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Facilitation of Neurotransmitter and Hormone Release by P2X Purinergic Receptors

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1. Introduction

Nucleotides are emerging as ubiquitous family of extracellular signaling molecules. Their effects are mediated through a specific class of plasma membrane receptors called purinergic P2 receptors that, according to the molecular structure, are further subdivided into two subfamilies: P2Y and P2X. Specifically, P2X receptors (P2XRs) are ligand-gated ion channels, whereas P2Y receptors (P2YRs) belong to the superfamily of G-protein-coupled receptors.

Purinergic P2XRs are expressed in a wide range of organisms from amoeba to humans (Fountain et al., 2007). In mammals, seven P2X subunits (termed P2X1-7) have been found (North, 2002). These receptors appeared early in evolution and have a widespread distribution on many neurons and non-neuronal cells. The P2XRs comprise the family of trimeric channels that use the energy of extracellular ATP binding to initiate a depolarizing flux of cations, including calcium, through the pore of channels. The extracellular actions of ATP are terminated by ectonucleotidases, leading to the generation of ADP, a primary agonist for some P2YRs, and adenosine, the common agonist for adenosine subtypes of receptor (Ralevic and Burnstock, 1998). Substantial progress has been made in elucidating the roles these receptors play under physiological and pathological conditions and in our understanding of the functional, structural, and pharmacological properties of seven P2X receptor subtypes. Purinergic signaling is involved in several basic physiological responses such as embryonic and stem cell development, pain sensation, regulation of renal blood flow, inflammatory responses, auditory neurotransmission etc., whereas pathophysiology of purinergic signalling includes stroke, thrombosis, osteoporosis, kidney failure, bladder incontinence, cystic fibrosis, dry eye, cancer and brain disorders (Khakh and North, 2006; Surprenant and North, 2009; Burnstock, 2011). In excitable cells, P2XR activation causes an increase in the cytosolic Ca^{2+} concentration via two distinct mechanisms: by membrane depolarization resulting in voltage-dependent Ca^{2+} entry and by Ca^{2+} entry through the P2XR itself. The role of P2XR involves fast synaptic transmission mediated by ATP in both the peripheral (Evans et al., 1992) and central nervous systems (Edwards et al., 1992), modulation of neuronal excitability (Khakh and Henderson, 1998), long-term potentiation (Sim et al., 2006), and stimulation of hormone secretion (luteinizing hormone, prolactin, oxytocin and vasopressin) (Kapoor and Sladek, 2000; Stojilkovic, 2009; Stojilkovic et al.,

2010b). ATP is also the dominant messenger for neuron-glia communication (Guthrie et al., 1999; Newman, 2003; Fields and Burnstock, 2006).

This review summarizes recent investigations and our contribution to the knowledge about the molecular structure and mechanism of function of P2X receptors, and also how do they facilitate neurotransmitter release in the brain and secretion of hormones in pituitary.

2. P2X receptors

The P2XRs are ATP-gated non-selective cation channels which are permeable to Na^+, K^+, Ca^{2+} and small organic cations (Valera et al., 1994; Egan and Khakh, 2004). The discovery of P2XRs introduced a third class of ligand-gated ion channels (North, 1996), distinct from the first class, represented by nicotinic acetylcholine receptors, γ-aminobutyric acid (GABA) receptors, glycine receptors and 5-hydroxytryptamine receptors, and the second class, represented by glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPAR) and N-methyl-D-aspartate receptors (NMDAR). In mammals, seven genes encode the P2XR subunits (North, 2002) which can form functional channels as homo- and heterotrimers (Lewis et al., 1995; Nicke et al., 1998; Stoop et al., 1999). The distinct P2XR subtypes are functionally differentiated by comparisons in their sensitivity to ATP and its analogs, antagonists and allosteric modulators. Prolonged application of ATP causes a decrease in the conductivity of some P2XRs, a process termed receptor desensitization, which is also receptor-specific (North, 2002). For P2X1R and P2X3R the rate of desensitization is very fast - within milliseconds, the P2X4R desensitizes within seconds. The P2X2R and P2X7R do not desensitize, but their pore diameter increases in the continuous presence of agonist (Virginio et al., 1999). Subunit P2X6 does not form functional homomeric channels as it is retained in the endoplasmic reticulum in the monomeric form (Barrera et al., 2005; Ormond et al., 2006), but its heteromeric assemblies are functional (Le et al., 1998; King et al., 2000). So far, three combinations of heteromeric P2X6R channels have been characterized in functional and biochemical studies: P2X1+P2X6 (Nicke et al., 2005), P2X2+P2X6 (King et al., 2000) and P2X4+P2X6 (Le et al., 1998).

Various subclasses of P2XRs are activated with different potencies to ATP and its analogs. Low micromolar concentrations of ATP activate most of P2XRs, only the P2X7R requires millimolar concetrations of ATP. The P2X1R and P2X3R, are also potently activated by αβ-methylene ATP (αβmeATP) and the P2X7R by 2′,3′-0′,3′-O-(4-benzoylbenzoyl)-ATP (BzATP) in micromolar range (Jacobson et al., 2002); even though BzATP is also an agonist for P2X1, P2X3 and P2X4 receptors. P2XRs are inhibited by pyridoxalphosphate-6-azophenyl-2', 4'-disulfonic acid (PPADS) and suramin, which are both non-selective P2X antagonists (Coddou et al., 2011). Pharmacological experiments revealed two distinct features of P2X4R: relative resistance to suramin and PPADS (Buell et al., 1996), and robust sensitivity to positive modulatory effect of ivermectin, a high molecular weight lipophilic compound used as an antiparasitic agent in human and veterinary medicine (Burkhart, 2000), whereas other subtypes of P2XR family are ivermectin-insensitive (Khakh et al., 1999; Jelinkova et al., 2006). Among P2XRs, the P2X7R is unique as it gradually develops permeability to organic cations, causing a sustained current growth accompanied with cell blebbing and death (Surprenant et al., 1996; Di Virgilio et al., 1998; Mackenzie et al., 2005; Adinolfi et al., 2010; Yan et al., 2010). The P2X7Rs are blocked by KN62, and reactive blue-2, and also by a more potent and selective antagonists *N*-[2-[[2-[(2–hydroxyethyl)amino]ethyl]

amino]-5-quinolinyl]-2-tricyclo[3.3.1.13,7]dec-1-ylacetamide dihydrochloride (AZ 10606120) (Michel et al., 2007) and 3-[[5-(2,3-dichlorophenyl)-1*H*-tetrazol-1-yl]methyl] pyridine hydrochloride (A 438079) (Nelson et al., 2006).

The P2XR subtypes are functionally differentiated also by comparisons in their calcium permeability which is relatively high, in the range from 2.7 % (P2X3R) to 12.4 % (P2X1R) of total ATP-induced current (Egan and Khakh, 2004).

2.1 Molecular structure of P2X receptors

Each P2XR subunit consists of a large extracellular domain which binds agonists, two α-helical transmembrane domains (TM1 and TM2) and cytoplasmic N- and C-termini (Valera et al., 1994; Egan et al., 2004). There are 10 conserved cysteine residues in the ectodomain that form five intrasubunit disulfide bonds (SS1-SS5) (Clyne et al., 2002; Ennion and Evans, 2002; Rokic et al., 2010). Using the baculovirus-Sf9 cell expression system, the P2X2R was expressed and purified, and its structure was observed using electron microscopy. These images showed that the P2X2R protein resembles an inverted three-sided pyramid 215 Å in height and 200 Å in side length (Mio et al., 2005), providing visual evidence of the trimeric composition of the P2XR family. This shape has been in principle confirmed by crystalization of zebrafish P2X4.1R (Kawate et al., 2009). Crystal structure shows that extracellular domain of P2XR trimer, rich in beta-strands, contains three non-canonical, intersubunit ATP-binding sites and three major subunit-to-subunit contacts, the role of which is still unknown. There are no contacts between the subunits at the base of the extracellular domain, proximal to the TM1 and TM2 domains; these lateral fenestration are suggested to provide a possible pathway for ions to enter and exit the channel pore (Kracun et al., 2010; Kawate et al., 2011). Crystal was solved in the absence of ATP and shows P2X4.1R in its closed state; subunit rearrangement after ATP binding and subsequent ion channel opening thus remains to be elucidated.

2.2 Role of TM2 in receptor function

Functional studies predict that both transmembrane helices move during gating (Li et al., 2004; Silberberg et al., 2005) and the P2XR apparently forms a parallel six-helix bundle, in the center of which is an aqueous cavity (Duckwitz et al., 2006; Li et al., 2011). While TM2 plays a key role in the formation of the ion pore and selectivity filter during receptor activation (Rassendren et al., 1997; Egan et al., 1998; Haines et al., 2001b; Haines et al., 2001a; Jiang et al., 2001; Migita et al., 2001; Li et al., 2004; Khakh and Egan, 2005; Silberberg et al., 2005; Kawate et al., 2009; Kracun et al., 2010) and is also critical as a hydrophobic anchor by which the receptor is fixed in the membrane (Torres et al., 1999), a contribution from TM1 to channel gating has also been suggested (Haines et al., 2001b; Haines et al., 2001a; Jiang et al., 2001; Samways et al., 2008; Jindrichova et al., 2009). In particular, TM2 residues Thr336, Thr339 and Ser340 (P2X2R numbering) contribute to formation of the selective filter, the narrow region in the channel pore, (Migita et al., 2001; Egan and Khakh, 2004). Conserved TM2 residue Asp355 (P2X5 numbering, human receptor form) has also been shown to be important for this function and it initiates oligomerization of subunits in the membrane (Duckwitz et al., 2006). It is clear that different residues are involved in the formation of selectivity filter of the other P2XR subtypes because their transmembrane helices are only 39-55% identical with the P2X2 subunit (North, 2002). For example, residues Gly340 and

Leu343 are important for P2X4R, in addition to Ser341 (position Thr336 in P2X2) and Ala344 (Thr339 in P2X2) (Jelinkova et al., 2008). The gating properties of P2X channels are affected by alanine mutation of conserved TM2 residue Gly342 that exhibited reduced sensitivity to ATP in the P2X2R (Li et al., 2004) and P2X4R (Jelinkova et al., 2008). This residue has been suggested to play a role in helix motion as a point of local flexibility, acting like a hinge between the lower and the upper part of TM2 (Khakh and Egan, 2005). The region between P2X2R residues Gly342 and Asp349 most probably contributes to formation of the channel pore gate (Egan et al., 1998). As mentioned above, the P2X2 and P2X7 receptors display a time- and activation-dependent increase in large cation permeability (Virginio et al., 1999). Dilation of the pore could proceed due to channel rearrangements that occur at the interface between TM1 and TM2 of neighboring subunits (Jiang et al., 2003; Khakh and Egan, 2005). Functional studies identified three TM1 residues (Phe31, Arg33 and Gln37) and six TM2 residues (Ile328, Ile332, Ser340, Gly342, Trp350 and Leu352) that might be involved in the increase of pore diameter at P2X2R (Khakh and Egan, 2005).

2.3 Role of TM1 and molecular basis of calcium conductivity

A study performed on several subtypes of P2X receptors revealed a key role for aromatic residues in the upper part of TM1 in sensitivity to agonist (Jindrichova et al., 2009). Out of several aromatic residues of TM1, Tyr42 (P2X4 numbering) is the only residue that is fully conserved among all species examined thus far (Bavan et al., 2009). Alanine or cysteine substitution of conserved TM1 tyrosine generated a constitutively active channel that exhibited enhanced ATP sensitivity in P2X2R (Haines et al., 2001a; Li et al., 2004), P2X3R (Jindrichova et al., 2009; Jindrichova et al., 2011) and P2X4R (Jelinkova et al., 2008; Jindrichova et al., 2009). This residue is important also for other receptor functions: it has been suggested to control Ca^{2+} permeability as an inter-pore binding site for Ca^{2+} in P2X2R (Samways and Egan, 2007), to link TM1 with TM2 of adjacent subunit to control P2X4R deactivation (Stojilkovic et al., 2010a) (Fig.1) or to stabilize desensitized states in P2X3R (Jindrichova et al., 2011). High Ca^{2+} permeability of P2X1R and P2X4R has been ascribed to negatively charged ectodomain residues glutamate and aspartate, localized near the membrane at the end of TM1 and at the beginning of TM2 (Samways and Egan, 2007). However, negatively charged residues are also present at the same positions in P2X3R and P2X7R which exhibit relatively low Ca^{2+} permeability (Samways and Egan, 2007) indicating that other residues are also involved in calcium conductivity of P2XRs. The dilatation of P2X2R and P2X7R channel pore is also accompanied by abnormal calcium influx.

These properties, particularly the high throughput for Ca^{2+} ions, account for numerous physiological functions stimulated by ATP and P2XR in the central nervous system. These involve an increase in neuronal activity (Khakh et al., 2003), potentiation of neurotransmitter release (Sperlagh et al., 2007) and stimulation of hormone secretion (luteinizing hormone, prolactin, oxytocin and vasopressin) (Kapoor and Sladek, 2000; Stojilkovic, 2009; Stojilkovic et al., 2010b).

A, Three-dimensional model and positions of the first transmembrane domains (C) and the second transmebrane domains (N) of the rat P2X4R as viewed from the extracellular side. Individual subunits are differentiated by color (gray, green and blue). The Tyr42 residue (red) is between TM1 and TM2 helices of adjacent subunits and its side chain is in close proximity to residues Ile333, I337 and Met336 (orange) from the adjacent subunit, forming a

network of hydrophobic interactions B, The structural model shows Tyr42 side chain interactions with residues in TM2 helix of neighboring subunit (blue). The distance between OH:Tyr42 and TM2 atoms Ile333, Ile337 and Met336 are indicated using dashed lines. The figure was made using PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.).
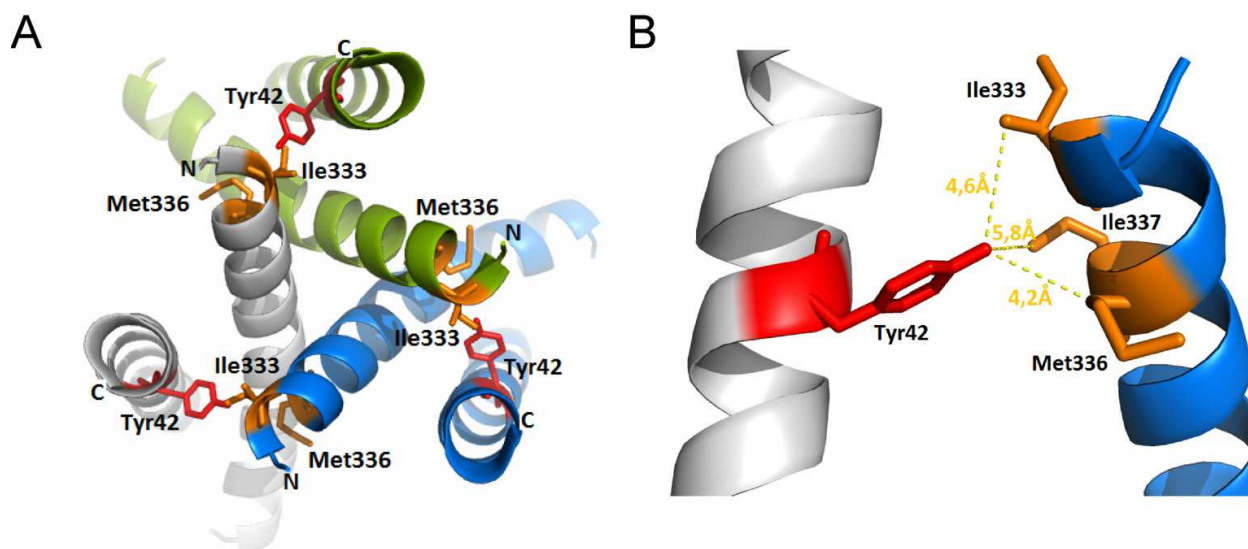

Fig. 1. Homology modeling of TM domains of P2X4 receptor.

3. Modulatory effect of P2X receptors on synaptic transmission

Following the discovery of purinergic neurotransmission in 1972 in non-adrenergic, non-cholinergic inhibitory nerves in guinea-pig taenia coli, ATP was identified as a co-transmitter in both sympathetic and parasympathetic peripheral nerves and latter also in the central nervous systems (Burnstock, 2011). Of all tissues investigated, the mammalian brain has the highest levels of purines and the greatest variety of the ATP-binding P2XRs (Buell et al., 1996; Collo et al., 1996; Seguela et al., 1996). Both neurons and glial cells release ATP and express P2XRs (Fields and Stevens, 2000; Raivich, 2005; Inoue et al., 2007) and it is common that several subtypes of P2XRs are expressed in the plasma membrane of one cell (Abbracchio et al., 2009). Neurons release ATP by exocytosis together with other neurotransmitters, such as GABA, glycine, glutamate and noradrenaline (Jo and Schlichter, 1999; Robertson et al., 2001; Sokolova et al., 2001; Jo and Role, 2002; Day et al., 1993). Glia have been shown to release ATP in response to mechanical and electrical stimulation (Newman, 2003; Burnstock, 2004), although the precise mechanisms have not been identified. The most frequent receptor forms in the brain are P2X2, P2X4, and P2X6 as well as heteromers composed of P2X2+X6, P2X4+X6, and perhaps P2X1+X4 receptors (Buell et al., 1996; Collo et al., 1996). Activation of P2X receptors by extracellular ATP acts mainly as a short-term signal but has also several long-term effects. The short-term effects involve fast synaptic transmission mediated by ATP in both the peripheral (Evans et al., 1992) and central nervous systems (Edwards et al., 1992), modulation of neuronal excitability (Khakh and Henderson, 1998) and long-term potentiation (Sim et al., 2006). Long-term (trophic) role comprise cell proliferation, differentiation and death, growth of axons during development and regeneration (Heine et al., 2006; Burnstock, 2011). Like other transmitters, ATP can

mediate both reciprocal interactions between neurons and glia, and intercellular communication in astrocytic networks (Guthrie et al., 1999; Newman, 2003; Fields and Burnstock, 2006; Burnstock, 2007).

3.1 Expression of P2X receptor subtypes in hypothalamus

Purinergic P2X receptors are expressed throughout the hypothalamus where ATP, co-released with neuropeptides, appears to be involved in the regulation of hormone secretion (Troadec et al., 1998; Kapoor and Sladek, 2000) and control of specific autonomic functions including the central mechanism of body temperature regulation (Gourine et al., 2002), for example. P2X2, P2X4 and P2X6, but not P2X5 receptor mRNAs have been identified in the rat supraoptic nucleus (SON), ventromedial nucleus, paraventricular nucleus, arcuate nucleus, suprachiasmatic nucleus, and several other hypothalamic areas using in situ hybridization; the P2X1, P2X3 and P2X7 receptors were not tested in this study (Collo et al., 1996). Another PCR analysis revealed that P2X2, P2X3, P2X4, P2X6, and P2X7 receptor mRNAs are expressed in the rat SON neurons; P2X1 and P2X5 mRNAs were not found (Shibuya et al., 1999). In the preoptic area of the rhesus monkey, the P2X2, P2X4 and P2X7 receptor mRNAs, but not P2X1, P2X3 and P2X5 receptor mRNAs, were identified; subunit P2X6 was not examined (Terasawa et al., 2005). Immunohistochemistry shows local differences in P2XR protein distribution in the rat hypothalamus. For example, the P2X2R immunoreactive neurons and nerve fibers have been found to be localized in the paraventricular nucleus, arcuate nucleus, retrochiasmatic area, periventricular nucleus, the ventral part of tuber cinereum area, supraoptic, circular, and ventral tuberomammillary nuclei, organum vasculosum, and median eminence (Xiang et al., 1998; Yao et al., 2003), but are absent in the ventromedial nucleus (Vulchanova et al., 1996; Xiang et al., 1998). The magnocellular neurons from paraventricular and supraoptic nuclei synthesize vasopressin and oxytocin and transport them to the axonal terminals in the posterior pituitary where they are secreted into the general circulation. Double-labeling fluorescence immunohistochemistry has shown that both vasopressin- and oxytocin-containing neurons in the SON express P2X2, P2X4 and P2X5 receptor proteins (Guo et al., 2009). P2X2R-positive immunoreactivity has been found both on SON somata and nerve fibers (Yao et al., 2003; Vulchanova et al., 1996; Xiang et al., 1998; Shibuya et al., 1999; Loesch and Burnstock, 2001). Our quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis showed the expression of mRNAs of all P2XR subunits and several P2YRs in SON-containing tissue; the expression of mRNAs for ionotropic P2X2R > P2X7R > P2X4R subunits was the most significant (Fig.2) (Vavra et al., 2011). Thus hypothalamus has a capacity to express both P2XR and P2YR.

Functional studies showed that application of ATP caused an increase in Ca^{2+} in cultured hypothalamic neurons (Chen et al., 1994), acutely isolated SON neurons and glial cells (Shibuya et al., 1999), SON neurons in hypothalamo-neurohypophyseal system explants (Song et al., 2007) and rat brain slices (Vavra et al., 2011). Application of ATP also stimulates the release of vasopressin and oxytocin in perfused explants of the hypothalamo-neurohypophyseal system (Gomes et al., 2009) and isolated neurohypophysial nerve terminals of the rat (Troadec et al., 1998). These ATP-induced effects were attenuated by the P2X receptor antagonists suramin and PPADS indicating that P2X2Rs or P2X3Rs play a role. Extracellular recordings showed that ATP application increases excitability of SON neurons in the hypothalamus (Day et al., 1993) and intracellular recordings from SON neurons in
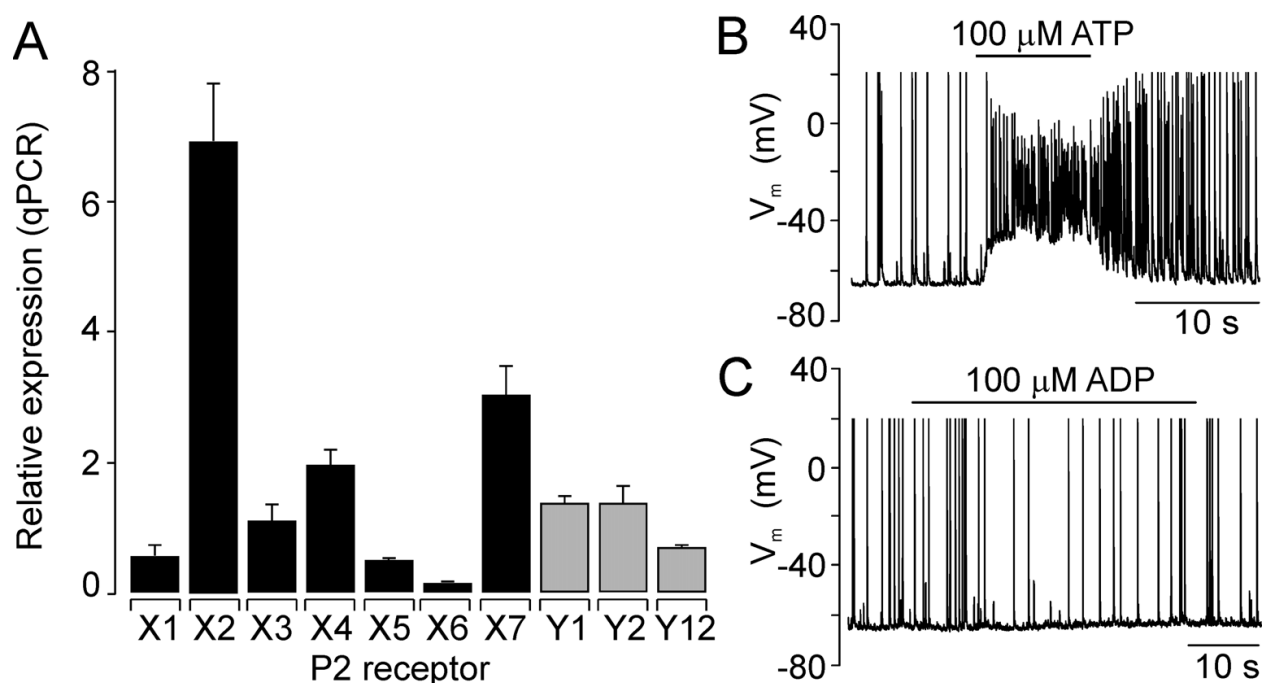
Fig. 2. Expression and function of P2X receptors in hypothalamus.

acute hypothalamic explants revealed that it was due to depolarization of membrane potential following exposure to ATP (Hiruma and Bourque, 1995). In voltage-clamped SON neurons of rat brain slices, application of ATP (10–100 µM) stimulated inward currents that were potentiated by acidic pH and attenuated by PPADS suggesting the involvement of P2X2R (Vavra et al., 2011). Similar ATP-evoked currents were recorded from neuronal somata of acutely dissociated SON neurons (Shibuya et al., 1999) and isolated terminals of the hypothalamic neurohypophysial system (Knott et al., 2005).

There are also evidences for the contribution of P2X4Rs, which are particularly prevalent in the central nervous system and hypothalamus (Illes and Ribeiro, 2004; Collo et al., 1996; Stojilkovic, 2009; Jo et al., 2011). Ivermectin, a receptor-specific allosteric modulator (Khakh et al., 1999) caused more than 2-fold augmentation of the maximum amplitude of ATP-evoked current and prolonged the ATP-stimulated calcium responses in SON neurons of slices (Vavra et al., 2011). A similar potentiating effect of ivermectin has been shown previously in neurons from the somato-sensory cortex (Lalo et al., 2007) and pituitary lactorophs that endogenously express homomeric or heteromeric P2X4Rs (Zemkova et al., 2010). The novel inhibitor of recombinant P2X4Rs, 5BDBD (Wu et al., 2011), failed to affect ATP-induced responses in neurons (Vavra et al., 2011), however, it may be ineffective on heteromeric P2X4Rs which prevail in the brain. The presence of the functional P2X2R and P2X4Rs on SON neurons was further corroborated by the qRT-PCR experiments, which demonstrated significant expression of mRNA transcripts for these receptors in SON tissue (Vavra et al., 2011). Significant level of mRNA for P2X7R was also observed but P2X1, P2X3, P2X5 and P2X6 mRNA expression levels were minor. The high mRNA level for P2X7 can be ascribed to P2X receptors expressed in non-neuronal cells, such as microglia (Collo et al., 1997), because the currents of SON neurons were not affected by BzATP, but non-neuronal SON cells in slices responded with an increase in intracellular calcium (Vavra et al., 2011).

ATP-evoked inward current mediated by P2X2 or P2X4 receptors, as observed in SON neurons, likely represents a particular example of a more general function of P2XRs: to make voltage-threshold more positive and to drive neurons to fire action potentials. We suppose the functional role of somatic P2XRs in the brain consists in controlling excitability close to the nominal resting potential of the neuron cell body, near − 60 mV (Vavra et al., 2011).

A, The presence of seven P2XRs (black columns) and three P2YRs (gray columns) mRNA transcripts in hypothalamic tissues from 16 day-old rats. Quantitative RT-PCR analysis shows significant expressions of ionotropic P2X2, P2X4 and P2X7 receptor mRNAs and moderate levels of mRNA for P2X3, P2Y1 and P2Y2 receptors. The mRNA levels of P2 receptor genes were related to the expression of GAPDH as a housekeeping gene /endogenous control. B,C Example recordings of action potentials from the SON neuron in slices before, during and after the application (bar) of 100 μM ATP (B) and 100 μM ADP (D). ATP induced depolarization and an increase in frequency of action potentials. Neither effect was observed after the application of ADP which had a tendency to decrease the frequency of action potentials. All neurons had resting membrane potential (Vm) lower than -50 mV when measured in current clamp mode. For details see: (Vavra et al., 2011).

3.2 Modulation of glutamate and GABA release by presynaptic P2XRs

ATP is viewed as a neurotransmitter in both the peripheral (Evans et al., 1992) and the central nervous system (Pankratov et al., 1998). It has been suggested that endogenously released ATP acts at postsynaptic P2XRs to mediate synaptic currents in the hippocampus (Edwards et al., 1992). This classical view, however, is challenged by a series of recent studies that showed that ATP acts rather as a modulator that stimulates the release of other neurotransmitters (Li et al., 1998; Sperlagh et al., 2002; Watano et al., 2004; Rodrigues et al., 2005; Kodama et al., 2007; Sperlagh et al., 2007; Donato et al., 2008). In the SON neurons, for instance, all spontaneous synaptic currents were inhibited by blockers of glutamate and GABAA receptors and application of ATP stimulated about 2300 % increase in frequency of both spontaneous inhibitory and excitatory postsynaptic currents without changes in their amplitude (Fig.3) (Vavra et al., 2011). This finding suggests that ATP does not act as a spontaneously released neurotransmitter, but as a modulator that stimulates the release of GABA or glutamate (or both) by activating purinergic receptors in the nerve terminals (Vavra et al., 2011). These experiments were performed in the presence of tetrodotoxin, which inhibits action potentials, thus P2XRs are implicated in presynaptic function by stimulating Ca^{2+} influx into the nerve terminals. Known presynaptic P2XR subtypes with a role in neurotransmission include P2X1, P2X2, P2X3 and possibly P2X4 and P2X7 receptors.

P2X1Rs have been localized and implicated in the function of sympathetic ganglion neurons (Calvert and Evans, 2004), cerebellum (Hervas et al., 2003) and spinal cord dorsal horn sensory neurons (Nakatsuka et al., 2003). Presynaptic P2X1R have been found to mediate agonist-evoked release of tritium-labeled glutamate from purified nerve terminals of the rat hippocampus (Rodrigues et al., 2005). Facilitation of both glutamate and GABA release mediated by presynaptic P2X1R was observed in the rat medial nucleus of the trapezoid body (Watano et al., 2004). Activation of presynaptic P2X1R elicits noradrenaline release from central catecholaminergic terminals in superfused rat hippocampal slices (Papp et al., 2004). These receptors are present in high concentrations on some smooth muscle cells

(Valera et al., 1994). Another role of the P2X1R in the brain thus might be in the smooth muscle of small arteries and arterioles, allowing the control of blood flow and pressure.
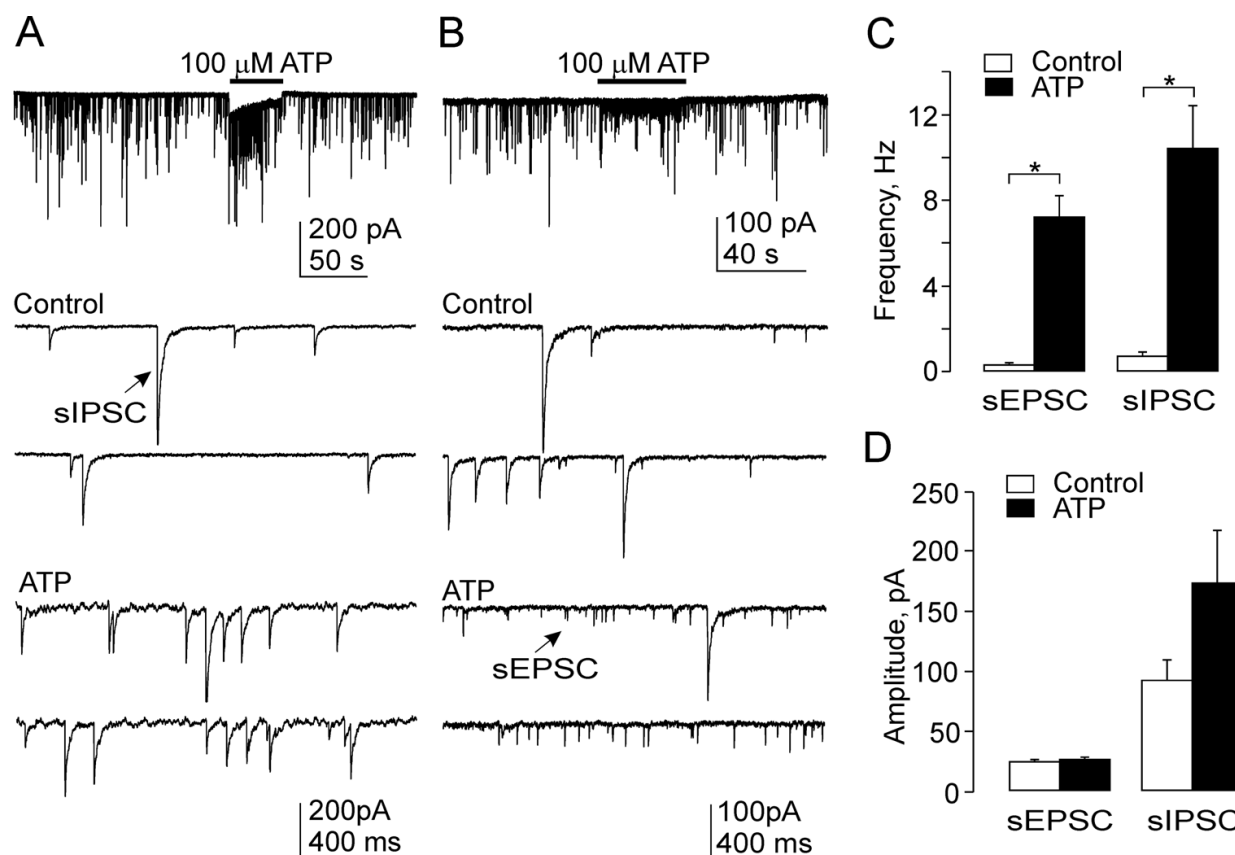

Fig. 3. Presynaptic effect of ATP on spontaneous glutamate and GABA release.

A, ATP (100 µM) induced inward current and increase in the frequency of spontaneous GABAergic inhibitory postsynaptic currents (sIPSC). B, ATP induced increases in the frequency of spontaneous glutamatergic excitatory postsynaptic currents (sEPSC). Traces in an expanded time scale show spontaneous synaptic currents before (Control) and after ATP application (ATP). Glutamatergic and GABAergic spontaneous postsynaptic currents differ in their amplitudes and duration. Experiments were performed on SON neurons voltage clamped at -60 mV. C,D, Summary histograms showing the effect of ATP on the frequency (C) and amplitude (D) of sEPSCs and sIPSCs. Analysis was performed on SON neurons in rat brain slices. (*) P < 0.01. For details see: (Vavra et al., 2011).

Numerous studies have shown that P2X2Rs act presynaptically to increase glutamate in several brain areas. Experiments with knockout mice showed that Ca^{2+} entry through presynaptic P2X2Rs increases the frequency of spontaneous AMPA receptor-mediated glutamatergic currents in GABAergic hippocampal interneurons (Khakh et al., 2003). Furthermore, inhibiting P2XRs by application of PPADS has been shown to abolish the glutamate-dependent postsynaptic currents evoked by focal application of ATP in dorsal horn neurons (Li et al., 1998 Nakatsuka and Gu, 2001). PPADS-sensitive P2X2Rs have been shown in glutamatergic terminals of neurons in trigeminal mesencephalic motor nucleus (Khakh and Henderson, 1998), the nucleus tractus solitari (Shigetomi and Kato, 2004) and the area postrema (Kodama et al., 2007). The presynaptic P2X2Rs have been shown to

underlie increase in GABA release in a subset of GABAergic interneurons in the spinal cord (Hugel and Schlichter, 2000) and in Purkinje cells in rat cerebellar slices (Donato et al., 2008). In SON neurons, the ATP-induced increases in the frequency of spontaneous excitatory and inhibitory postsynaptic currents were also inhibited by PPADS and potentiated by pH 6.5, indicating the involvement of presynaptic P2X2Rs in the release of both neurotransmitters (Vavra et al., 2011).

The P2X3Rs are almost exclusively expressed in dorsal root ganglion (DRG) neuronal processes that are involved in acute pain (Vulchanova et al., 1997; Dunn et al., 2001; North, 2004). Immunocytochemistry for P2X3 subunits revealed that these receptors are expressed in small- and medium-sized neurons but are absent from large diameter neurons (Vulchanova et al., 1997; Vulchanova et al., 1998; Novakovic et al., 1999). Stimulation of presynaptic P2XRs in DRG neuronal processes by ATP application induced glutamate release that activated postsynaptic glutamatergic receptors expressed in the dorsal horn neurons (Gu and MacDermott, 1997) and that was inhibited by PPADS. Presynaptic P2X3R have been also found in other brain areas, including purified nerve terminals of the rat hippocampus (Rodrigues et al., 2005), in cranial afferent neurons in nodose ganglia (Kato and Shigetomi, 2001; Jin et al., 2004), in the midbrain periaqueductal gray (Xing et al., 2008), in the rat medial nucleus of the trapezoid body (Watano et al., 2004). ATP-stimulated GABA release from rat midbrain terminals are mediated through the activation of P2X receptors with an abundance of P2X3 subunits (Gomez-Villafuertes et al., 2001). Activation of presynaptic P2X3R receptors contributes to noradrenaline release in superfused rat hippocampal slices (Papp et al., 2004).

Evidence for the presence of P2X4Rs in the nerve terminals is less obvious because selective antagonists are lacking. We showed that the ATP-induced effect on frequency of spontaneous inhibitory postsynaptic currents in the SON declined during prolonged ATP application, which indicates the involvement of a desensitizing P2X4R (Vavra et al., 2011).

BzATP has been reported to enhance release of glutamate by acting at P2X7Rs in purified rat neocortex synaptosomes (Patti et al., 2006). Possible presynaptic mechanisms underlying enhancement of excitatory transmitter release by P2X7R has been also suggested for hypoglossal motoneurons in brainstem slices (Ireland et al., 2004). However, neuronal origin of P2X7R is still uncertain. The P2X7Rs are expressed on satellite glial cells enwrapping the peripheral neurons (e.g., DRG neurons) and on microglia, astrocytes and oligodendrocytes in the brain. After repeated or prolonged exposure to ATP, P2X7R are able to form pores permeable to small molecules, and could mediate release of cytokines from satellite glial and microglial cells (Ferrari et al., 1997b; Ferrari et al., 1997a; Ferrari et al., 2006; Matute et al., 2007) or release of neurotransmitters from astrocytes. Several reports have shown that the P2X7R, and not other P2XRs, is responsible for IL-1β release from activated myeloid cells (Mehta et al., 2001; Le Feuvre et al., 2002). LPS-primed macrophages from transgenic mice lacking P2X7 receptors fail to release IL-1β in response to ATP, although synthesis of pro-IL-1β and caspase-1 is unaltered (Solle et al., 2001; Labasi et al., 2002). Activation of P2X7Rs has been reported to elicit the release of glutamate (Duan et al., 2003), GABA (Wang et al., 2002), 2-arachidonoylglycerol (Walter et al., 2004), and purines (Ballerini et al., 1996) from cultured astrocytes. In neurological diseases or injuries extracellular ATP may activate P2X7Rs further enhancing purine release, with important pathophysiological consequences (Ballerini et al., 1996). In paraventricular nucleus of hypothalamus, P2X7 receptor activation results in insertion of AMPA receptors into postsynaptic neuronal membrane (Gordon et al., 2009).

In the rat suprachiasmatic nuclei, ATP content and extracellular ATP levels (Yamazaki et al., 1994; Womac et al., 2009) negatively correlate with electrical activity and arginine vasopressin (AVP) secretion rhythm: the frequency of action potentials and the level of AVP is high during the day and low during the night, but ATP release peaks during the night. This might indicate that ATP is primarily stored and released from distinct pool of vesicles and/or that ATP and AVP are released from different cells. How ATP release from hypothalamic cells is regulated and what is the reason for local differences in distribution of P2X receptor subtypes is still being elucidated.

4. Potentiation of hormone secretion in pituitary gland

Depolarization and Ca^{2+} influx stimulated by extracellular ATP has numerous functions also in endocrine cells. These involve stimulation of luteinizing hormone, prolactin, oxytocin and vasopressin hormone secretion by pituitary gland (Kapoor and Sladek, 2000; Stojilkovic, 2009; Stojilkovic et al., 2010b). The anterior pituitary is a heterogeneous gland with multiple cell types that secrete six major peptide hormones necessary for reproduction, lactation, growth, development, metabolic homeostasis, and the response to stress: FSH and LH-producing gonadotrophs, prolactin (PRL)-producing lactotrophs, GH-producing somatotrophs, TSH-producing thyrotrophs, and ACTH-producing corticotrophs. This lobe also contains the non-hormone-producing folliculostellate cells, which are glia-like cells, and endothelial cells that line the capillaries. Pituitary hormone secretion is under control of hypothalamic neurohormones and is also modulated by extracellular ATP (Chen et al., 1995) that is released by the anterior pituitary itself in a regulated manner, or coreleased with hypothalamic peptides (Tomic et al., 1996; Lazarowski et al., 2000; Stojilkovic and Koshimizu, 2001; He et al., 2005). The mRNA transcripts for several P2X subunits (P2X2R, P2X3R, P2X4R, and P2X7R) were identified in anterior pituitary cells from neonatal (Zemkova *et al.*, 2006) and adult rats (Koshimizu et al., 2000a; Koshimizu et al., 2000b; Stojilkovic and Koshimizu, 2001), including two spliced forms of the P2X2R subunit. Experiments with the plasma membrane-targeted luciferase expressed in HEK cells or ACN neuroblastoma cells indicated that endogenous extracellular ATP concentrations are in the range of 100-200 μM, which is more than sufficient to activate all types of P2X receptors (Pellegatti et al., 2005). *In vivo*, the ATP action on gonadotroph functions could be controlled by ectonucleotidases 1-3, which are expressed in pituitary cells (He et al., 2005) and provide an effective pathway for the control of extracellular ATP concentrations.

Detection and localization of mRNA transcripts for P2X2, P2X3 and P2X4 receptors in pituitary gland by in situ hybridization. *PP,* posterior pituitary, *AP,* anterior pituitary. For details see: (Stojilkovic et al., 2010b).

Most anterior pituitary cells express functional P2X receptors (Fig.4) (Carew et al., 1994; Villalobos et al., 1997; Koshimizu et al., 2000a). The P2X2Rs were identified in pituitary gonadotrophs (Tomic et al., 1996; Koshimizu et al., 2000b; Zemkova et al., 2006) and somatotrophs (Koshimizu et al., 2000a). Lactotrophs express functional P2X4Rs (Carew et al., 1994; He et al., 2003; Zemkova et al., 2010) as well as P2X7R subtypes (Chung et al., 2000; He et al., 2003). Corticotrophs seem not to respond to extracellular ATP directly by stimulation of any P2X receptor (Zhao et al., 2006), and identification of P2X receptors in remaining anterior pituitary cell type, thyrotrophs, has not yet been done. Functional studies showed that ATP application induces inward slowly desensitizig current and increases
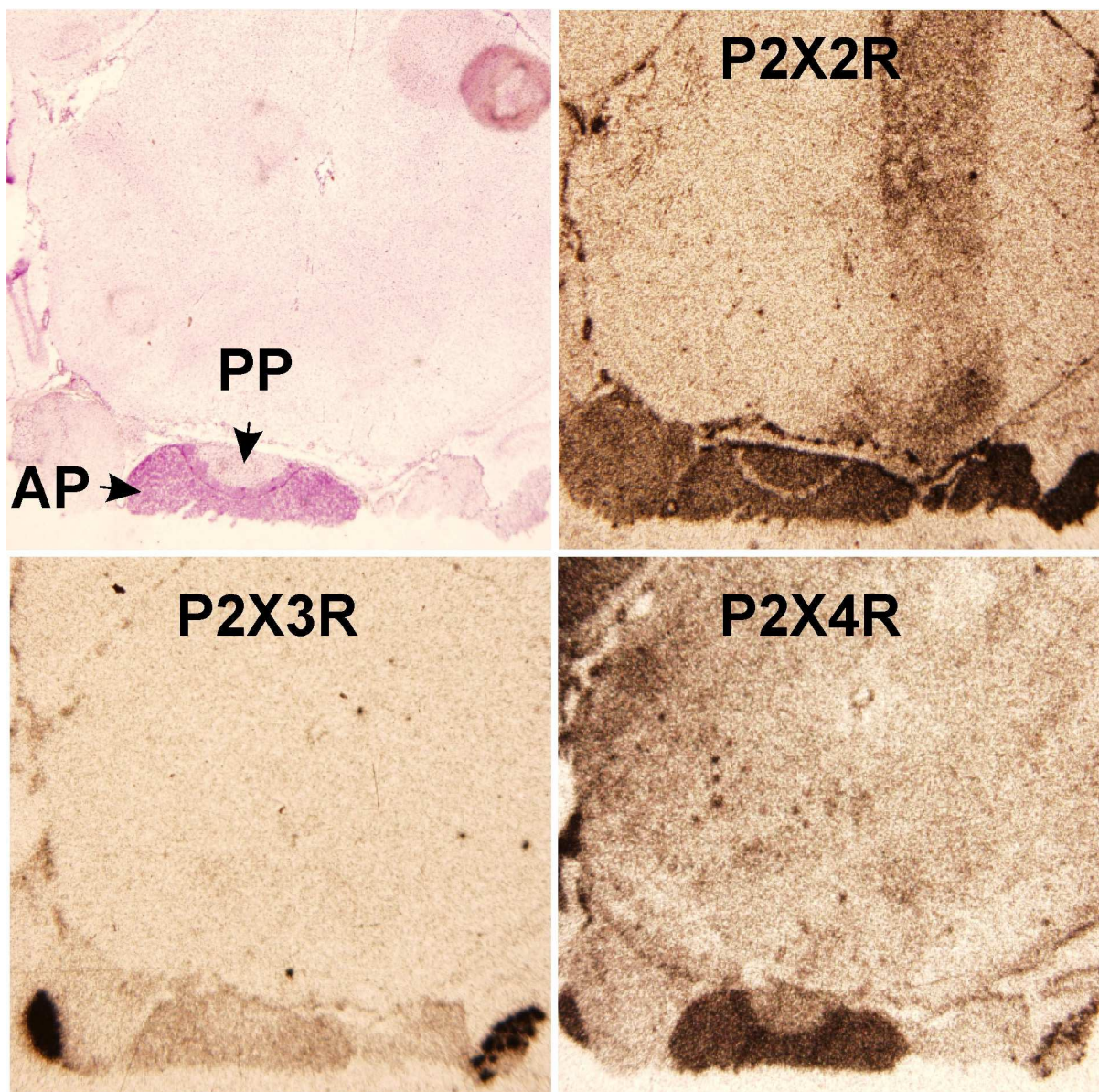
Fig. 4. Expression of P2XR in pituitary gland.

frequency of action potentials (Fig.5) in pituitary gonadotrophs. These responses were sensitive to PPADS indicating that P2X2Rs could operate as depolarizing channels in the pituitary (Zemkova et al., 2006). ATP could play the role of modulator in the anterior pituitary lactotrophs (Stojilkovic and Koshimizu, 2001), P2X4R potentiator ivermectin per se augmented ATP-induced prolactin secretion and slightly potentiated the effect of TRH in lactotrophs which express P2X4Rs (Zemkova et al., 2006). Thus in pituitary, ATP may serve to synchronize of spontaneous electrical activity, to initiate intercellular Ca^{2+} waves, as observed in other cell types (Guthrie et al., 1999) and modulate G-protein coupled receptor-stimulated Ca^{2+} signaling by refilling of intracellular stores from calcium influx through P2XR pore (Zemkova et al., 2006). Such an action of extracellular ATP could provide a mechanism for the amplification of the effects of hypothalamic peptides on pituitary hormone secretion. Further experiments should clarify to what extent these capacities of P2X receptors are utilized *in vivo*.
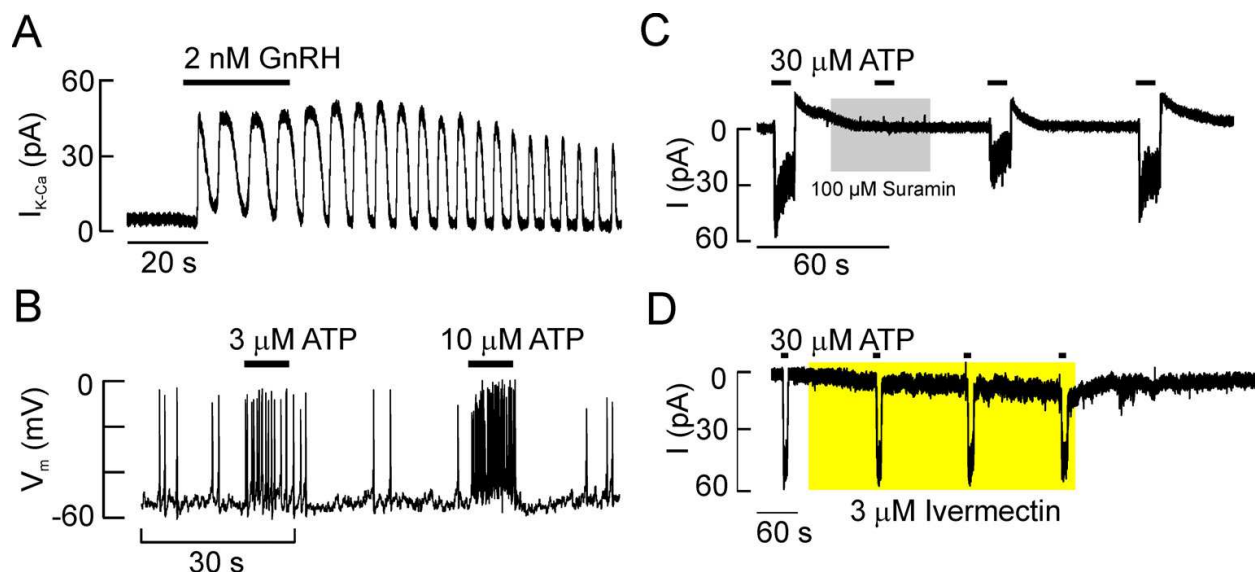
Fig. 5. Electrophysiological characterization of the P2X2R in pituitary gonadotrophs. A, Gonadotrophs can be identified in a mixed population of pituitary cells because of their specific GnRH-induced calcium oscillations monitored as outward calcium-activated potassium current (I_{K-Ca}). B, Stimulation of electrical activity by application of ATP to identified gonadotrophs. C, The sensitivity of ATP-induced current to suramine, general P2XR blocker. D, Insensitivity of ATP-induced current to ivermectin indicates that gonadotrophs express the P2X2 receptor. For details see: (Zemkova et al., 2006).

5. Conclusion

To summarize, the cellular actions of extracellular ATP and P2XRs in the brain range from modulation of resting membrane potential and electrical activity to stimulation of neurotransmitter and hormone release, including release of anterior pituitary hormones. It is clear that the effects of ATP and its analogs are realized by membrane depolarization resulting in voltage-dependent Ca^{2+} entry and by Ca^{2+} entry through the pore of P2XR itself. Nonetheless, the molecular and neurochemical mechanisms underlying these effects are far from being fully understood and likely involve multiple receptor systems and various signaling pathways. Experimental evidences gathered so far suggest that whereas neurotransmitter release seems to be linked to the activation of presynaptic P2X1, P2X2, P2X3 and perhaps P2X4 and P2X7 receptors expressed in nerve terminals, neuropeptide and hormone secretion more likely involves P2X2 and P2X4 receptors on the surface of neuronal somata and pituitary cells. Thus, extracellular ATP together with P2XRs comprise a new excitatory system in the mammalian central nervous system and an increasing number of studies support also an indirect role of P2XRs in inhibitory system owing to its ability to facilitate GABA release. However, surprisingly few studies confirmed the role of ATP as neurotransmitter in the central nervous system, whereas its modulatory roles are well established. Further studies aimed at analyzing the cellular and molecular actions of ATP in various brain regions and under different physiological states would be required for a comprehensive understanding.

6. Acknowledgements

7. References

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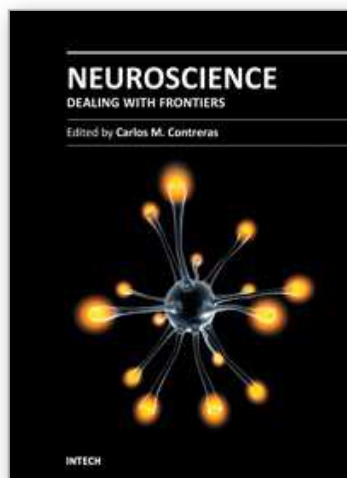
Neuroscience - Dealing With Frontiers
Edited by Dr. Carlos M. Contreras

The Neuronal Doctrine recently reached its 100th year and together with the development of psychopharmacology by the middle of 20th century promoted spectacular developments in the knowledge of the biological bases of behavior. The overwhelming amount of data accumulated, forced the division of neuroscience into several subdisciplines, but this division needs to dissolve in the 21st century and focus on specific processes that involve diverse methodological and theoretical approaches. The chapters contained in this book illustrate that neuroscience converges in the search for sound answers to several questions, including the pathways followed by cells, how individuals communicate with each other, inflammation, learning and memory, the development of drug dependence, and approaches to explaining the processes that underlie two highly incapacitating chronic degenerative illnesses.

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