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A Novel Neuronal P_{2x} ATP Receptor Ion Channel with Widespread Distribution in the Brain

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There is strong evidence that ATP acts as an excitatory neurotransmitter in the periphery, yet little is known about fast central ATP-mediated transmission. We report here the molecular cloning of a novel neuronal ionotropic ATP receptor of the P_{2x} subtype (P_{2x3}) isolated from rat brain. This central P_{2x} channel subunit has significant amino acid homology with two recently cloned ATP-gated channels from rat smooth muscle (47%) and pheochromocytoma PC12 cells (37%). P_{2x3} receptor contains the characteristic 10 conserved cysteines of ATP-gated channels, a putative extracellular region homologous to the Walker type A motif found in various nucleotide-binding proteins, and two potential sites for phosphorylation by protein kinase C. Homomeric receptor P_{2x3} channels expressed in *Xenopus* oocytes produce rapid cation-selective purinergic currents that are potentiated by zinc ions and reversibly blocked by the P_{2x} antagonists suramin, Reactive Blue 2, and pyridoxalphosphate-6-axophenyl-2*U*,4*U*-disulfonic acid. P_{2x3}-receptor subunit mRNA is found in the Purkinje cells and the granule cells of the cerebellum as well as in CA3 pyramidal cells of the hippocampus that are innervated by zinc-rich axon terminals of mossy fibers. Our results suggest that fast excitatory synaptic transmission mediated by zinc-sensitive ATP-gated channels is widespread in mammalian brain.

Key words: ATP-gated channel; purinergic; nucleotide; cerebellum; hippocampus; purinoceptor

Despite extensive data regarding the role of extracellular ATP as a fast transmitter at neuro-neuronal and neuro-effector synapses in the peripheral nervous system (Krishtal et al., 1988; Bean, 1990; Bean et al., 1990; Fieber and Adams, 1991; Evans et al., 1992; Silinsky and Gerzanich, 1993) (for review, see Bean and Friel, 1989; Surprenant et al., 1995), central ATP-mediated neurotransmission has been reported only in the rat medial habenula (Edwards et al., 1992) and in dissociated rat nucleus solitarii neurons in culture (Ueno et al., 1992). These ATP-induced excitatory responses have pharmacological and electrophysiological properties consistent with the activation of ionotropic ATP receptors of the P_{2x} subtype (Burnstock, 1990). Because of the lack of specific pharmacological tools discriminating between metabotropic (P_{2y}, P_{2u}) and ionotropic P_{2x} ATP receptors, the identification of central synapses in which ATP mediates fast purinergic responses rests on the molecular characterization and localization of neuronal ATP-gated channels expressed in the brain. Expression-cloning experiments revealed that P_{2x} ATP-gated channels isolated from vas deferens smooth muscle (P_{2x1}) (Valera et al., 1994) and PC12 cells (P_{2x2}) (Brake et al.,1994) are structurally closer to inward rectifier K channels and amiloride-sensitive Na channels than to receptor channels of the nicotinic receptor gene family or of the glutamate-gated channel family. Cloned peripheral ATP-gated channels thus belong to a new gene family of neurotransmitter receptor channels showing poor selectivity for small cations and marked electrophysiological differences in their desensitization and recovery profiles. They share a unique global topology, with only 2 transmembrane domains and 10 conserved cysteines likely to be involved in the proper folding of the large putative extracellular domain (Surprenant et al., 1995). We describe in this report the primary structure and functional expression of a novel central subtype of rat P_{2x} ATP receptor (P_{2x3}) with a widespread neuronal distribution and a high level of transcription in the cerebellum.

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MATERIALS AND METHODS

Degenerate PCR and molecular cloning. Two degenerate PCR primers were designed to correspond to the conserved amino acid sequences (Q,K)VWDV(A,E)(D,E) (forward primer ARGTNTGGGAYGTNGMNG) and G(F,Y)NFRFA (reverse primer GCRAANCKRAARTTRWANCC) of smooth muscle (Valera et al., 1994) and PC12 cell (Brake et al., 1994) ATP-gated channel subtypes. A 645-bp-long PCR product was amplified using the following conditions: 2 μM primers, 2.5 U of *Taq* DNA polymerase, 2 min of initial denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 55°C for 40 sec, and extension at 72°C for 40 sec. The single-strand template was composed of 50 ng of random-primed reverse-transcribed cDNA from adult rat brain total RNA. A novel partial-length cDNA sequence was identified by restriction pattern and sequencing. The full-length P_{2x3} clone was isolated by plaque screening of a size-selected adult rat brain cDNA library in λZAP (Frech and Joho, 1989) (cDNA library kindly provided by R. Joho, University of Texas Southwestern Medical Center, Dallas, TX) probed with the radiolabeled PCR product in conditions of high stringency (last wash in 0.05× SSC at 65°C). The P_{2x3} cDNA was dideoxy-sequenced manually on both strands using Sequenase (United States Biochemicals, Indianapolis, IN) and automatically with fluorescently 5′ end-labeled primers using an ALF automatic sequencer (Pharmacia, Piscataway, NJ).

Electrophysiology. Fragment *Hin*dIII–*Xba*I of P_{2x3} plasmid, corresponding to nucleotide (nt) 1–1249 of the clone, was integrated directionally in eukaryotic CMV promoter-driven expression vector pCDNAI (Invitro-

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                  AAGCTTCAGCAGGCGGGGACATGCCTCCTAGGTCTTGGAGTCAGTAGCGGCCAAGGCGCGGAGCGGTCGGCGGAGCC    -1

ATGGCGGGCTGCTGCTCCGTGCTCGGGTCCTTCCTGTTCGAGTACGACACGCCGCGCATCGTGCTCATCCGCAGCCGTAAAGTGGGGCTC    90
MetAlaGlyCysCysSerValLeuGlySerPheLeuPheGluTyrAspThrProArgIleValLeuIleArgSerArgLysValGlyLeu
                                          TMDI
ATGAACCGCGCGGTGCAGCTGCTCATCCTGGCTTACGTCATCGGGTGGGTGTTCGTGTGGGAAAAGGGCTACCAGGAAACGGACTCCGTG   180
MetAsnArgAlaValGlnLeuLeuIleLeuAlaTyrValIleGlyTrpValPheValTrpGluLysGlyTyrGlnGluThrAspSerVal

GTCAGCTCGGTGACAACCAAAGCCAAAGGTGTGGCTGTGACCAACACCTCTCAGCTTGGATTCCGGATCTGGGACGTGGCGGACTATGTG   270
ValSerSerValThrThrLysAlaLysGlyValAlaValThrAsnThrSerGlnLeuGlyPheArgIleTrpAspValAlaAspTyrVal

ATTCCAGCTCAGGAGGAAAACTCCCTCTTCATCATGACCAACATGATCGTCACCGTGAACCAGACACAGAGCACCTGTCCAGAGATTCCT   360
IleProAlaGlnGluGluAsnSerLeuPheIleMetThrAsnMetIleValThrValAsnGlnThrGlnSerThrCysProGluIlePro
                                                           Δ
GATAAGACCAGCATTTGTAATTCAGACGCCGACTGCACTCCTGGCTCCGTGGACACCCACAGCAGTGGAGTTGCGACTGGAAGATGTGTT   450
AspLysThrSerIleCysAsnSerAspAlaAspCysThrProGlySerValAspThrHisSerSerGlyValAlaThrGlyArgCysVal

CCTTTCAATGAGTCTGTGAAGACCTGTGAGGTGGCTGCATGGTGCCCGGTGGAGAACGACGTTGGCGTGCCAACGCCGGCTTTCTTAAAG   540
ProPheAsnGluSerValLysThrCysGluValAlaAlaTrpCysProValGluAsnAspValGlyValProThrProAlaPheLeuLys
      Δ
GCTGCAGAAAACTTCACCCTCTTGGTAAAGAACAACATCTGGTACCCCAAGTTTAACTTCAGCAAGAGGAACATCCTCCCCAACATCACC   630
AlaAlaGluAsnPheThrLeuLeuValLysAsnAsnIleTrpTyrProLysPheAsnPheSerLysArgAsnIleLeuProAsnIleThr
         Δ                                            Δ
ACGTCCTACCTCAAATCGTGCATTTACAATGCTCAAACGGATCCCTTCTGCCCCATATTCCGTCTTGGCACAATCGTGGAGGACGCGGGA   720
ThrSerTyrLeuLysSerCysIleTyrAsnAlaGlnThrAspProPheCysProIlePheArgLeuGlyThrIleValGluAspAlaGly

CATAGCTTCCAGGAGATGGCAGTTGAGGGAGGCATCATGGGTATCCAGATCAAGTGGGACTGCAACCTGGATAGAGCCGCCTCCCTTTGC   810
HisSerPheGlnGluMetAlaValGluGlyGlyIleMetGlyIleGlnIleLysTrpAspCysAsnLeuAspArgAlaAlaSerLeuCys

CTGCCCAGATATTCCTTCCGGCGCCTGGACACCCGGGACCTGGAACACAATGTGTCTCCTGGCTACAATTTCAGGTTTGCCAAGTACTAC   900
LeuProArgTyrSerPheArgArgLeuAspThrArgAspLeuGluHisAsnValSerProGlyTyrAsnPheArgPheAlaLysTyrTyr
                                                    Δ
AGGGACCTGGCCGGCAAAGAGCAGCGCACACTCACCAAGGCGTACGGCATCCGCTTTGACATCATCGTGTTTGGAAAGGCTGGGAAGTTT   990
ArgAspLeuAlaGlyLysGluGlnArgThrLeuThrLysAlaTyrGlyIleArgPheAspIleIleValPheGlyLysAlaGlyLysPhe
                    H5                                          TMDII
GACATCATCCCTACCATGATCAACGTTGGCTCTGGCTTGGCGCTCCTCGGGGTGGCGACGGTGCTCTGTGACGTCATAGTCCTCTACTGC  1080
AspIleIleProThrMetIleAsnValGlySerGlyLeuAlaLeuLeuGlyValAlaThrValLeuCysAspValIleValLeuTyrCys

ATGAAGAAGAAATACTACTACCGGGACAAGAAATATAAGTATGTGGAAGACTACGAGCAGGGTCTTTCGGGGGAGATGAACCAGTGACGC  1170
MetLysLysLysTyrTyrTyrArgAspLysLysTyrLysTyrValGluAspTyrGluGlnGlyLeuSerGlyGluMetAsnGln...
CTAAAGTTACATTTCCACCCCGCTCAGCCCGCGAAGCAGAAAGATGGGGAGAGATGGCTACTGCGTCTGTCACTCTAGAGAAAGCTCCAG  1260
AGTTTCAGCTCAGTTCTCCACTCCACAAATACTCAGGGTTGCCCAAGCACATCTTGTTGAGCCCGGTTCTTGCTCTGCTGCTCAGATGGG  1350
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CATTTTTTACATTTTACCTTTCCTTTGTATACATCTAAGGCTGGCCTCAGACGCAAGACGTTCTTCCACCCTATACACCCTTTTAATCTC  1530
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AGCGTATGGAGGTAGGAAGGGTCTAAGAGCAGAGACACTGCTGTGGCTTACGGACAGGCCCAGGCTCTGTCCACGCACTTTATTTCTCCG  1710
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Figure 1. Primary structure of neuronal P_{2x3} ATP-gated channel from rat brain. Nucleotide and deduced amino acid sequence of rat P_{2x3} cDNA. Putative transmembrane domains and H5-related regions are *boxed*. Two potential phosphorylation sites by protein kinase C (Thr^{17}, Ser^{25}) in the putative intracellular N-terminal domain are *underlined*. In the extracellular loop, 10 Cys residues (in *bold*) conserved in all known ATP-gated channels and five putative sites of N-glycosylation (Δ) are indicated.

gen, San Diego, CA). Oocytes were prepared, injected, and recorded from as described previously (Bertrand et al., 1991). ATP-gated currents were recorded with a two-electrode voltage clamp (built by J. Knowles, McGill University) 2–5 d after nuclear injection of 2–5 ng of P_{2x3} cDNA. The membrane currents were filtered with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and then sampled, displayed, and stored on-line with a 386 PC. We used Patchkit (Alembic Software, Patterson, NJ) for data acquisition and analysis; we also analyzed data with Igor (Wavemetric, San Francisco, CA). All agonists and antagonists were dissolved in perfusion solution at concentrations indicated in the text. The control perfusion solution consisted of the following: NaCl, 95 mM; KCl, 2.5 mM; $CaCl_2$ or (as indicated) $BaCl_2$, 1.8 mM; $MgCl_2$, 1 mM; atropine, 1 μM; and HEPES, 10 mM, pH 7.4. In some experiments, we recorded from oocytes in a high K solution in which KCl was increased to 95 mM and NaCl was reduced to 2.5 mM. Both the current and the voltage electrodes were filled with 3 M KCl, and all recordings were made at 22–24°C.

In situ *hybridization.* Sense and antisense [^{35}S]uridine triphosphate (UTP)-labeled *in vitro* transcribed cRNA probes comprising nt 324–968 of P_{2x3} cDNA subcloned in pBluescript II SK (Stratagene, La Jolla, CA) were hybridized on formaldehyde-fixed rat brain sections in conditions of high stringency according to the previously described protocol (Soghomonian et al., 1994). Briefly, sections were treated for 10 min with 0.25% acetic anhydride and triethanolamine (0.1 M, pH 8.0) and for 30 min with Tris-glycine (1 M, pH 7.0), dehydrated in graded ethanol, and air-dried. Each section was covered with 3–8 ng of radiolabeled cRNA probe (specific activity, 4 × 10^5 cpm/ng) diluted in hybridization solution containing 40% formamide, 10% dextran sulfate, 4× SSC, 10 mM dithiothreitol, 1% sheared salmon sperm DNA, 1% yeast tRNA, and 1× Denhardt's solution containing 1% RNase-free bovine serum albumin. The

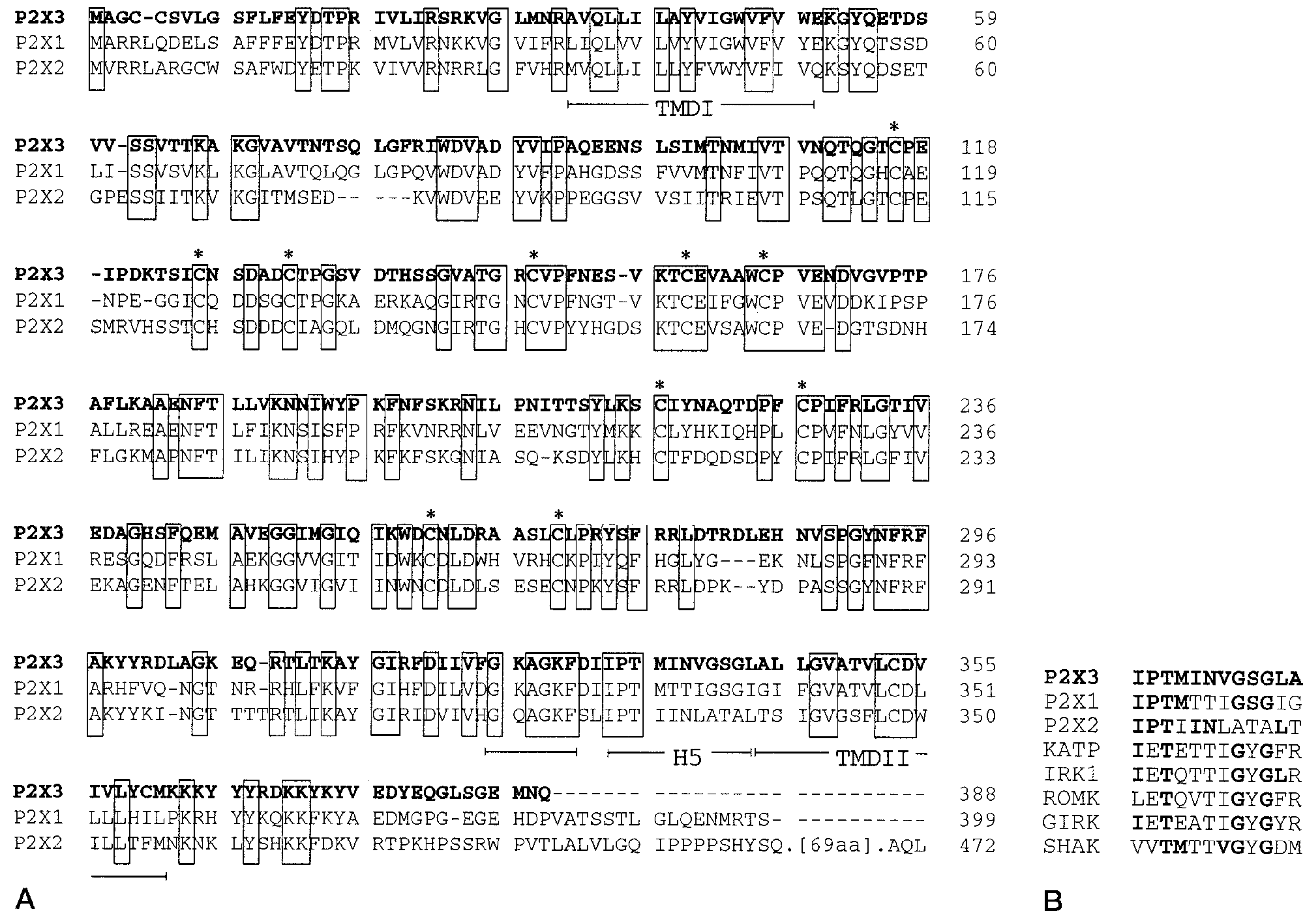


Figure 2. Alignment of amino acid sequences of three known subtypes of ATP-gated channels. *A*, Putative transmembrane domains (*TMD*), pore regions (*H5*), and nucleotide-binding domains are *underlined*. *B*, Alignment of ATP-gated channels with representative members of the inward rectifier and voltage-sensitive K-selective channel families in the H5 region. *KATP*, Rat ATP-sensitive K channel (Ashford et al., 1994); *IRK1*, mouse inward rectifier K channel (Kubo et al., 1993a); *ROMK*, rat ATP-regulated K channel (Ho et al., 1993); *GIRK*, rat G-protein-coupled muscarinic K channel (Kubo et al., 1993b); *SHAK*, *Drosophila Shaker* K channel (Tempel et al., 1987).

sections were incubated for 4 hr at 50°C. Sections were then rinsed in 50% formamide and 2× SSC at 52°C for 25 min and treated in 100 μg/ml RNase A (Sigma, St. Louis, MO) and 2× SSC for 30 min at 37°C. After a final rinse in 50% formamide and 2× SSC for 5 min at 52°C, the sections were left overnight at room temperature under mild agitation in 2× SSC and 0.05% Triton X-100. Sections were then dehydrated in ethanol, defatted, and dried until processing for x-ray film (X-OMAT AR; Kodak, Rochester, NY) and nuclear emulsion (NTB3; Kodak) radioautography. Films and sections were developed in D-19 at 14°C after 10 and 30 d exposure, respectively. Emulsion radioautographs were lightly counterstained with hematoxylin and eosin before mounting.

RESULTS

Primary structure

By using degenerate oligonucleotide primers corresponding to conserved domains of cloned ATP-gated channels (Brake et al., 1994; Valera et al., 1994) (see details in Materials and Methods), a novel P_{2x} receptor-channel subunit was identified by sequencing reverse-transcribed (RT)-PCR products obtained from rat brain random-primed cDNA. A full-length clone, including the RT-PCR probe sequence, was isolated by high-stringency screening of a size-selected directional adult rat brain cDNA library. The clone pP_{2x3} is a 1.8-kb-long cDNA (cDNA sequence deposited in Genbank database under accession number U32497), including a main open-reading frame coding for 388 amino acids flanked by 77 bp of a 5′-noncoding region and 543 bp of a 3′-noncoding region (Fig. 1). The predicted initiation codon conforms to an efficient Kozak consensus sequence (Kozak, 1986), and the nonglycosylated P_{2x3} channel subunit has a predicted M_r of 43.5 kDa. At the amino acid level, P_{2x3} has 47 and 37% homology with the rat smooth muscle subtype (P_{2x1}) (Valera et al., 1994) and the PC12 subtype (P_{2x2}) (Brake et al., 1994), respectively (Fig. 2*A*). According to the hydropathy profile, the subunit would consist of small intracellular N- and C-terminal domains and two transmembrane domains separated by a large extracellular loop of 275 amino acids containing five potential sites of N-glycosylation. The initial part of the N-terminal domain diverges significantly from the positively charged homologous region in P_{2x1} and P_{2x2}, but it does not define a leader sequence and, thus, does not change the predicted transmembrane topological organization of the receptor subunit.

Functional characterization

To characterize the functional properties of the homomeric P_{2x3}-receptor subtype, the full-length P_{2x3} cDNA was expressed in

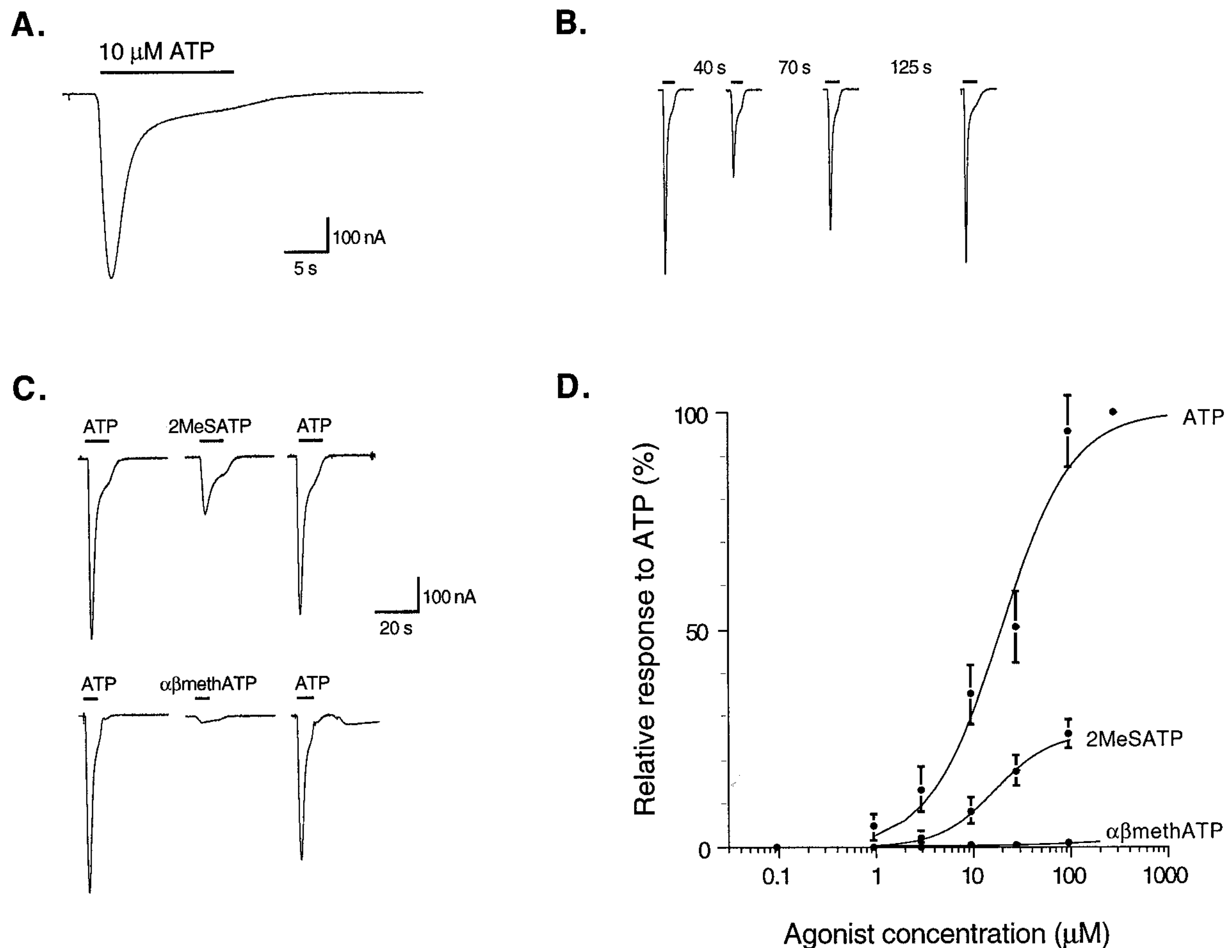


Figure 3. Functional expression of P_{2x3} ATP receptors in *Xenopus* oocytes. *A*, Whole-cell currents recorded from an oocyte nuclear-injected with P_{2x3} cDNA on application of 10 μM ATP. The oocyte was voltage-clamped (Vc) at −40 mV. *B*, Recovery from desensitization. Whole-cell currents in response to repeated application of 100 μM ATP at different intervals: 40, 70, and 125 sec; Vc = −60 mV. *C*, Activation by P_{2x} agonists. Comparisons of currents evoked by 30 μM of ATP, 2-methylthio-ATP (*2MeSATP*), or αβ-methylene-ATP (*αβmethATP*). Each set shows three consecutive recordings from the same oocyte. *Top*, ATP, 2MeSATP, ATP; Vc = −40 mV. *Bottom*, ATP, αβmethATP, ATP; Vc = −80 mV. For each set, the agonists were applied 2 min apart. *D*, Dose–response relationships for the three agonists shown in *C*. The mean peak agonist-evoked currents were normalized to the response to 300 μM ATP. Each point is the mean ± SEM ($n = 3$) response to a particular agonist concentration. The oocytes were voltage-clamped at −30 to −40 mV to minimize possible contributions of the Ca^{2+}-activated Cl^- current present endogenously in *Xenopus* oocytes. Replacement of extracellular Ca^{2+} with Ba^{2+} had no effect on the agonist sensitivities. A least-square curve-fitting routine fit the data to a Hill equation: for ATP, EC_{50} = 20 μM and n = 1.2; for 2MeSATP, EC_{50} = 12.7 μM and n = 1.5.

Xenopus oocytes by nuclear injection. When activated by ATP, homomeric P_{2x3} receptors produce rapid inward currents that desensitize in the continual presence of agonist (Fig. 3*A*); complete recovery from desensitization occurs in 2–3 min (Fig. 3*B*). This inward current is reduced markedly by substituting Ca^{2+} with Ba^{2+}, indicating that these receptors have a significantly high Ca^{2+} permeability. The order of agonist potency for these receptors is ATP > 2-methylthio-ATP > αβ-methylene-ATP (Fig. 3*C*), and the dose–response curves (Fig. 3*D*) indicate that EC_{50} for ATP is ~20 μM, similar to the EC_{50} for ATP of PC12 P_{2x2} receptors. Neither UTP (100 μM, $n = 3$) nor GTP (100 μM, $n = 3$) produced any detectable response. The ATP-evoked currents were inhibited reversibly by 50–200 μM concentrations of the characteristic purinergic antagonists suramin, pyridoxalphosphate-6-axophenyl-2*U*,4*U*-disulfonic acid (PPADS), and Reactive Blue 2 (Fig. 4*A*). Consistent with the behavior of neuronal ATP-gated channels (Cloues et al., 1993; Li et al., 1993), coapplication of 10 μM Zn^{2+} with 0.3–10 μM ATP potentiated the evoked current reversibly; the average potentiation is 1.8 ± 0.35-fold (mean ± SEM, $n = 11$; Fig. 4*B*). The current–voltage relationship for P_{2x3} receptors indicates that currents reverse at ~0 mV (Fig. 5) and show much less inward rectification compared with that reported for P_{2x1} and P_{2x2} receptors. Replacing most of the extracellular Na^+ with K^+ has little effect on the reversal potential (Fig. 5), demonstrating that P_{2x3} receptors are cation-selective and discriminate poorly among small ions.

Central distribution

Brain sections that were incubated with the P_{2x3} sense probe did not exhibit significant labeling in any of the levels examined. On the contrary, sections processed with the antisense probe (Fig. 6) exhibited consistent labeling in the cerebellum, hippocampus, and piriform cortex at all levels examined. In addition, at brainstem level the pontine nuclei and the lateral nucleus of the cerebellum were also labeled. Figure 6, *A* and *B*, shows that P_{2x3} mRNA encodes a neuronal receptor subunit transcribed at a high level in the cerebellar cortex. Accumula-

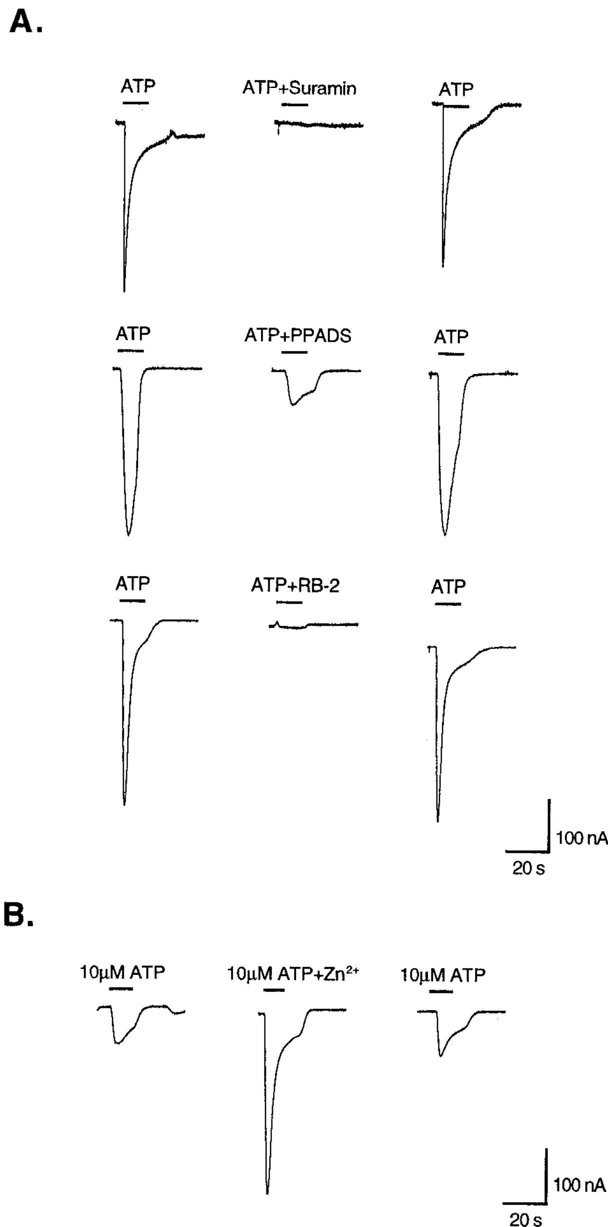


Figure 4. Blockade of P_{2x3} ATP-gated channels by P_{2x} antagonists and potentiation by Zn^{2+}. *A*, Blockade by P_{2x} antagonists. Each set shows three consecutive currents from the same oocyte. *Top*, 100 μM ATP, 100 μM ATP + 100 μM suramin, 100 μM ATP; Vc = −70 mV. *Middle*, 1 μM ATP, 1 μM ATP + 100 μM PPADS, 1 μM ATP; Vc = −40 mV. *Bottom*, 1 μM ATP, 1 μM ATP + 100 μM Reactive Blue 2 (*RB-2*), 1 μM ATP; Vc = −50 mV. Similar results were observed in five other oocytes. With suramin and PPADS, we found that it was necessary to preincubate the oocyte for at least 90 sec to inhibit the ATP currents. *B*, Potentiation by Zn^{2+}. Three consecutive currents from the same oocyte: 10 μM ATP, 10 μM ATP + 10 μM Zn^{2+}, 10 μM ATP; Vc = −40 mV.

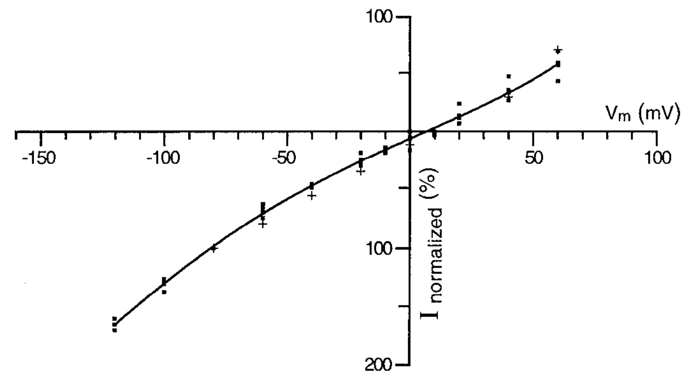
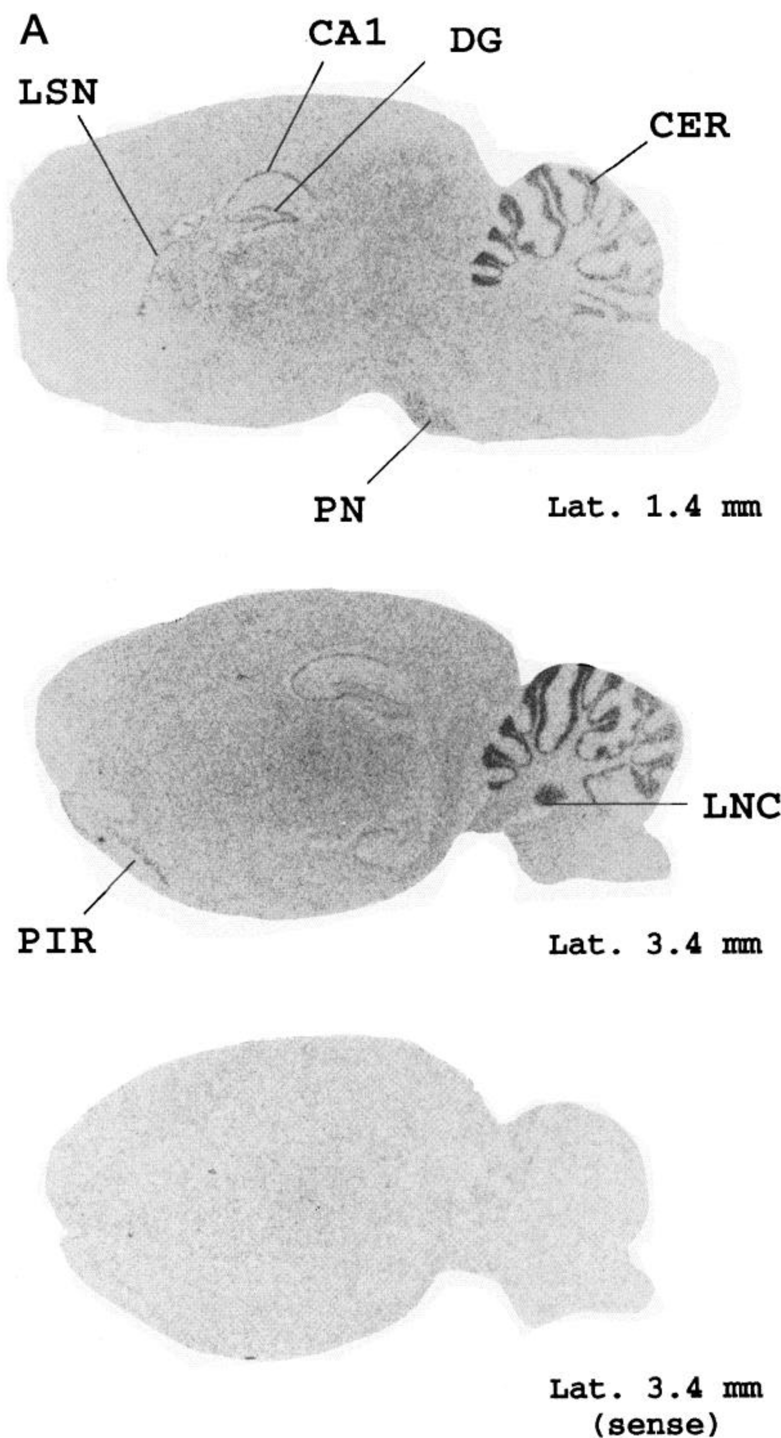


Figure 5. Current–voltage relationship of homomeric P_{2x3} ATP-gated channels. Peak currents evoked by 10 μM ATP at various holding potentials and normalized to that evoked at −80 mV. ATP was applied at 3 min intervals; between applications the membrane potential was held at −40 mV. The *filled squares* are data obtained in control solution from four different oocytes. The *crosses* are data obtained in high K (95 mM) solution. The *solid line* represents a fifth-order polynomial fit to the data.

tion of silver grains was visible in the Purkinje cells and in neurons throughout the granular layer (Fig. 6*B*). In the hippocampus, labeling was found consistently at moderate levels in the pyramidal cell layer of all CA fields and in the granule cell layer of the dentate gyrus. In addition to these regions, the emulsion radioautographs also revealed a low but significant level of transcription in other forebrain regions, in the thalamus, and in the brainstem. In the forebrain, labeled neurons were observed mainly in the presubiculum and in the dorsal endopiriform nucleus. In the thalamus, neurons in the geniculate nuclei, the reticular thalamic nucleus, and the ventralis posteriolateralis also seemed to be labeled. In the brainstem, motoneurons in the facial nucleus, in the nucleus ambiguus, and in the trigeminal motor nucleus exhibited labeling. Some diffuse hybridization signal suggests glial localization. In the peripheral nervous system, RNase protection assays indicate that P_{2x3} mRNA levels are 10- to 20-fold lower than P_{2x2} in sympathetic and nodose neurons (data not shown). The P_{2x3} receptor gene thus seems to encode a central subtype of ATP-gated channel with a higher level of transcription in the CNS than other known subtypes (Surprenant et al., 1995).

DISCUSSION

P_{2x} receptors constitute a novel class of ligand-gated channels with a transmembrane topology similar to that of inwardly rectifying K channels, and they include a domain homologous to the H5 region, which is believed to form the selectivity gate of the ion pore of many types of channels (MacKinnon, 1995). In K-selective channels, this region contains a highly conserved GYG motif (Yellen et al., 1991; Yool and Schwartz, 1991). It has been proposed that P_{2x1} receptors contain a GSG motif in this region, and our results indicate that this structure is conserved in P_{2x3} (Fig. 2*B*). There is some controversy, however, regarding the correct alignment of the H5 region of P_{2x2} receptors (Kerr and Sansom, 1995; Surprenant et al., 1995). By aligning all three P_{2x} subtypes and taking into consideration the conservation of the second transmembrane domain (Fig. 2*B*), it becomes apparent that P_{2x2}-receptor subunits are atypical in this region and contain a unique motif with Ala residues instead of the highly conserved Gly residues. As suggested by the homology with K-selective channels, the motif (G,A)(S,T)(G,A) found in P_{2x} receptors could underlie the poor selectivity of these channels for small cations. Between the two putative transmembrane domains, 10 Cys residues are conserved in the three subtypes (Fig. 1). By presumably forming disulfide bridges, these Cys residues would maintain the structural constraints needed to couple the ATP-binding do-



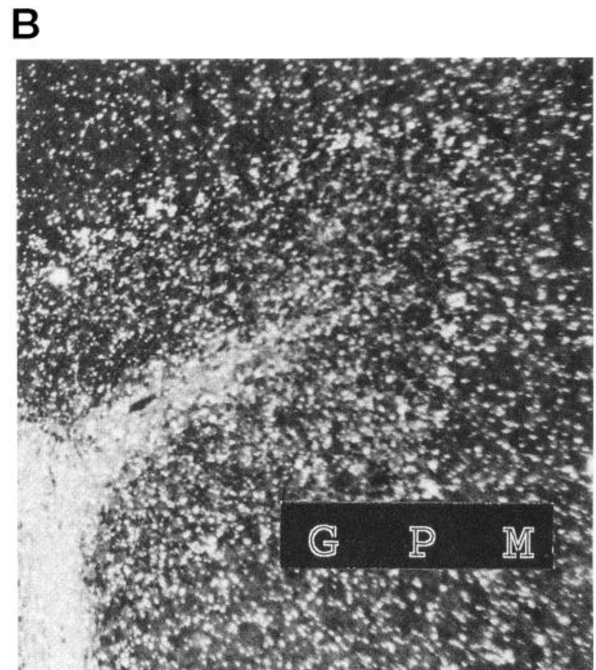


Figure 6. Distribution of P_{2x3} ATP-receptor mRNA in adult rat brain. *A*, Regional localization of P_{2x3} mRNA in sagittal sections of adult rat brain—coordinates according to Paxinos and Watson (1986). *CA1*, Hippocampal Ammon's horn area 1; *CER*, cerebellar cortex; *DG*, dentate gyrus; *LNC*, lateral nucleus of cerebellum; *LSN*, lateral septal nuclei; *PIR*, piriform cortex; *PN*, pontine nuclei. Negative control sections (*bottom*) were hybridized with sense riboprobe (*sense*). Magnification, 5×. *B*, Dark-field photomicrograph showing the neuronal localization of P_{2x3} ATP-gated channels in the cerebellar cortex. Purkinje cells show dense accumulation of silver grains. *G*, Granular cell layer; *P*, Purkinje cell layer; *M*, molecular cell layer. Magnification, 45×.

main to the ion pore. Interestingly, the proposed extracellular domain immediately adjacent to the H5 region in all P_{2x} subtypes contains a conserved region that resembles a Walker type A ATP-binding domain (Walker et al., 1982); it is tempting to speculate that this region forms the nucleotide-binding site of the channel, in physical proximity to the ion pore mouth. The two potential sites of phosphorylation (Thr^{17} and Ser^{25}; Fig. 1) by protein kinase C found in the N-terminal domain of P_{2x3} channel subunits indicate that the activity of this neurotransmitter-gated channel may be regulated heterologously by receptors linked to second-messenger systems. The conservation of Thr^{17} in central and peripheral subtypes of P_{2x} receptors (Fig. 2*A*) suggests a tonic regulation of ATP-gated channels in various cell types.

The functional properties of P_{2x3} receptors differ from those of P_{2x1} and P_{2x2} receptors. The relative agonist sensitivities of P_{2x3} receptors are closer to those for neuronal P_{2x2} than for muscle P_{2x1}. For example, $\alpha\beta$-methylene-ATP, which is a potent agonist for P_{2x1} receptors, is much less effective for P_{2x3} receptors (Fig. 3*D*). On the other hand, the desensitization

properties of P_{2x3} are similar to those of P_{2x1}. Both P_{2x3} and P_{2x1} receptors desensitize in hundreds of milliseconds; however, P_{2x3} receptors recover from desensitization 2 to 3 times more rapidly than P_{2x1} receptors. In contrast, P_{2x2} receptors show little or no desensitization. P_{2x3} receptors also differ from the other subtypes of ATP-gated channels in their rectification properties. P_{2x2} receptors show strong inward rectification, whereas P_{2x3} receptors have weak rectification properties (Fig. 5). Conceivably, determinants in the C-terminal region of P_{2x2} receptors, which is 84 residues longer than the homologous domain of P_{2x3}, are involved in the channel rectification. P_{2x3} receptors have a significantly high Ca^{2+} permeability, as do P_{2x1} receptors; however, the permeability of P_{2x2} receptors to divalent ions is low (Brake et al., 1994). The atypical H5 region found in P_{2x2} channels may underlie this lack of permeability for Ca^{2+} (Fig. 2*B*).

To date there has been only one report demonstrating that ATP mediates fast excitatory synaptic transmission in the CNS. Edwards et al. (1992) reported that neurons from the medial habenula showed evoked and miniature synaptic currents mediated by a purinergic receptor with pharmacological and electrophysiological properties of the P_{2x} type. Our *in situ* hybridization results indicate that P_{2x3} mRNA is present throughout the brain, suggesting that fast purinergic transmission mediated by P_{2x3}-containing ATP receptors could occur at many central synapses. Our anatomical data corroborate the widespread distribution of P_{2x} receptors estimated by the density of high-affinity binding sites for tritiated $\alpha\beta$-methylene-ATP in rat brain (Michel and Humphrey, 1993; Bo and Burnstock, 1994). The marked expression of P_{2x} receptors in the cerebellar cortex, mainly in the Purkinje and the granular cell layers, correlates with quantitated autoradiographic data (Bo and Burnstock, 1994). Interestingly, the existence of a purinergic excitatory transmission in the cerebellum has not been reported so far. It is possible that the ubiquity of the P_{2x3} receptor, in the cerebellum in particular and in rat brain in general, reflects a generic role of ATP in the presynaptic modulation of neurotransmitter release rather than the involvement of ATP as a mediator of direct purinergic transmission in various postsynaptic elements (Li and Perl, 1995). The presence of P_{2x3}-receptor mRNA in hippocampal CA3 pyramidal cells is of particular functional relevance, because the mossy fibers that synapse on these neurons contain one of the highest concentrations of Zn^{2+} in the brain (Crawford and Connor, 1972). Zn^{2+}, which potentiates P_{2x3} receptors, is released from these terminals in a calcium-dependent manner (Assaf and Chung, 1984). It is possible, therefore, that part of the neuronal hyperexcitability of the hippocampus induced by zinc (Reece et al., 1994) relates to a direct effect of this metal on P_{2x3} ATP-gated channels expressed in CA3 pyramidal cells. Furthermore, kainate-induced epileptic seizures, which cause CA3 pyramidal cell death (Nadler et al., 1978), lead to Zn^{2+} release (Assaf and Chung, 1984). In this context, the upregulation of the P_{2x1} ATP-gated channel expression in thymocytes during apoptosis (Owens et al., 1991; Zheng et al., 1991) could underlie a general relationship between the expression of P_{2x} receptors and cell death. The identification of central subtypes will help elucidate possible roles for excitatory ATP-gated channels in signaling, in neuronal death during brain development, and in epileptogenic conditions.

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