One of these cDNAs (570 bp) displayed complete sequence identity to part of the PC12 P_{2X} purinoceptor cDNA, confirming the presence of the latter in the brain (Fig. 3, *lane 5*). Likewise, from neonatal rat cerebellum poly(A)$^+$ RNA (the region with the highest levels of transcripts for the vas deferens P_{2X} purinoceptor), a single product of the predicted size (597 bp) was obtained (Fig. 3, *lane 2*) by PCR with primers specific for the vas deferens P_{2X} purinoceptor sequence; this product corresponded exactly to a section of the vas deferens P_{2X} purinoceptor sequence of Valera *et al.* (29), confirming the *in situ* evidence described above for the presence of this mRNA in the cerebellum. PCR

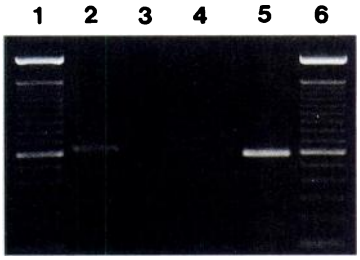

Fig. 3. Agarose gel electrophoresis of the isolated cDNAs. Gel (1.2%) was stained with ethidium bromide. *Lanes 1* and *6*, 100-bp DNA ladder (GIBCO, Paisley, UK). *Lane 2*, The single PCR amplification product obtained from neonatal rat cerebellum poly(A)$^+$ RNA with vas deferens P_{2X} purinoceptor-specific primers. This product corresponded in sequence to 597 bp of the rat vas deferens P_{2X} purinoceptor cDNA. *Lanes 3* and *4*, Control reactions performed either in the absence of template cDNA (*lane 3*) or without reverse transcriptase in the first-strand reaction (*lane 4*). *Lane 5*, cDNA obtained after amplification from rat whole brain-derived template with PCR primers based on the two known P_{2X} purinoceptor cDNAs (see Materials and Methods). This product corresponded in sequence to 570 bp of the rat PC12 cell P_{2X} purinoceptor cDNA.

amplification with poly(A)$^+$ RNAs extracted from adult cerebellum gave the same results. However, the intensity of the amplified cDNAs was much lower, reflecting a lower level of expression (data not shown).

These findings significantly extend those of Brake *et al.* (27), who detected PC12 P_{2X} purinoceptor mRNA in whole adult rat brain in a Northern blot. However, the presence of vas deferens P_{2X} purinoceptor transcripts in the adult and neonate brains is not in keeping with the findings of Valera *et al.* (29), who did not find any evidence by Northern blot analysis for vas deferens P_{2X} purinoceptor mRNA in adult rat brain. Our *in situ* hybridization study with ^{35}S-labeled oligonucleotides specific for the rat vas deferens P_{2X} purinoceptor cDNA revealed the presence of this mRNA in certain discrete regions of the neonate brain. However, in adult brain, much lower levels of this transcript were found in the same areas (data not shown). Thus, it is likely that these would be undetectable by Northern blot analysis of mRNA isolated from whole brain. The high expression seen in the neonate could be due to a specific role of these receptors in the developing brain. However, it is also possible that P_{2X} purinoceptors are present in the adult and the neonate brain but that their expression is much more easily detected in the developing brain, where overall protein synthesis is much greater.

The distribution pattern of the two P_{2X} purinoceptor transcript types described in the present study is in agreement with some of the functional studies that show ATP-evoked excitatory responses in the SCG (15, 16), NG (16), and hippocampus (12, 13). However, surprisingly, there was no evidence for mRNA for either receptor in areas such as the locus ceruleus, nucleus tractus solitarius, and supraoptic nucleus (data not shown), where electrophysiological studies have provided strong evidence for the presence of an ATP-gated ion channel (5–9, 11). It is possible that the receptor protein may be synthesized elsewhere and transported to these areas. The use of selective antibodies or antagonists for these receptor proteins would be necessary to investigate this possibility. A more plausible explanation for the apparent lack of labeling in the locus ceruleus, nucleus tractus solitarius, and supraoptic nucleus in the present study is that other P_{2X}

purinoceptor types (not recognized by the probes used here) are present in these areas. The neonate medial habenula contained only relatively low levels of transcripts for the PC12 P_{2X} purinoceptor and none for the vas deferens P_{2X} purinoceptor, whereas no transcripts for either receptor were detectable in the adult brain, probably due to the very low levels of expression. Because $\alpha\beta$-methylene ATP is ineffective at the expressed recombinant PC12 P_{2X} purinoceptor (27) but is a potent agonist at the receptor in the medial habenula (10), these results provide further support for the existence of other, as yet unidentified, P_{2X} purinoceptor types in the nervous system. The discrete localization of P_{2X} purinoceptors described in the present study should facilitate future studies on the role of ATP in synaptic transmission in the brain.

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