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The molecular mechanisms and role of purinergic signaling in hypothalamus

Molekulární mechanismy a význam purinergní signalizace v hypotalamu

Doctoral thesis

Prague, 2021

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Proclamation**:**

In accordance with the guidelines for the Ph.D. dissertation publication at the Faculty of Sciences at Charles University in Prague, I, Milorad Ivetic, declare that the research described within this dissertation has been conducted solely by me, under supervisor RNDr. Hana Zemková, CSc, head of the the Department of Cellular and Molecular Neuroendocrinology, Institute of Physiology, Czech Academy of Sciences. I have created and presented all of the figures, unless otherwise noted, the data has been collected during the course of Ph.D. study. Credit has been given for any data or experimental findings that have been referenced from other studies and publications. This work is not used to obtain any other academic degree.

Prague, November 30, 2021.

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Milorad Ivetic

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Abstract (EN)

Extracellular ATP and purinergic P2 receptors (P2X and P2Y) are involved in a signaling network called "purinergic signaling" which is widely exploited in both somatic and neuronal tissues, and is also operative in endocrine system. The main focus of my thesis is on the role and expression of P2X and P2Y receptors in hypothalamic supraoptic nuclei (SON) producing hormones vasopressin and oxytocin, and the suprachiasmatic nuclei (SCN), the principal circadian pacemaker in mammals. In the first part of my thesis, we tested the hypothesis that P2X and P2Y receptors play a role in the enhanced release of hormones from magnocellular SON neurons stimulated through fasting/refeeding experimental protocol. We studied the effect of 2 h of refeeding after 48 h of fasting on hormone, P2X and P2Y mRNA expression in the SON tissue of 30-day-old rats, and the effect of ATP on electrophysiological properties of SON neurons in brain slices from control and fasted/refed rats. Quantitative real-time PCR revealed that the expression of P2X2 and arginine-vasopressin (AVP) mRNA was increased and P2Y1 mRNA expression was decreased in fasted/refed rats compared to controls, whereas P2X4, P2X7, P2Y2 and oxytocin mRNA levels were not significantly changed. Whole-cell patch clamp recordings showed that the amplitude of the ATP-stimulated current and the ATP-induced increases in the frequency of miniature GABAergic inhibitory postsynaptic currents were significantly increased in a subpopulation of SON neurons, most probably AVP neurons, from fasted/refed rats. These results revealed that the increase in expression and function of P2X2 receptors is significantly linked with the complex stimulation of SON neurons and synthesis of AVP under conditions of physiologically stimulated secretion of this hormone. In the second part of my thesis, we tested a hypothesis that activation of P2X7 and P2Y receptors, which have been previously shown to be expressed in the SCN tissue at transcriptional level, contribute to rhythmical release of ATP from SCN astrocytes. This hypothesis was tested using ATP bioluminescent assays, immunohistochemistry, patch-clamping and calcium imaging. We showed that circadian rhythm in extracellular ATP accumulation in medium with SCN organotypic cultures is inhibited by application of AZ10606120, A438079 and BBG, specific blockers of P2X7 receptor, and potentiated by GW791343, a positive allosteric modulator of this receptor. Double-immunohistochemical staining revealed high expression of the P2X7 protein in astrocytes of SCN slices. MRS2179, specific P2Y1 receptor antagonist, also abolished extracellular ATP accumulation, and the pannexin-1 hemichannel blocker carbenoxolone displayed a partial inhibitory effect. These results showed that SCN astrocytes utilize multiple purinergic P2 receptors and pannexin-1 hemichannels to release ATP. In conclusion, the results of this thesis indicate that the unique properties of P2X receptors could facilitate various hypothalamic functions the receptors are involved in.

These investigations are summarized in two peer-reviewed publications, one first-authored, that both form the body of my thesis.

Abstract (CZ)

Extracelulární ATP a purinerginí P2 receptory (P2X a P2Y) hrají roli v signalizačním systému zvaném "purinergní signalizace", který je hojně využíván jak somatickými tak nervovými tkáněmi, a působí také v endokrinním systému. Středem zájmu mé disertační práce je úloha a výskyt P2X a P2Y receptorů v hypotalamických supraoptických jádrech (SON) produkujících hormony vasopresin a oxytocin, a v suprachiasmatických jádrech (SCN), která představují centrum biologických hodin u savců. V první části mé práce jsme testovali hypotézu, že P2X a P2Y receptory se účastní zvýšené tvorby a sekrece hormonů v magnocelulárních SON neuronech stimulovaných prostřednictvím experimentálního protokolu hladovění/ dokrmování. Studovali jsme účinek 2 hodinového dokrmování po 48 hodinovém hladovění na expresi hormonální, P2X a P2Y mRNA v SON tkáni u 30ti denních potkanů, a účinek aplikace ATP na elektrofyziologické vlastnosti SON neuronů v mozkových řezech kontrolního a experimentálního potkana. Kvantitativní real-time PCR analýza ukázala, že exprese mRNA pro P2X2 a arginin vasopresin (AVP) je zvýšena a exprese P2Y1 mRNA snížena u potkanů vystavených hladovění/dokrmování ve srovnání s kontrolami, zatímco hladiny mRNA pro P2X4, P2X7, P2Y2 a oxytocin nebyly významně změněny. Snímání z celé buňky technikou patch clamp ukázala, že amplituda ATP-evokovaného proudu a ATP-stimulované zvýšení frekvence miniaturních GABAergních inhibičních postsynaptických proudů jsou významně zvýšeny v subpopulaci SON neuronů, pravděpodobných AVP neuronů, u potkanů dokrmovaných po vyhladovění. Tyto výsledky prokázaly, že zvýšená exprese a funkce P2X2 receptorů je úzce spřažena s komplexní stimulací SON neuronů a tvorbou AVP za podmínek fyziologicky stimulované sekrece tohoto hormonu. Ve druhé části mé disertační práce jsme testovali hypotézu, že aktivace P2X7 a P2Y receptorů, které mají v SCN tkáni relativně vysoké zastoupení na úrovni mRNA, přispívá k rytmickému uvolňování ATP z SCN astrocytů. Tuto hypoyézu jsme testovali pomocí bioluminiscenčního měření ATP, imunohistochemie, elektrofyziologie a měření intracelulárního vápníku. Zjistili jsme, že rytmická akumulace extracelulárního ATP v mediu SCN organotypické kultury je inhibována AZ10606120, A438079 a BBG, specifickými blokátory P2X7, a zvýšena GW791343, pozitivním alosterickým modulátorem tohoto receptoru. Dvojité imunohistochemické značení prokázalo expresi P2X7 proteinu v astrocytech mozkového SCN řezu. Dále bylo nalezeno, že MRS2179, specifický antagonista P2Y1 receptoru, a carbenoxolon, blokátor kanálu pannexin-1, také částečně inhibovali akumulaci extracelulárního ATP. Tímto jsme zjistili, že SCN astrocyty využívají několika typů P2 receptorů a panexinový kanál k uvolňování ATP. Celkově tyto výsledky ukazují, že unikátní vlastnosti P2X receptorů mohou podporovat různé hypotalamické funkce, do kterých jsou zapojeny.

Tyto výsledky jsou shrnuty ve dvou recenzovaných publikacích, jedné prvoautorské, které tvoří základ mé disertační práce.

Podíl práce Milorada Ivetice v autorských publikacích

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Prováděl všechna elektrofyziologická měření, krájel mozkové řezy, analyzoval data a podílel se na přípravě manuskriptu.

II. Svobodova, I., Bhattaracharya, A., **Ivetic, M.,** Bendova, Z., Zemkova, H. (2018). Circadian ATP release in organotypic cultures of the rat suprachiasmatic nucleus is dependent on P2X7 and P2Y receptors. Front Pharmacol 9:192. doi: 10.3389/fphar.2018.00192
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IF=5.372 (rok 2020)

Prováděl elektrofyziologická měření na mozkových řezech, analyzoval data

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IF=4.294 (rok 2020)

Prováděl elektrofyziologická měření na mozkových řezech, analyzoval data

Praha, 23. prosince 2021.

RNDr. Hana Zemková, CSc. školitelka

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Abbreviation list

ACSF – artificial cerebrospinal fluid
ADP – adenosine-5'-diphosphate
AMP – adenosine-5'-monophosphate
ATP – adenosine-5'-triphosphate
2MeSATP – 2-methylthio-adenosine-5′-triphosphate
αβmeATP – α,β-methyleneadenosine-5′-triphosphate
βγmeATP – β,γ-methyleneadenosine-5′-triphosphate
AVP – arginine vasopressin
AZ10606120 – negative allosteric modulator of P2X7
A438079 – negative allosteric modulator of P2X7
BBF – brilliant blue G
BIC – bicuculline
BzATP – 3´-O-(4-benzoyl) benzoyladenosine-5´-triphosphate
EC_{50} – effective concentration of agonist producing half maximal effect
GABA – γ-aminobutyric acid
$GABA_A$ – GABA receptor type A
GW791343 – positive allosteric modulator of P2X7
HEPES - N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid
mIPSC – miniature inhibitory postsynaptic current
mEPSC – miniature excitatory postsynaptic current
$NMDG^+$– N-methyl-D-glucamine ion
MRS2179 - specific antagoonist for P2Y1
MRS2365 - specific agoonist for P2Y
MRS2768 - specific agoonist for P2Y2
OT – oxytocin
P1 – purinergic receptor type 1
P2X – purinergic receptor type 2 (ionotropic)
P2Y – purinergic receptor type 2 (metabotropic)
PPADS – pyridoxalphosphate-6-azophenyl-2', 4'-disulfonic acid
PVN – paraventricular nucleus
SCM gavage – sweetened condensed milk gavage; experimental protocol
SCN – supraschiasmatic nucleus
SON – supraoptic nucleus
TTX – tetrodotoxin

1. Introduction

The hypothalamus is functionally associated with the limbic system and pituitary gland, and contains a number of small nuclei that are involved in a variety of endocrine functions and regulation of circadian rhythms. Main function of hypothalamic-neurohypophyseal system, producing oxytocin and vasopressin, is to maintain body fluid homeostasis, food intake, lactation and parturition. Through the actions of its hormones at the level of the central nervous system it is also involved in control of related behavior such as maternal and parental care (Taylor et al., 2017; Iovino et al., 2021), for example. The hypothalamic-neurohypophyseal system exhibits considerable plasticity as a function of endocrine state, and is often used as a model system for investigation of neuropeptide secretion. Food deprivation for 48h and refeeding for 2-6 h reliably stimulate synthesis and release of the vasopressin although changes in the oxytocin might be also observed (Burlet et al., 1992).

Regulation of endocrine system is very complex and extracellular adenosine-5´-triphosphate (ATP) and its degradable products adenosine-5´-diphosphate (ADP) and adenosine contribute to its regulation (Stojilkovic and Zemkova, 2013; Burnstock, 2014). Extracellular ATP-mediated signaling attracted scientific interest in research of neuroendocrine system after the early observations that locally applied ATP excites neurosecretory vasopressin neurons (Day et al., 1993) and evokes vasopressin, but no significant oxytocin, release from isolated neurohypophysial nerve terminals (Troadec et al., 1998). Neuroendocrine cells release ATP and express numerous P2X and P2Y receptors (Stojilkovic, 2009; Stojilkovic and Zemkova, 2013; Burnstock, 2014), but physiological importance of purinergic signaling in hypothalamus is still not well understood.

1.1. Purinergic signaling

Extracellular ATP and purinergic receptors are involved in network called "purinergic signaling" which is widely exploited in both somatic and neuronal tissues (Khakh and North, 2006; Illes et al., 2021). ATP and its metabolites act as extracellular ligands for two specific membrane receptor families named P1, if activated by adenosine, or P2, if activated by ATP or ADP (Burnstock, 2006a). Family of P2 receptors has been further divided into family of P2X ionotropic receptors, consisting of 7 subtypes, and family of P2Y metabotropic G protein-coupled receptors, consisting of 8 subtypes (Burnstock, 2006c).

1.1.1. ATP release and extracellular ATP accumulation

ATP is a purine nucleotide with that plays a key role in intracellular energy metabolism. It acts as an extracellular messenger involved in a variety of physiological and pathophysiological functions of the cardiovascular, endocrine, immune, central and peripheral nervous systems, as well as in inflammation and cancer (Burnstock, 1977, 1986, 2006b; Khakh and North, 2006; Illes et al., 2021). ATP may be released by cells under normal or pathological conditions (Fig. 1). The cytoplasm of most neurons contains 2-5 mM ATP while higher concentrations (up to 100 mM) can be found in the synaptic vesicles. Transport of ATP into synaptic vesicles can be distinguished from other neurotransmitter transport systems in terms of its mechanism and energy requirements (Zalk and Shoshan-Barmatz, 2003). Large amounts of ATP have also been detected in the dense-core vesicles and in the lysosomes of astrocytes

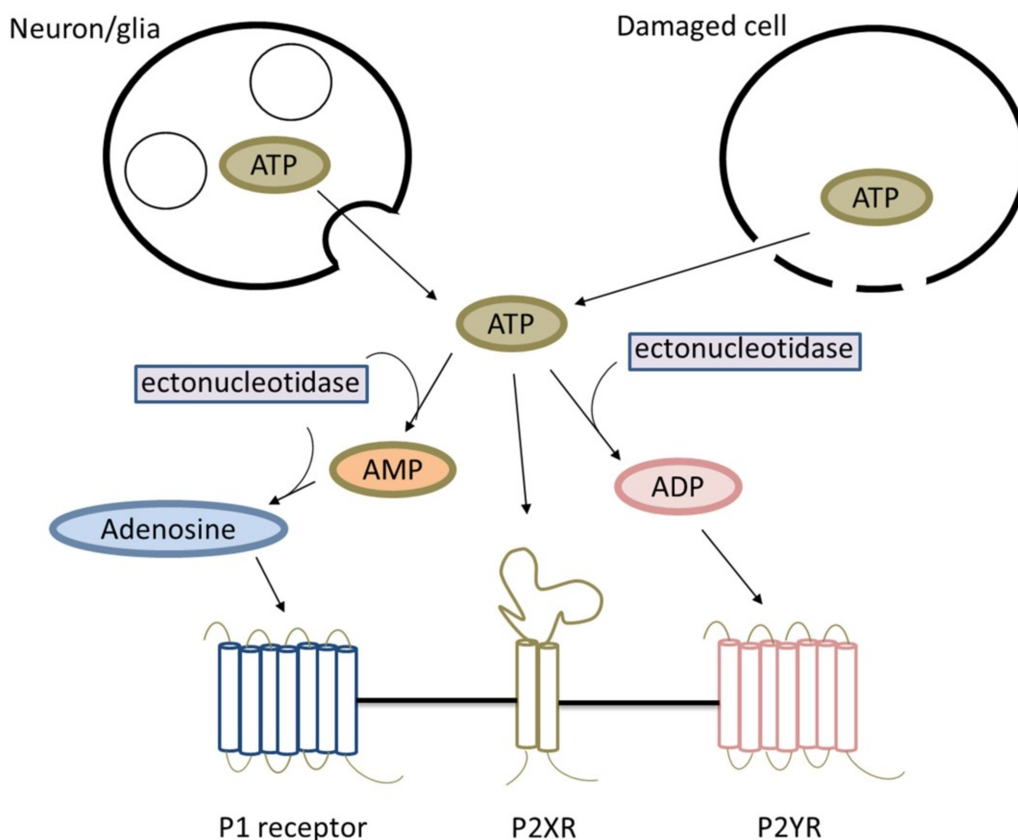

Figure 1. Scheme of purinergic signaling involving ATP, ADP, AMP, adenosine, and P1, P2X and P2Y receptors, and ectonucleotidases. ATP is released by excitable cells, as well as non-excitable cells such as glia, alone or together with different neurotransmitters. In general, excitable cells secrete ATP trough exocytosis or "kiss and run" mechanism and non-excitable cells through pannexin or connexin channels and ABC transporters. Large amounts of ATP are also released from damaged or dead cells. Outside of the cell, ectonucleotidases decompose ATP. Speed of decomposition varies in different tissues. Half-life of ATP in blood is in between 10-30 minutes and a mere 0,2 seconds in heart or lungs.

(Hong et al., 2016). Evidence for the presence and release of ATP from synaptic and secretory vesicles was described in many papers showing that it is released from both peripheral and central neurons (Holton and Holton, 1953; Burnstock et al., 1970) and from secretory pituitary cells (Li et al., 2011b). ATP is usually co-released with other neurotransmitters such as catecholamines (Burnstock, 1986), acetylcholine, glutamate and γ-aminobutyric acid (GABA) (Jo and Schlichter, 1999; Pankratov et al., 2006), and hormones vasopressin and oxytocin (Custer et al., 2012) by a mechanism of Ca^{2+}-stimulated exocytosis.

Main source of extracellular ATP in the central nervous system are astrocytes. ATP is released from astrocytes into the synaptic cleft where it can act on neuronal pre- or postsynaptic purinergic P2 receptors (Burnstock et al. 2011; Lazarowski et al., 2011; Lazarowski, 2012). ATP is also released by glial cells in PVN (Gordon et al., 2005) and suprachiasmatic nucleus (SCN) of hzpothalamus (Womac et al., 2009). Extracellular ATP accumulation in the SCN is time-of-day dependent, with maximum during the night (Womac et al., 2009). Cultured SCN2.2 cells (Womac et al., 2009) and mouse cortical astrocytes (Marpegan et al., 2011) also exhibit circadian rhythm in ATP release. ATP can also be released from astrocytes and it was thought to be vesicular and dependent on Ca^{2+} (Fumagalli et al., 2003; Pascual et al., 2005; Pangrsic et al., 2007). Later studies in rat SCN2.2 cells have shown that this is not the case and that the changes in intracellular Ca^{2+} levels are inversely related to the circadian variations in extracellular ATP accumulation (Burkeen et al., 2011). Additionally, there was no effect of genetic disruption of the vesicular release on circadian ATP release in cultured mouse cortical astrocytes (Marpegan et al., 2011) which indicated the possibility of a non-vesicular pathway for circadian ATP release. In this case the release might occur through different conductive mechanisms involving pore-forming molecules such as pannexin-1 hemichannels (Stout et al., 2002; Schenk et al., 2008; Iglesias et al., 2009; Li et al., 2011b) or purinergic P2X7 receptor channel (Khakh and Sofroniew, 2015).

The duration and extent of ATP actions in the extracellular environment are limited by several ectonucleotidases (Zimmermann, 2000), which hydrolyze ATP to ADP, AMP, and adenosine (Fig. 1). The activity of these enzymes is important for proper timing of activation of purinergic P2 receptors, P2X and P2Y, involved in signaling network called “purinergic signaling”. P2 receptor signaling can functionally be differentiated in two ways: a) short-term effects such as transmission, modulation, and inflammation, mostly mediated by P2X (Abbracchio and Burnstock, 1998), and b) long-term effects such as cell proliferation, development, regeneration, mostly mediated by P2Y (Neary et al., 1996; Mishra et al., 2006; Del Puerto et al., 2013; Guzman and Gerevich, 2016). Thus, both purinergic receptors

and ectonucleotidases regulate brain development in embryonic and postnatal periods (Guo et al., 2013; Burnstock and Dale, 2015; Rodrigues et al., 2019).

1.1.2. Purinergic P2X receptors

Extracellular ATP and its metabolic products, ADP and adenosine, act as agonists or extracellular messengers on purinergic (P) receptors (Burnstock, 2006c) that have been categorized based upon their preferential agonist. P1 receptors activate primarily to adenosine and P2 agonists are activated by ATP and ADP (Fig. 1). There are ionotropic and metabotropic ATP-sensitive purinergic receptors, a fact used to further classify the P2 receptors as P2X and P2Y, respectively (Burnstock, 1977, 1986). In 1994, the first P2X was isolated, cloned and investigated functionally (Brake et al., 1994; Valera et al., 1994). Since then, seven genes have been identified for P2X receptor family, P2X1-7 (North, 2002).

The P2X purinergic receptors are ion channels which activate in response to ATP causing a rapid and selective permeability to cations. They form functional homo or heterotrimers (Nicke et al., 1998), and sometimes even interact with other ion channels, for example P2X7 interacts with panexin type 1 channel (Pelegrin and Surprenant, 2006) and P2X4 with $GABA_A$ receptor (Jo et al., 2011). Heteromers which have been successfully expressed include P2X2/3, P2X1/5, P2X2/6 and P2X4/6, although various other types may occur. P2X7 has not yet been found or expressed as a heteromer (Torres et al., 1999). Analyses of functional homomeric receptors of each subunit expressed in *Xenopus* oocytes and human embryonic kidney (HEK) cells have revealed the distinct functional profiles of the P2X1-7 receptors (North, 2002) (Fig. 2).

The amino acid structure of the P2X receptor subunits maintain a 26 - 47% sequence homology and can vary in size from 379 to 595 amino acids long (Khakh, 2001). Each of the receptor subunits has two transmembrane structures, TM1 and TM2, with both the N- and C-terminus located intracellularly. The extracellular domain, extending from residue 51-329 for P2X2 (North, 2002), has been shown to account for ATP binding (Jiang et al., 2000b). The TM2, which extends from residues 330-353 for P2X2, has been shown to line the pore and residues near either end of the TM2 region contribute to the ion selectivity of the channel (Haines et al., 2001). TM1, extending from residue 30-50 for P2X2, is located at periphery and is believed to contribute to the gating mechanism (Rassendren et al., 1997a). The C-terminus region displays the most variable part of P2X receptors. It varies considerably in

length, and contributes to the desensitization kinetics of the receptors (Koshimizu et al., 1999).

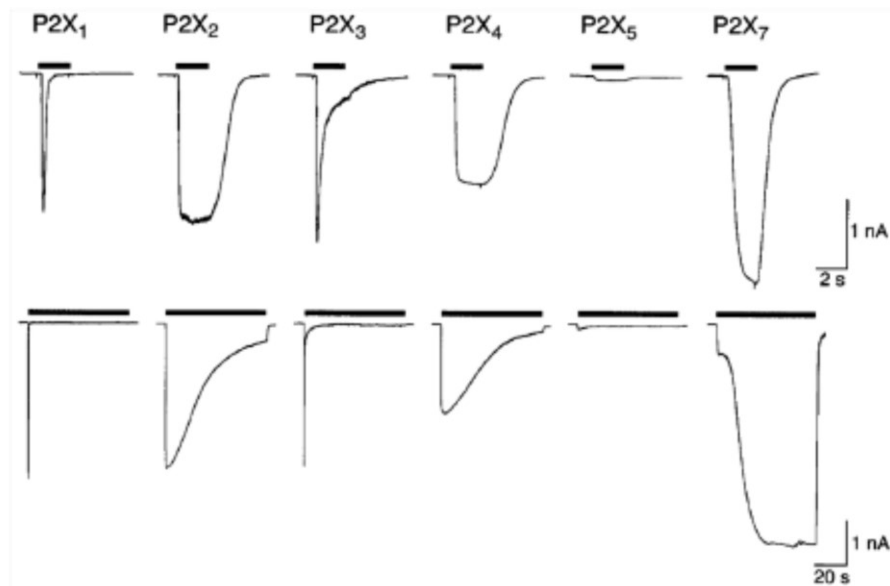

Figure 2. Representative P2X currents demonstrating the different functional properties of each P2X receptor. Membrane currents induced by application of supramaximal agonist concentration for 2 s (upper row) or 60 s (lower row) are shown. The time courses of these currents demonstrate that P2X1 and P2X3 exhibit very fast desensitization, the P2X4 and P2X2 show moderate desensitization rates, and the P2X5 has significantly lower amplitude so that its desensitization cannot be determined. The P2X7 show no desensitization but prominent secondary current growth after long-term agonist application. Horizontal lines demonstrate 30 µM ATP, in the case of P2X7 1mM ATP, application. The P2X6 receptor homomer is nonfunctional, therefore is not shown here. Taken from North 2002.

Very long C-terminus of P2X7, that constitutes 40% of the whole protein, has been shown to be involved in many types of cellular responses, not associated with ion channel function, such as activation of lipases, kinases, and transcription factors, as well as cytokine release and apoptosis (Kopp et al., 2019). Phosphorylation site in the N-terminus has been also shown to acts as a determinant of desensitization kinetics (Boue-Grabot et al., 2000). Crystal structures (Fig. 3) have been solved for truncated zebrafish P2X4 (Kawate et al., 2009), full-length human P2X3 (Mansoor et al., 2016) and truncated panda P2X7 receptor that has been expressed and purified from Sf9 insect cells (Karasawa and Kawate, 2017). Cryoelectron microscopy structures have been generated for full-length rat P2X7 (McCarthy et al., 2019). Analysis of apo (closed) and ATP-bound (open) states enable detailed understanding of the structure and molecular physiology of these receptors.

To better understand the physiological role of P2X it is important to the gating properties of these receptors. P2X1 and P2X3 rapidly activate and desensitize, whereas P2X2 and P2X4 slowly desensitize. P2X7 does not show an obvious desensitization but exhibit the secondary current growth (Fig. 2). Rat P2X5 generates low amplitude non-desensitizing currents, and P2X6 does not express well at the plasma membrane. Among these receptors,

P2X1 and P2X3 have the highest affinity for ATP with an effective concentration of agonist producing half maximl effect (EC_{50}) in a submicromolar concentration range. ATP is also a full agonist for P2X5 with estimated EC_{50} values ranging from submicromolar to low micromolar concentration range. P2X2 and P2X4 are also fully activated by ATP but less sensitive, with EC_{50} values in a low micromolar concentration range. The P2X7 is the least sensitive member of the P2X family with the EC_{50} value for ATP in a high micromolar to low millimolar concentration range (North, 2002; Coddou et al., 2011b).

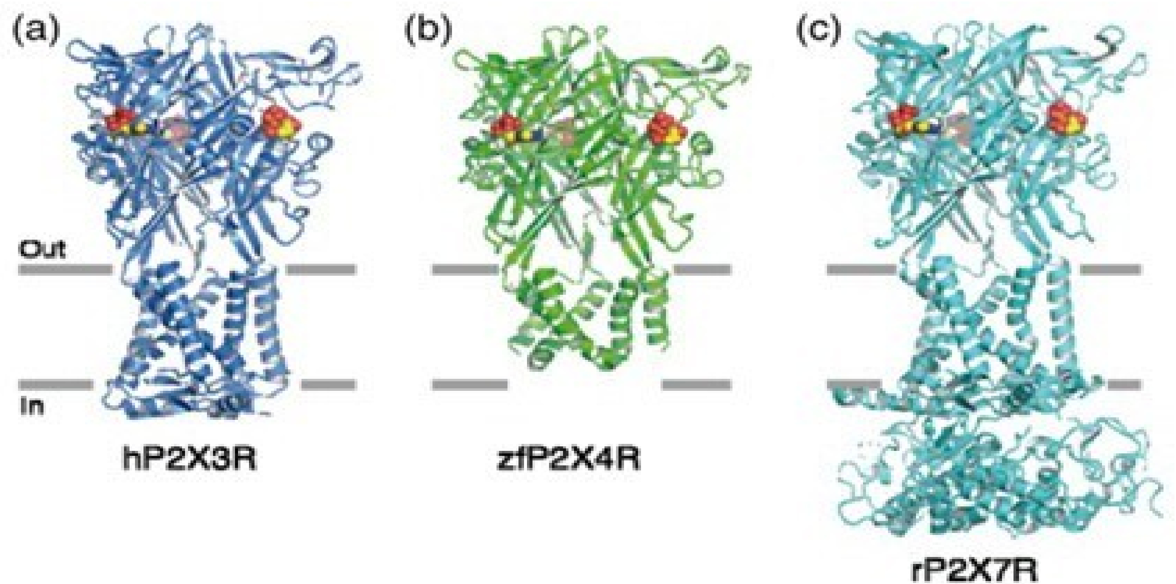

Figure 3. Structures of selected P2X receptors. (a) Crystal structure of human P2X3 receptor (hP2X3) bound to ATP (PBD ID: 5SVK) (Mansoor et al., 2016). The hP2X3 receptor is shown in blue cartoon representation, and ATP is shown as spheres (carbon is yellow, oxygen is red, nitrogen is blue, and phosphorus is orange). Horizontal grey bars indicate the approximate location of the membrane bilayer defining the extracellular (out) and intracellular (in) milieu. (b) Crystal structure of zebrafish P2X4 receptor (zfP2X4) bound to ATP (PBD ID: 4DW1) (Hattori and Gouaux, 2012). The zfP2X4 receptor is shown in green cartoon representation, and ATP is shown as spheres. (c) Models of full-length rat P2X7 receptor (rP2X7) bound to ATP (PBD ID: 6U9W) (McCarthy et al., 2019). The rP2X7 receptor is shown in cyan cartoon representation, and ATP is shown as spheres. Adapted from (Illes et al., 2021).

Although P2X receptors tend to be equally permeable to small cations such as Na^+, K^+ and Ca^{2+}, larger cations such as N-methyl-D-glucamine ion ($NMDG^+$) can permeate through the channel depending on the subunit composition. The P2X7 subtype is the most permeable to $NMDG^+$ and its permeability may be increased by sustained activation of the receptor (Virginio et al., 1999). Finally, P2X exhibit a 'run-down' effect by which the ATP response is inhibited after repeated or prolonged activation. The effect is believed to be due to receptor internalization that is linked to channel opening (Jensik and Cox, 2002). Although ATP is the main ligand for all P2X receptors, some of them can be activated by other substances. P2X7 is more sensitive to 3´-O-(4-benzoyl)benzoyladenosine-5´-triphosphate (BzATP) than ATP itself, and α,β-methyleneadenosine 5′-triphosphate (αβmeATP) or β,γ- methyleneadenosine 5′-triphosphate (βγmeATP) are agonists with high potency for the fast- desensitizing P2X3

and P2X1, respectively (North, 2002).

Most of P2X display sensitivity to inhibitory effects of suramin and pyridoxalphosphate-6-azophenyl-2', 4'-disulfonic acid (PPADS) (North, 2002). Due to lack of specific drugs it was difficult to thoroughly investigate the physiology of P2X receptors. Recenly, there are multiple new selective agonists and antagonists available for specific receptor subtypes, for review see (Coddou et al., 2011a; Illes et al., 2021). P2X receptors can be modulated by a wide array of molecules and allosteric modulators, protons, trace metals like zinc and copper, phosphoinositides, alcohols, reactive oxygen species and neurosteroids, for example. Well known modulator is ivermectin which potentiates selectively only the P2X4 subtype (Khakh et al., 1999b; Jelinkova et al., 2006; Mackay et al., 2017).

1.1.3. Specific properties of P2X2

The P2X2 cDNA was originally isolated from rat cerebellum (Simon et al., 1997), rat pheochromocytoma PC12 cells (Ralevic and Burnstock, 1998) and pituitary (Koshimizu et al., 1998). P2X2 receptors are defined as nondesensitizing receptors, with respect to P2X1 and P2X3, due to minimal decline in current during prolonged application of agonist (Fig. 2). Like other P2X, the P2X2 is a cation-selective channel permeable to small monovalent and divalent cations. The P2X2 has a relatively high permeability to Ca^{2+}, P_{Ca}/P_{Na} 2.5, but not as high as the P2X1 or P2X4 (North, 2002). P2X2 receptors display a time-dependent increase in permeability to large cations. With sustained agonist applications, P2X2 are seen to undergo pore formation allowing for the permeation of large organic cations, such as $NMDG^+$ (Khakh et al., 1999a).

P2X2 exhibits lower sensitivity to ATP (EC_{50} is about 10 μM) in comparison with P2X1 and P2X3. So far, no selective agonists have been identified. The potency profile for P2X2 varies based on species differences, but generally is accepted to be ATP $\geq$ 2-methylthio-adenosine triphosphate (2MeSATP) = ATPγS > BzATP, although BzATP has been found to act only as a partial agonist (North, 2002; Li et al., 2004). The potency order for P2X2 antagonists is reactive blue 2 > TNP-ATP $\geq$ PPADS > suramin (King et al., 1997). To date, no selective antagonist has been identified.

Acidic extracellular pH potentiates P2X2, opposite to the effect seen for most P2X receptors. Acidic pH, approximately 6.5, appears to reduce the EC_{50} of the agonist response, but does not affect the maximal response (Clyne et al., 2002). A potentiating effect is caused

also by the extracellular Zn^{2+} and Cu^{2+} (North, 2002) and steroid dehydroepiandrosterone, DHEA, (De Roo et al., 2003). We have found recently that synthetic testosterone derivatives also potentiate P2X2 (Sivcev et al., 2019) while endogenous steroid, lithocholic acid, exhibits allosteric inhibitory effect (Sivcev et al., 2020).

The P2X2 is widely distributed in the nervous system and throughout various tissues. The receptor was found in the bladder, brain, spinal cord, cochlea, adrenal medulla and intestine, with highest quantities in the pituitary gland and vas deferens (Ralevic and Burnstock, 1998; Khakh et al., 2001; Zemkova and Stojilkovic, 2018). The unique properties of the P2X2 facilitate the various physiological roles the receptor is involved in. For instance, it has been implicated in the regulation of respiration, a role that is amplified by the high pH sensitivity (Gourine et al., 2003). Another research confirmed that activation of nondesensitizing P2X2 in pituitary somatotrophs (Koshimizu et al., 1998) and gonadotrophs (Zemkova et al., 2006) modulates electrical activity and action potential-dependent Ca^{2+} transients.

1.1.4. Specific properties of P2X4

The P2X4 displays a number of similarities to the P2X2 and falls into the category of moderately desensitizing P2X receptors. P2X4 cDNAs were independently isolated by five different groups of investigators from various rat tissues and have also been isolated from human, mouse, chick and *Xenopus* (for review see (North, 2002)). Although the desensitization is significantly slower than the P2X1 and P2X3, complete desensitization occurs between 5-10 seconds at saturating ATP concentrations (North, 2002). Recovery from complete desensitization takes between 10-15 minutes (Jones et al., 2000). The human P2X4 desensitizes more rapidly in comparison to the rat P2X4 (Ralevic and Burnstock, 1998). With a short application of agonist, the P2X4 activates rapidly opening a cation-selective channel with approximately equal Na^+ and K^+ permeability and a relatively high Ca^{2+} permeability, P_{Ca}/P_{mono} 4.2 (Soto et al., 1996). Like P2X2 and P2X7, P2X4 displays a time-dependent shift to a high-permeability state (Virginio et al., 1999). ATP, the most potent P2X4 agonist, displays higher potency (EC_{50} 7.4 μM), in comparison with P2X2 receptors (Garcia-Guzman et al., 1997). The potency profile of the P2X4 is ATP > 2MeSATP > αβmeATP > BzATP. The most unusual characteristics of the P2X4 in relation to other P2X is its low sensitivity to general P2X antagonists such as suramin and PPADS, with 100 μM applications resulting in approximately 20% and 10% inhibition, respectively (Soto et al., 1996). On the other hand,

P2X4 displays higher sensitivity to some other related antagonists, such as 5-BDBD (Donnelly-Roberts et al., 2008).

An important allosteric modulator is ivermectin which selectively potentiates only the responses mediated by P2X4, serving as an important tool to distinguish this receptor from other P2X (Khakh et al., 1999b). Zn^{2+} mediates a potentiating effect but Cu^{2+} displays an inhibitory effect on P2X4 (Soto et al., 1996; Xiong et al., 1999; Coddou et al., 2003). Other modulatory effects that have been investigated are the inhibition by ethanol (Xiong et al., 2000; Popova et al., 2010), and the Cd^{2+}-induced potentiation (Coddou et al., 2005; Rokic et al., 2014)

The P2X4 subunit is expressed widely throughout the central and peripheral nervous system. The receptors containing this subunit have also been localized in the lungs, bronchial epithelium, thymus, bladder, salivary gland, adrenal gland, and vas deferens (Ralevic and Burnstock, 1998; Khakh, 2001). High expression of P2X4 in the brain might indicate its role in synaptic modulation via increases in Ca^{2+} uptake (Virginio et al., 1999). Another potentially important role of brain-localized P2X4 is its sensitivity to ethanol (Xiong et al., 2000) which inhibits P2X4 responses in rat hippocampal neurons within the range of pharmacological concentrations (Li et al., 2000). It is supposed that P2X4 could be a possible therapeutic target for tactile allodynia since it was established that it plays a role in the hypersensitivity of nociceptive transmission in injured primary sensory neurons (Inoue et al., 2004).

1.1.5. Specific properties of P2X7

The P2X7 has a number of features which distinguish it from the other P2X receptors (Surprenant et al., 1996). The P2X7 has been found only as a homomer, and structurally, the P2X7 contains a significantly longer, 240 amino acid, intracellular C-terminus region. The P2X7 activates with slower kinetics than other P2X receptor, and after maximal activation, the receptor shows no desensitization (North, 2002). Brief agonist application to the P2X7 yields permeability to small cations, similar to the other P2X receptors, but with prolonged application, the pore appears to permeabilize and allow the movement of larger cations. The time-activated permeabilization allows for the bi-directional transport of cations and small molecules, up to 900 daltons (Ralevic and Burnstock, 1998). P2X7 can form a large pore itself (Khakh and Lester, 1999; Jiang et al., 2021) or after interaction with another transmembrane molecule such as panexin type 1 channel (Pelegrin and Surprenant, 2006; Locovei et al., 2007). The increased permeability of the receptor may lead to cell swelling,

membrane disruption and cell lysis. (Rassendren et al., 1997b).

ATP activates the P2X7 with extremely low potency, the EC_{50} falling into the > 100 μM range (North, 2002). BzATP is not a selective agonist for P2X7, but activates P2X7 with a potency approximately 30 times higher than ATP. The agonist potency profile of the receptor is BzATP >> ATP = 2MeSATP > ATPγS > αβmeATP = ADP (Khakh and Lester, 1999).

The P2X7 appears to be relatively insensitive to P2X antagonists suramin and PPADS. However, brilliant blue G (BBG) (Jiang et al., 2000a) blocks P2X7 with a remarkably high sensitivity, at least 100 times more effectively than other P2X (North, 2002). Another highly selective antagonist for the receptor is KN-62, oxidized ATP (oATP) and calmidazolium. The most interesting modulators to the P2X7 are divalent cations which have strong inhibitory effects on agonist-evoked currents. The potency profile of the cations is $Cu^{2+} > Cd^{2+} = Zn^{2+} > Ni^{2+} >> Mg^{2+} = Co^{2+} > Mn^{2+} > Ca^{2+} = Ba^{2+} >> Sr^{2+}$ (Virginio et al., 1997). Protons also inhibit the current evoked by agonist at pH 6.1. (North, 2002). Well documented selective negative allosteric modulator acting at low concentrations (1-10 nM) is AZ10606120 (Michel et al., 2007), and the structurally unrelated A438079 (Donnelly-Roberts et al., 2009). GW791343 (100 nM) is a positive allosteric modulator of rat P2X7 (Michel et al., 2008).

P2X7 is involved in inflammation and originally was supposed to be expressed namely on cells of hemopoietic origin, such as mast cells, macrophages, fibroblasts, and erythrocytes (Ralevic and Burnstock, 1998), for review see (Bautista-Perez et al., 2020). More recently, P2X7 expression has been found in retinal cells (Puthussery and Fletcher, 2004), epithelial and glandular cells (Sim et al., 2004), and astrocytes (Narcisse et al., 2005; Sperlagh et al., 2006; Hamilton et al., 2008; Kamatsuka et al., 2014; Zhao et al., 2016). Evidences for P2X7 expression in neurons is still lacking (Illes et al., 2017). Other physiological roles of the P2X7 include regulation of bone formation and resorption (Ke et al., 2003), neuronal protection against glutamate toxicity (Suzuki et al., 2004) and regulation of salivary gland secretion (Turner et al., 1999). The P2X7 has been detected in complexes with pannexins that are supposed to promote Ca^{2+}-independent gliotransmitter release (Ballerini et al., 1996; Wang et al., 2002; Duan et al., 2003; Hamilton et al., 2008; Carrasquero et al., 2009; Norenberg et al., 2011b).

1.1.6. Purinergic P2Y receptors

The G-protein coupled P2Y receptors have been further subdivided into eight subclasses, P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14 (Abbracchio et al., 2003)

based upon the pharmacology and G-protein subunit coupling of the receptors. ATP and ADP can activate the various subclasses of P2Y receptors with different potencies. Some P2Y receptors, such as P2Y4 and P2Y6, are most effectively activated by UTP causing some references to the receptors as pyrimidinergic receptors (O'Connor et al., 1991).

1.2. Hypothalamo-neurohypophyseal system

Hypothalamic-neurohypophyseal system is represented by magnocellular neurosecretory cells (in short magnocellular neurons) in hypothalamus and their nerve endings in posterior pituitary (Fig. 4). The hypothalamic part of the system consists of the SON and PVN (Fig. 5) nuclei containing magnocellular neurons, and in the case of PVN with addition of other cell types. Principal role of this system is synthesis and secretion of oxytocin and vasopressin, also

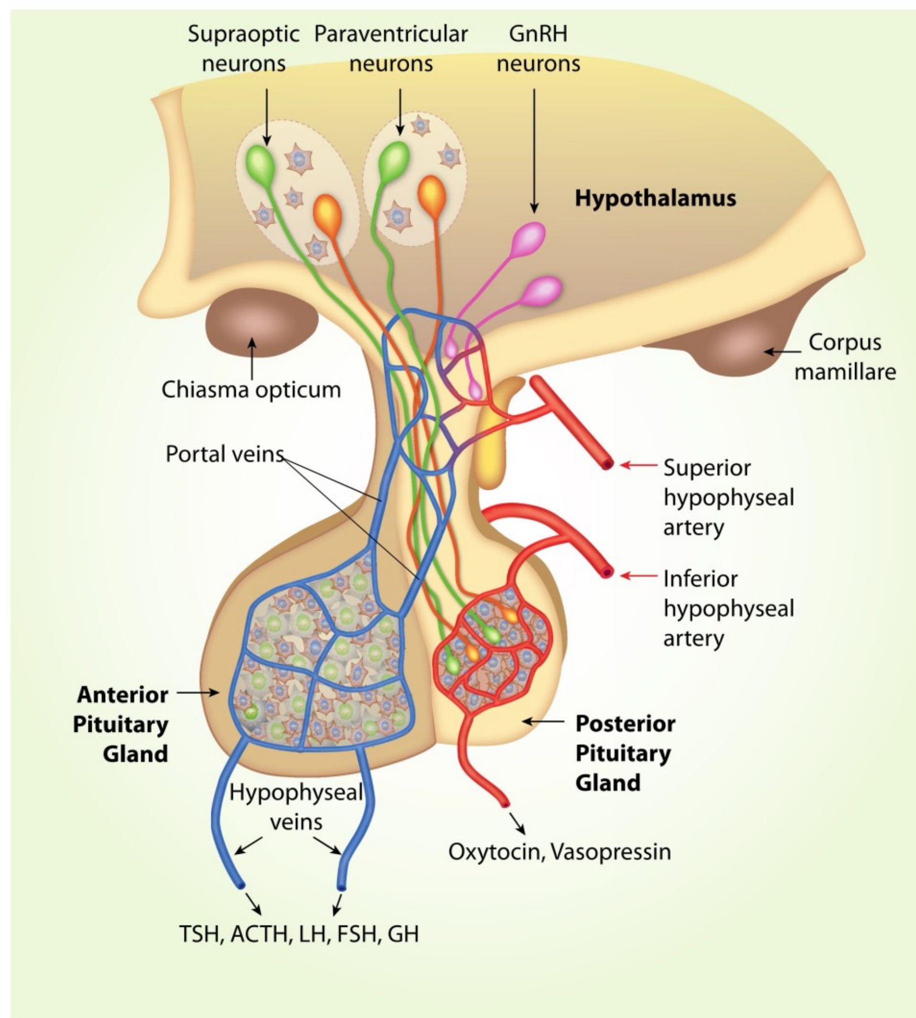

Figure 4. Communication between hypothalamus and posterior pituitary. (Stojilkovic presentation, personal communication).

called arginin-vasopressin (AVP), which is the most common form of vasopressin found in mammals.

Magnocellular neurons from SON and PVN project to the posterior pituitary and secrete oxytocin or vasopressin into the circulation. These neurons can independently secrete both hormones centrally via dendritic release (Pow and Morris, 1989; Sabatier et al., 2003). These two peptides are also released within the PVN and SON nuclei themselves, where they have local regulatory actions (Ludwig, 1998; Kombian et al., 2002; Leng and Ludwig, 2008). There is also a number of smaller nuclei located usually between SON and PVN (Fig. 5 and Fig.6) which secrete oxytocin and vasopressin, and which can vary in number, shape and size across species.

Figure 5. Scheme of coronal section of rat hypothalamus. Regions of interest are coloured . Optical chiasma (**OX**), supraoptic nucleus (**SON**; blue), medial amygdaloid nucleus (**MeAD**), nucleus of lateral olfactory tract (**LOT**), anterior amygdala area, ventral (**AAV**), magnocellular preoptic nucleus (**MCPO**), ventrolateral hypothalamus (**VLH**), lateral hypothalamus (**LH**), suprachiasmatic nucleus (**SCN**; pink), lateroanterior hypothalamic nucleus (**LA**), anterior hypothalamic area (**AHA**), medial preoptic nucleus (**MPO**), periventricular hypothalamic nucleus (**Pe**), third ventricle (**3V**), BST medial posteolateral (**BSTMPL**), rcircular nucleus (**Cir**), fornix (**f**), ventral reuniens thalamic nucleus. (**VRe**), reuniens thalamic nucleus (**Re**), anterior part of paraventricular nucleus (**PVA**; orrange), antromedial thalamic nucleus, ventral (**AMV**), stria medularis (**sm**), median forebrain bundle (**mfb**), substantia innominate (**SI**), nucleus of stria medularis (**SM**), basal nucleus (**B**). Adapted from Paxinos et Watson: The Rat Brain Atlas, 4th edition (1998) Academic Press, USA.

These smaller nuclei, together with single neurons scattered around them, are sometimes called accessory magnocellular neurosecretory system (Fig.6); the most notable minor nucleus is the circular nucleus which is present in most species (Moller et al., 2018). Accessory magnocellular neurosecretory system is hypothesized to secrete hormones directly into the blood vessels inside the brain (Moller et al., 2018).

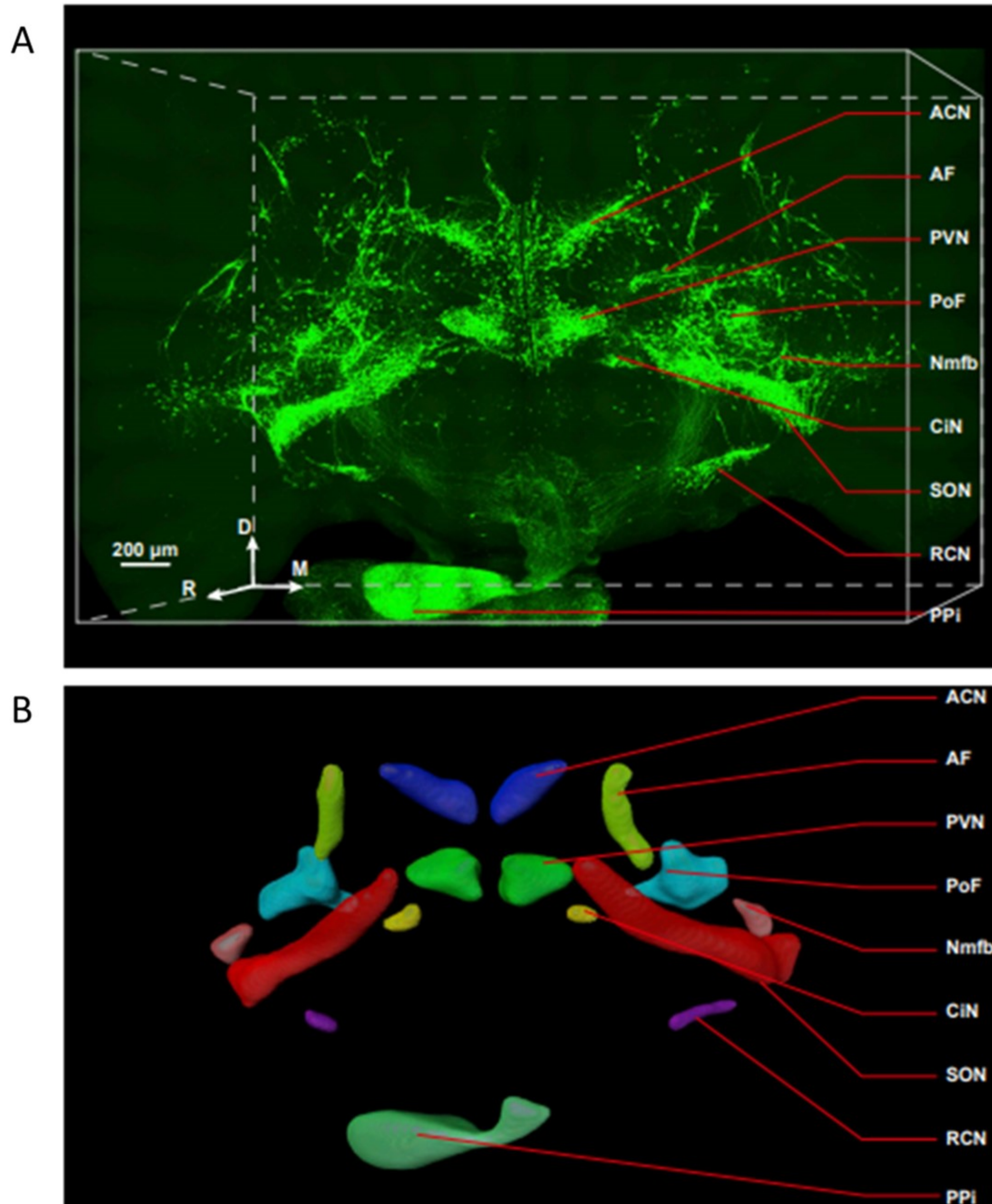

Figure 6. 3D reconstruction of hypothalamo-neurohypophyseal system using fluorescent micro-optical sectioning tomography (fMOST) imaging. (A) 3D projections of the hypothalamo-neurohypophyseal system derived from 1,700 fMOST images (thickness of 3,400 mm). (B) Schematic representation of different nuclei containing magnocellular neurons: **ACN**-anterior commissural nucleus (dark blue), **AF**-anterior perifornical nucleus (light green), **PVN**-paraventricular nucleus (green), **PoF**-posterior perifornical nucleus (light blue), **Nmfb**-nucleus of the median forebrain bundle (pink), **CiN**-circular nucleus (yellow), **SON**-supraoptic nucleus (red), **RCN**-retrochiasmatic nucleus (purple), **PPi**- posterior pituitary (dark green). Adapted from (Zhang et al., 2021).

Hormonal levels in peripheral blood circulation fluctuate in a small optimal range, reported plasma concentration of vasopressin in human is 0.012 – 0.105 pg/ml and plasma concentration of oxytocin is 0.006 – 0.548 pg/ml (Minutillo et al., 2021), but the level of hormones in the brain is several times higher than that in the periphery, reported AVP concentration in hypothalamus of mice is 6.1±1.3 ng/mg (Jiang et al., 2017), and oxytocin was found in high concentrations (>0.1 M) in the neurosecretory granules of the posterior pituitary (Minutillo et al., 2021). Beside the forementioned hormones, magnocellular neurons co-secrete a wide range of other molecules: dynorphin, opioids, nitric oxide synthase and heme oxygenase-1 (that generates carbon monoxide), for example, and their vesicles contain ATP (Brown, 2016).

Neurohypophysial part of the system contains fenestrated capillary-type of blood vessels and terminals of magnocellular neurons (Fig. 4). The axons of the hypothalamic magnocellular neurons localized in SON and PVN transport secretory dense-core vesicles from the soma to the posterior pituitary, releasing oxytocin and vasopressin near these fenestrated capillaries.

1.2.1. SON, synaptic inputs and outputs

The SON is located on both sides of the chiasma opticum (Fig. 5) and in the vicinity of hypothalamic blood vessels. Its shape and size is species dependent and it consists only of magnocellular neurosecretory cells without presence of other neuronal cell types. It can be oval shaped or elongated, and in humans it stretches through most of the ventral side of hypothalamus (Moller et al., 2018). SON neurons are tightly packed with oxytocin and vasopressin subpopulation evenly distributed (Fig. 7).

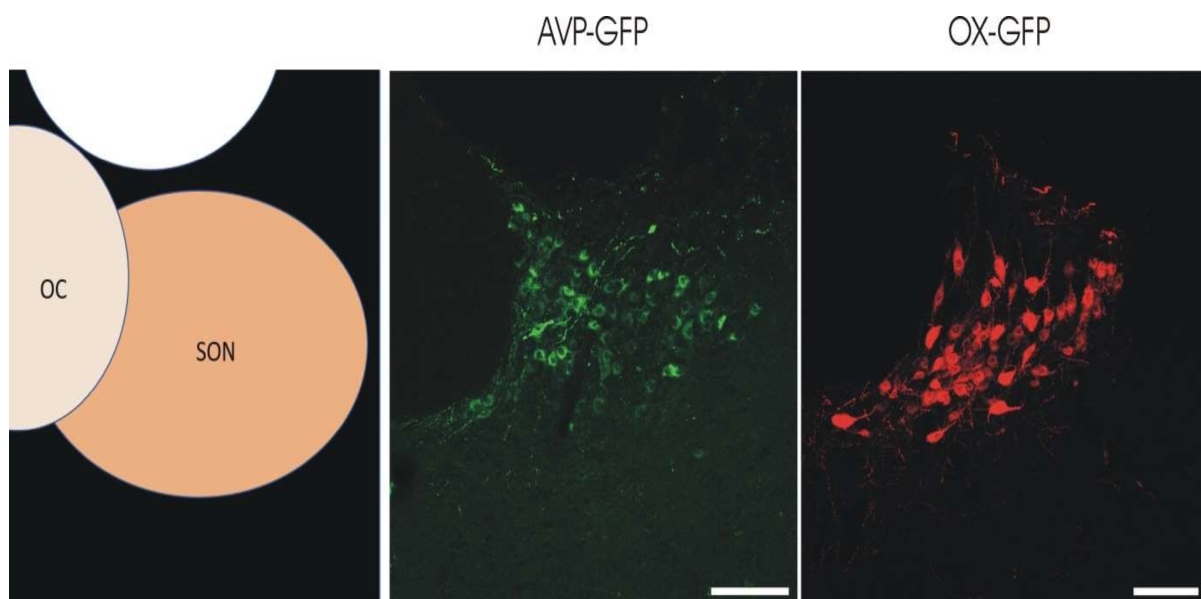

Figure 7. Distribution of oxytocin and vasopressin neurons in the SON of rat. Two subpopulations of SON neurons in transgenic rats expressing an AVP-enhanced green fluorescent protein fusion gene (AVP-eGFP), and an oxytocin-monomeric red fluorescent protein (OX-RFP); **OC**, chiasma opticum. Scale 100 µm (Vavra and Zemkova, unpublished).

Electrical activity of neurons dictates the secretion of both hormones (Sladek and Kapoor, 2001; Armstrong, 2007; Li et al., 2007; Leng et al., 1999; Choe et al., 2015). Their activity is in turn regulated by various excitatory and inhibitory synaptic inputs (Hu and Bourque, 1991; Wuarin and Dudek, 1993; Shibuya et al., 2000; Israel et al., 2010). Figure 8 shows some of the major peripheral and afferent inputs to magnocellular neurosecretory cells. These inputs use particularly glutamate (Decavel and Curras, 1997; Boudaba et al., 2003; Iremonger et al., 2010; Vilhena-Franco et al., 2018) and GABA (Brussaard and Kits, 1999) as neurotransmitters.

Both oxytocin and vasopressin secreting neurons possess R,S- alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor subtypes, and in both cell types, synaptically released glutamate often coactivates both AMPA and NMDA receptors (Armstrong and Stern, 1998). The AMPA receptor channel on both cell types is characterized by a relatively high calcium permeability and voltage-dependent rectification, which indicate a diminished presence of its GluR2 subunit. However, AMPA-mediated synaptic transients are larger and decay faster in oxytocin compared with vasopressin neurons, suggesting a potential difference for synaptic integration. The characteristics of NMDA-mediated synaptic transients are similar in oxytocin and vasopressin neurons, but some data suggests that NMDA receptors may be less involved in the glutamatergic activation of oxytocin neurons (Armstrong and Stern, 1998). Potentiation of excitatory inputs provides the main mechanism through which neuroendocrine output can be enhanced (Kombian et al., 2000). For example, magnocellular neurosecretory neurons receive glutamatergic input from dorsal raphe nucleus and perinuclear zone which are sensitive to noradrenaline that stimulates hormone secretion (Fig. 8). However, situation is even more complicated. Although A1 noradrenaline neurons of the caudal medulla provide a direct, excitatory visceral and somatic inputs to neurosecretory neurons in SON (Fig. 8), they do not use noradrenaline but ATP as their primary excitatory transmitter to stimulate secretion of vasopressin (Day et al., 1993).

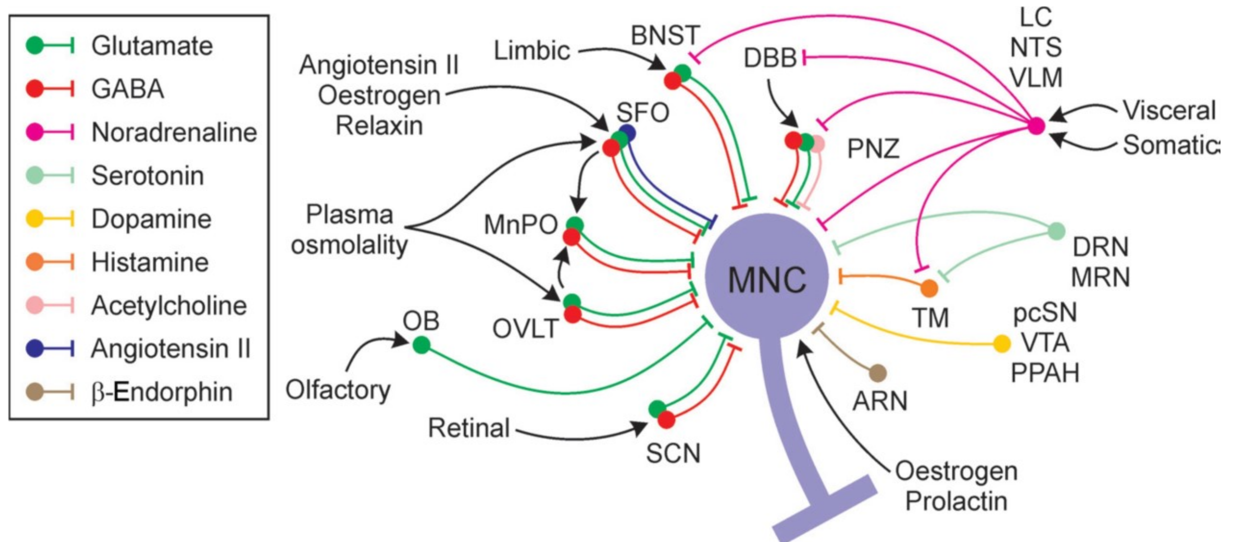

Figure 8. Schematic representation of some of the major peripheral and afferent inputs to magnocellular neurosecretory cells. ARN: arcuate nucleus; **BNST**: bed nucleus of the stria terminalis; **DBB**: diagonal band of Broca; **DRN:** dorsal raphe nucleus; **LC**: locus coeruleus; **MNC**: magnocellular neurosecretory cell (magnocellular neuron); **MnPO**: median preoptic nucleus; **MRN**: median raphe nucleus; **NTS**: nucleus tractus solitarius; **OB**: olfactory bulb; **OVLT**: organum vasculosum of the lamina terminalis; **pcSN:** pars compacta of the substantia nigra; **PNZ**: perinuclear zone; **PPAH**: preoptic periventricular /anterior hypothalamic region; **SCN**: suprachiasmatic nucleus; **SFO**: subfornical organ; **TM**: tuberomammillary nucleus; **VLM**: ventrolateral medulla; **VTA**: ventral tegmental area. Adapted from (Brown, 2016). Noradrenaline afferents secrete also **ATP** (Day et al., 1993).

In electrophysiological experiments on rat brain slices, spontaneous miniature glutamatergic excitatory postsynaptic currents (mEPSCs) can be only observed in a subpopulation of SON neurons, while the GABAergic inhibitory postsynaptic currents (mIPSCs) can be recorded practically from all SON neurons (Vavra et al., 2011). In agreement with this finding, quantitative analysis of glutamate decarboxylase or GABA immunostaining, combined with oxytocin and vasopressin immunolocalization, revealed that GABAergic innervation within the SON is very extensive and uniformly distributed within the nucleus, and that GABAergic nerve terminals contact vasopressin and oxytocin neurons to a similar extent (Theodosis et al., 1986; Meeker et al., 1993). The SON receives GABAergic afferent inputs from osmosensitive neurons of the circumventricular subfornical organ (Fig. 8) that communicate chronic changes in plasma osmolality through direct projections to the vasopressinergic neurons (Cobbett and Weiss, 1990). Some inputs to SON could originate from GABA-containing cells in the locus coeruleus (Jones and Moore, 1977; Leng et al., 1999) or interneurons within the perinuclear zone, located in a close vicinity of SON (Tappaz et al., 1983). The extent of interneuron contribution to the GABAergic innervation of the SON is not known. There is a possibility that interneurons within the SON and perinuclear zone couls mediate the projections from other regions and regulate excitatory and inhibitory inputs into the SON neurons (Leng et al., 1999). The GABAergic neurons in the perinuclear zone

are thought to mediate rapid inhibition of vasopressinergic neurons following transient body hypertension (Jhamandas et al., 1989; Nissen et al., 1993). The perinuclear zone also contains cholinergic neurons thought to innervate the SON (Wang et al., 2015). Vasopressinergic neurons receive inputs from baroreceptors located in carotid sinus, aortic arch and left atria (Iovino et al., 2017; Renaud & Bourquet, 1991; Tribollet et al., 1985). Important part of their physiological function represents the ability of dendritic release of peptides within the SON since the locally released peptides can regulate the electrical activity of magnocellular neurons (Kombian et al., 2000).

Oxytocinergic stimuli originate in sensory receptors in cervix (during parturition) or nipples (during lactation) and affect oxytocin production in SON and PVN through multisynaptic pathways (Iovino et al., 2021). Other regions of the brain involved in oxytocin secretion are the amygdala, the locus coeruleus, the dorsal raphe nuclei, the retrotrapezoid nucleus, the lateral parabrachial nucleus, the diagonal band of Broca, nucleus tractus solitarius and the bed nucleus of the stria terminalis (Fig. 8) (Raby and Renaud, 1989; Brown, 2016). Endogenous oxytocin, released from the dendrites of magnocellular neurosecretory cells, decreases evoked excitatory synaptic transmission by inhibiting glutamate release from the presynaptic terminals by modulating voltage-dependent calcium channels, mainly N-type and to a lesser extent P/Q-type channels, located on glutamatergic terminals (Kombian et al., 2002). Although evidence is less conclusive, it is possible that endogenous vasopressin has similar effect on excitatory transmission (Kombian et al., 2002). Regulation by gonadal and adrenal steroids is specific for oxytocin neurons, and production of oxytocin is significantly regulated by estrogen (Gimpl and Fahrenholz, 2001).

Synapses onto SON neurons constitute the final integration step before the exocytosis of the hormones, and play an important role in generation of the specific pattern of electrical activity in vasopressin and oxytocin neurons (Voisin et al., 1995; Moos, 1995; Jourdain et al., 1998; Brussaard and Kits, 1999; Israel et al., 2010; Choe et al., 2015).

1.2.2. PVN and SCN, cell composition, synaptic inputs and outputs

The PVN is located on the sides of the third ventricle of the brain (Fig. 5 and Fig. 6) (Moller et al., 2018). It is composed of magnocellular neurons secreting oxytocin and vasopressin, and parvocellular neuroendocrine cells that project to the median eminence where they secrete neurohormones into the hypophyseal portal system. These include corticotrophin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH), growth hormone-releasing hormone (GhRH), dopamine and somatostatin. The portal

system runs into the anterior lobe of the pituitary gland, where these hypothalamic neurohormones modulate secretory activity of specialized anterior pituitary cells (Fig. 4). Population of magnocellular neurosecretory cells in the PVN release vasopressin and oxytocin in posterior pituitary, and shows similar pattern of projections as the SON neurons in other areas of the brain. It is supposed that neurohypophyseal hormones produced in the PVN and released within the brain affect many types of behavior: social bonding, aggression and stress related behavior (Taylor et al., 2017; Iovino et al., 2021). Magnocellular neurosecretory cells in the PVN receive numerous GABAergic and glutamatergic inputs, similarly as proposed for the SON neurons (Fig. 8), and exhibit a high frequency of quantal release of glutamate that is sensitive to noradrenaline (Boudaba et al., 2003; Gordon and Bains, 2003, 2005).

Magnocellular neurons are also present in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, but do not have neuroendocrine function here. The SCN is the principal circadian pacemaker in mammals (Moore and Eichler, 1972; Stephan and Zucker, 1972). In most species, including rat, the SCN has two subdivisions that differ in neuronal input and neuropeptide contents. The ventrolateral part of the SCN receives glutamatergic inputs from retinal ganglion cells and produces vasoactive intestinal polypeptide, whereas the dorsomedial part does not receive a direct visual input and produces AVP (Moore and Card, 1985; Jacomy et al., 1999). Circadian rhythm in AVP secretion parallels rhythms of electrical activity in SCN neurons (Inouye and Kawamura, 1979; Groos and Hendriks, 1982; Pennartz et al., 2002) and both these rhythms are mediated by the expression of clock genes (Reppert, 1998). Retrograde tracer analysis demonstrated that the projection of AVP neurons from SCN to the PVN connects light reception to feeding behavior. Light exposure acutely suppresses food intake and elevates c-Fos expression in the AVP neurons of SCN and the oxytocin neurons of PVN. The light-induced suppression of food intake was mostly abolished by blockade of the oxytocin receptor in the brain (Santoso et al., 2018). Furthermore, intracerebroventricular injection of AVP suppressed food intake and increased c-Fos in PVN oxytocin neurons. Intra-PVN injection of AVP exerted a stronger anorexigenic effect than intracerebroventricular injection. AVP also induces intracellular Ca^{2+} signaling and increases firing frequency in oxytocin neurons in PVN slices (Santoso et al., 2018).

1.2.3. Characteristics of magnocellular neurosecretory neurons

Oxytocin and vasopressin secreting neurons share many properties and a roughly similar morphology with slightly oval neuronal body approximately 20-30 microns in diameter. Magnocellular neurosecretory cells are intrinsically osmosensitive and can be activated by

increases in blood osmolality, triggering the release of antidiuretic hormone vasopressin to promote water retention (Mason, 1980). The cells undergo dynamic changes that require expression of transient receptor potential vanilloid type-1 channels, which act as a mechanoreceptors, and cytoskeletal elements connected to the receptor (Barad et al., 2020). During the response to osmotic pressure, the cells shrink which leads to depolarization (Barad et al., 2020).

Another characteristic of magnocellular neurosecretory cells is a wide range of plasticity in response to different physiological demands which require adjustment in hormone secretion. For example, the dendritic arbors of oxytocin and vasopressin neurons in female rats differs from one another as a function of endocrine state. In virgin rats, oxytocin neurons have more dendritic branches and a greater total dendritic length compared with lactation, when the arbor is much less extensive. A complementary change occurs in vasopressin dendrites, which are more extensive during lactation. This reorganization suggests that communication between oxytocin neurons may be more effective during lactation as compared to a naive state (Armstrong and Stern, 1998).

Glia cells in SON and PVN play an important role in regulation of magnocellular neuron activity and hormone secretion as they express glutamate and GABA, which regulate autocrine neuronal activity (Tasker et al., 2012).

1.2.4. Electrophysiological properties of magnocellular neurosecretory neurons

Many neurons in the central nervous system display rhythmic patterns of activity to optimize excitation-secretion coupling. However, the mechanisms of rhythmogenesis are only partially understood. Early electrophysiological recordings from SON and PVN showed that oxytocin and vasopressin secreting neurons exhibit different firing patterns (Armstrong, 1995). *In vivo*, oxytocin neurons are characterized by an intermittent high frequency discharge during suckling that leads to the pulsatile release of oxytocin into the blood and to subsequent milk ejection. Vasopressin neurons are characterized by their asynchronous phasic activity (bursting) during maintained vasopressin release and the subsequent regulation of water balance. In both cases, it is the clustering of spikes, albeit with different time courses for each peptide, that facilitates hormone release (Lemos and Wang, 2000).

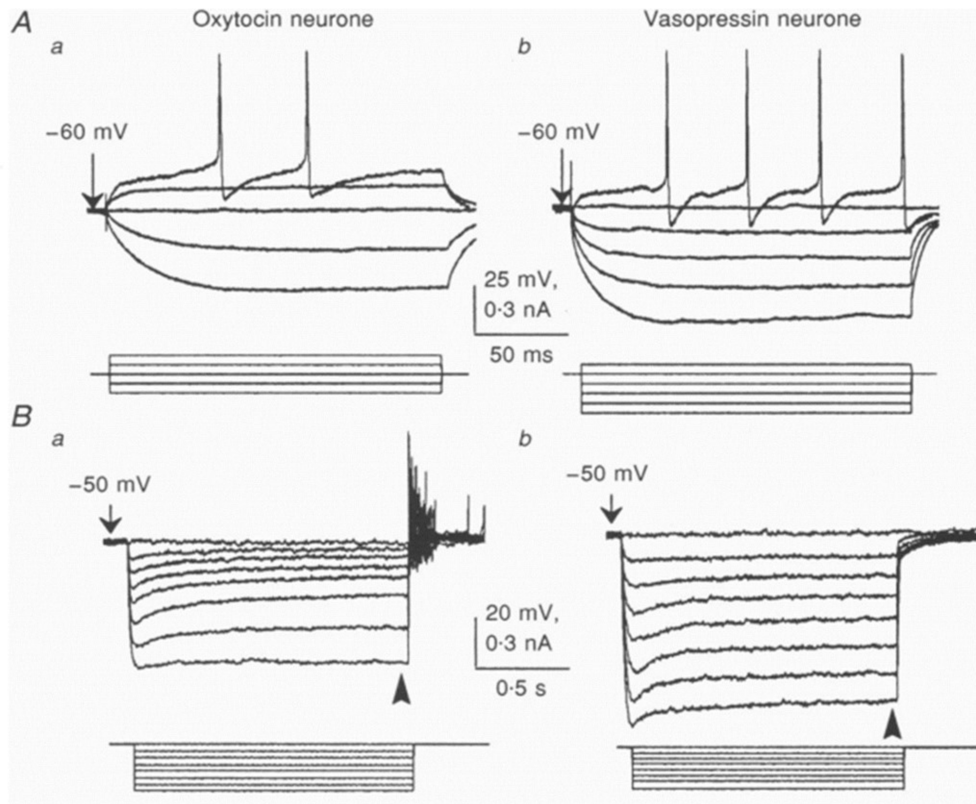

Figure 9. Differences in intrinsic membrane properties between oxytocin and vasopressin neurons. (A) Both oxytocin and vasopressin neurons exhibit a transient outward rectification underlain by an IA-type K^+ current. (B) This transient rectifier delays spiking to depolarizing stimuli from a relatively hyperpolarized baseline, and is more prominent in vasopressin neurons (arrows) (Armstrong and Stern, 1998).

Originally it has been proposed that vasopressin cell rhythmicity depends on intrinsic membrane properties (Armstrong, 1995; Bourque and Renaud, 1991). Experiments of Stern and Armstrong in 1998 showed significant differences in the intrinsic membrane properties, probably due to differences in K^+ currents, between oxytocin and vasopressin secreting SON neurons in both lactating and virgin female rats (Armstrong and Stern, 1998). Oxytocin, but not vasopressin neurons, are characterized by a depolarization-activated, sustained outward rectifier channels which turns on near spike threshold, and which can produce prolonged spike frequency adaptation (Fig. 9). Others investigated possibility of more electrophysiologically distinct types of magnocellular neurons, but so far there is no conclusive evidence on the matter (Tasker and Dudek, 1991; da Silva et al., 2015).

Different firing patterns of oxytocin and vasopressin neurons cannot be observed in acutely isolate slices in vitro, suggesting that phasic activity in vasopressin secreting neurons is completely dependent on synaptic transmission and is controlled by complex glutamatergic and GABAergic synaptic inputs rather than by intrinsic neuronal properties (Israel et al., 2010; Armstrong et al., 2018).

Secretion of hormone is stimulated when a burst of action potentials depolarizes neurosecretory membrane which leads to opening of voltage dependent Ca^{2+} channels (Bourque, 1991; Armstrong et al., 2018). Nerve terminals of magnocellular neurosecretory cells contain several ligand-gated ion channels, including ATP-stimulated P2X receptors, which can further depolarize the membrane, increase intracellular calcium levels (Xiang et al., 1998) and stimulate hormone release (Troadec et al., 1998).

1.2.5. Neurohypophyseal hormones

Neurohypophyseal hormones oxytocin and vasopressin are oligopeptides (9 amino acids, nonapeptides) with a single disulfide bridge between cystein residues (Fig. 10). Inside the cell,

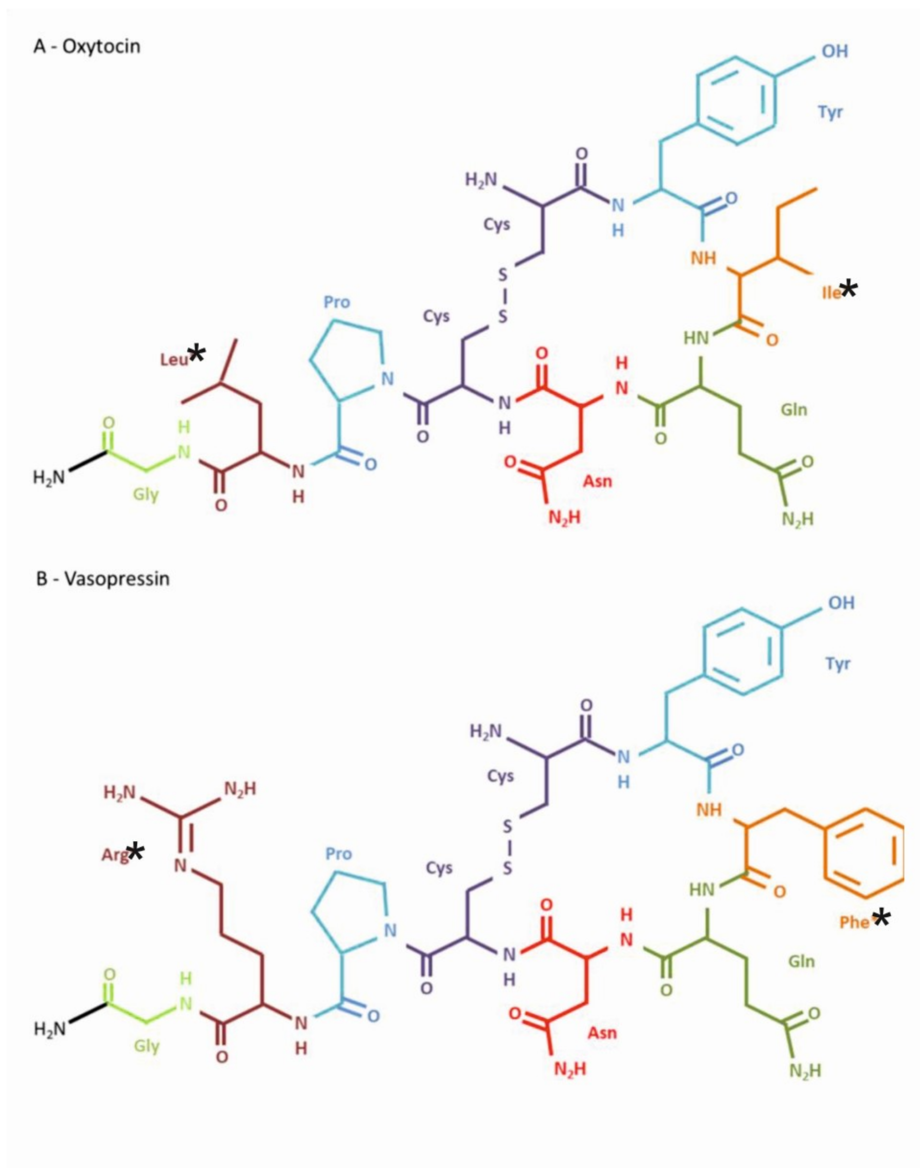

Figure 10. Chemical structure of neuroypophyseal hormones. (A) Structure of vasopressin. (B) Structure of oxytocin. Both hormones are nonapetides that differ only in two amino acids (marked with *).

they are connected to the protein carrier called neurophysin; for oxytocin it is called neurophysin 1 and for vasopressin neurophysin 2 (Renaud and Bourque, 1991). As such they are transported to nerve terminals where they are packed in the vesicles together with other molecules such as neurotransmitters, neuromodulators (for example acetylcholine), ATP and other peptides. The disulfide bridge is cleaved before a hormone is secreted into the blood vessels. Hormones are secreted through Ca^{2+}-dependent exocytosis. Released hormones bind to their respective receptors which belong to a family of G protein coupled receptors (Gimpl and Fahrenholz, 2001).

Oxytocin, vasopressin and their respective receptors are highly conserved across species. It is known that vasopressin receptor allows for binding of oxytocin and oxytocin receptor can bind vasopressin but with low affinity. Therefore, high concentration of one hormone can produce the effects of the other. Genes coding these hormones are located fairly close to each other and are probably result of duplication (Rao et al., 1992).

1.2.6. Vasopressin

Most common form of vasopressin found in mammals is arginine vasopressin (Fig. 10A). AVP release increases as a function of plasma osmolality and water status of the body (Oliet and Bourque, 1993; Israel et al., 2010; Choe et al., 2015). It is known as a regulator of serum osmolality but more recent studies imply many other functions such as caregiving behaviour in fathers (Taylor et al., 2017), effect on stress, pair-bonding, blood pressure, temperature regulation, circadian rhythm etc. Chronic exposure to high dietary salt leads to excessive activation of vasopressin neurons, thereby elevating levels of circulating vasopressin, which can cause an increase in blood pressure contributing to salt-dependent hypertension (Barad et al., 2020). There are many studies implicating role of vasopressin in different disorders such as schizophrenia, diabetes and other, for review see Iovino et al., 2018.

Concentration of vasopressin in circulation is normally ~0.1 pg/ml. (Minutillo et al., 2021). In humans, these levels are sufficient to cause renal reabsorption of ~30 l of water from the urine per day, but are below the concentrations required to induce vasoconstriction (Yosten and Samson, 2012). The level of vasopressin in the brain is several times higher than that in the periphery, reported AVP concentration in hypothalamus of mice is 6.1±1.3 ng/mg (Jiang et al., 2017).

Vasopressin receptors are divided into three groups according to their expression in different tissues: V1, V2 and V3. Sometimes V1 and V3 are classified as V1A and V1B

because of their structural similarity. Subtype V1 is coupled to Gs class GTP binding protein stimulating adenylate cyclase activity.

1.2.7. Oxytocin

Oxytocin (Fig. 10B) was the first peptide hormone artificially synthesized. The classical actions of oxytocin are stimulation of uterine smooth muscle contraction during labor and milk ejection during lactation (Voisin et al., 1995; Moos, 1995; Jourdain et al., 1998; Brussaard and Kits, 1999). The fact that oxytocin is found in equivalent concentrations in the neurohypophysis and plasma of both sexes suggests that it has further physiological functions (Gimpl and Fahrenholz, 2001). Oxytocin is critical for sexual behavior and maternal care (Kendrick, 2000), but both oxytocin and vasopressin are important for caregiving behaviors in fathers (Taylor et al., 2017). Oxytocin is also a key modulator of a variety of socio-emotional behaviors such as fear, trust and empathy (Althammer and Grinevich, 2017). Other studies showed indirect effects of oxytocin on many neuropsychiatric disorders such as autism, depression and anorexia, for example (Iovino et al., 2018; Aulinas et al., 2019; Iovino et al., 2021). Estrogen can induce production of oxytocin through its action on estrogen β receptors in hypothalamus (Hrabovszky et al., 2004).

Under basal physiological conditions, oxytocin plasma concentration is is 0.006 – 0.548 pg/ml (Minutillo et al., 2021), with higher levels during sleep. The level of oxytocin in the brain is several times higher than that in the periphery (Jiang et al., 2017), and very high concentration of oxytocin (>0.1 M) was found in the neurosecretory granules of the posterior pituitary (Minutillo et al., 2021). Under physiological stress, for example during birth and lactation, large pulses in oxytocin secretion occur every few minutes to start rhythmic contraction of the uterus and episodic milk duct contraction during suckling-induced reflex milk ejection.

Peripherally and centrally injected oxytocin inhibits food intake to a similar extent and with similar time course. Peripherally injected oxytocin induces c-Fos expression in PVN largely in oxytocinergic neurons and inhibits food intake (Iwasaki et al., 2019). The intraperitoneal oxytocin-induced inhibition of food intake was blunted in oxytocin KO mice, by intracerebroventricular injection of oxytocin receptor antagonist, and by vagotomy. These results demonstrate that intraperitoneal oxytocin injection activates PVN oxytocin neurons via vagal afferent nerves, thereby inhibiting food intake. This vagal afferents-mediated oxytocin peripheral-to-central coupling may serve to promote satiety and possibly a series of neural functions of oxytocin and to treat their disorders (Iwasaki et al., 2019). During oxytocin

administration in sated animals, oxytocin induces c-Fos changes in a broader area of hypothalamus and brain stem compared to those affected in the hungry state. Fasted animals have significantly lower oxytocin receptor mRNA levels than their ad libitum-fed counterparts (Head et al, 2019).

Only one oxytocin receptor has been identified so far (Danoff et al., 2021). Oxytocin receptor belongs to G protein-coupled receptors, it is functionally coupled to Gq/11a class GTP binding proteins that stimulate the activity of phospholipase Cβ (Gimpl and Fahrenholz, 2001). As signal transduction mechanism it utilizes IP_3 and DAG, its activation results in intracellular calcium mobilization.

1.3. Purinergic signaling in hypothalamus

Hypothalamus is one of the brain regions with highest density of P2X receptors (Bo and Burnstock, 1994; Collo et al., 1996) and with well characterized role of extracellular ATP in neuron-glia interaction (Gordon et al., 2009).

1.3.1 Expression and function of P2X in hypothalamus

Earliest evidence for the expression of P2X receptors in magnocellular neurons comes from work of Shibuya et al., in 1999. RT-PCR and in situ hybridization have demonstrated the expression of mRNAs for P2X2, P2X3, P2X4, P2X6 and P2X7 in the SON and the PVN (Shibuya et al., 1999b). Immunoreactivity, RT-PCR and ultrastructural studies have shown P2X2 expression in the PVN, arcuate nucleus, retrochiasmatic area, periventricular nucleus, the ventral part of tuber cinereum area and a subpopulation of SON neurons (Kidd et al., 1995; Vulchanova et al., 1996; Xiang et al., 1998). Later studies showed more specific localizations of these receptors, particularly the presence of P2X2 receptors on orexinergic neurons in lateral hypothalamus (Wollmann et al., 2005) or the presence of P2X2, P2X4, P2X5 and P2X6 on GnRH neurons in preoptic area of hypothalamus (Fu et al., 2009). The P2X2 has been found also in PVN parvocellular CRH, TRH and CART neurons that are associated with the regulation of food intake (Collden et al., 2010). High colocalization of P2X5 and vasopressin neurons was reported in PVN (~ 80%) (Xiang et al., 2006). Injection of the retrogradely transported tracer, rhodamine-tagged microspheres, into the rostral ventrolateral medulla showed several P2X subtypes in the PVN that innervates this region (Cham et al., 2006). Different combinations of P2X subunits have been found on the perikarya and axon terminals of GnRH neurons using double-labeling fluorescence immunohistochemistry (Fu et al., 2009). Multiple types of P2X are present in the SCN as well

(Xiang et al., 1998). High expression level of P2X2, P2X4 and P2X7 subtypes has been documented in the SON and SCN at the level of mRNA and protein (Vavra et al., 2011; Bhattacharya et al., 2013).

Multiple studies showed that αβmeATP induces cardiovascular responses through its actions on P2X1, P2X3, P2X2/3 and/or P2X1/5 in PVN (Busnardo et al., 2013; Ferreira-Neto et al., 2013). This was the first evidence that P2X receptors in PVN can modulate sympathetic nervous system (Ferreira-Neto et al., 2013). A subset of P2X is activated following hyperosmotic stimulation (Ferreira-Neto et al., 2017). Table 1 and Table 2 summarize the most important findings about expression of different P2X subtypes in SON and PVN, respectively.

Table 1. Expression of P2X receptors in SON

Receptor	**Methodology**	**Reference**
P2X2,3,4,6, 7	RT-PCR, in situ hybridization, Ca^{2+} imaging and electrophysiology (patch-clamp)	(Shibuya et al., 1999b)
P2X2	PCR	(Kidd et al., 1995)
P2X2	Immunohistochemistry	(Xiang et al., 1998)
P2X2	Electrophysiology (patch-clamp)	(Wollmann et al., 2005)
P2X2,4,5,6	Double-labeling fluorescence immunohistochemistry	(Guo et al., 2009)
P2X2,4,7	Electrophysiology (patch-clamp), Ca^{2+} imaging and RT-PCR	(Vavra et al., 2011)

Table 2. Expression of P2X receptors in PVN

Receptor	**Methodology**	**Reference**
All subtypes	Injection of the retrogradely transported tracer, rhodamine-tagged microspheres, immunohistochemistry	(Cham et al., 2006)
P2X4,5,6	Double-labeling fluorescence immunohistochemistry	(Guo et al., 2009)
All subtypes	Electrophysiology, histochemistry	(Ferreira-Neto et al., 2013)
P2X1,2,3,5	Histochemistry	(Busnardo et al., 2013)
P2X2	Immunohistochemistry	(Ferreira-Neto et al., 2017)
P2X7	RT-PCR, immunofluorescence	(Du et al., 2015)

Numerous studies showed that P2X are functionally expressed in both somatic and presynaptic membranes of hypothalamic neurons. In the SON, activation of somatic receptors induces depolarizing currents and extracellular Ca^{2+} entry (Shibuya et al., 1999a). Functional P2X2 have been identified in isolated nerve terminals of magnocellular neurosecretory cells in posterior pituitary (Troadec et al., 1998), as indicated by cytosolic calcium measurements (Troadec et al., 1998). ATP application induced increase in cytosolic calcium and peptide release from nerve terminals (Song et al., 2006; Gomes et al., 2009). In identified hypocretin cells in hypothalamic slices of transgenic mice, ATP application induced small inward current and increased frequency of action potentials exclusively in hypocretin-producing cells. ATP-induced current was inhibited by suramin and PPADS, and potentiated by a decrease in bath pH, suggesting P2X2 involvement (Wollmann et al., 2005). Functional P2X2 and P2X4 subunits are expressed in GnRH neurons in olfactory placode cultures where ATP application leads to synchronization of calcium oscillations (Terasawa et al., 2005). Electrophysiological experiments also showed the presence of ATP-induced P2X2-mediated inward current in unidentified neurons of the arcuate nucleus (Wakamori and Sorimachi, 2004). Contribution of both P2X2 and P2X4 receptors to ATP-induced somatic current was reported in SON neurons (Vavra et al., 2011). Whole-cell patch-clamp recordings from neurons in slices revealed that extracellular ATP application increased the frequency of mIPSCs and mEPSCs without changes in their amplitudes indicating that ATP activates presynaptic P2X2 receptors to regulate synaptic transmission within the SON (Vavra et al., 2011) and SCN (Bhattacharya et al., 2013). Little evidence was found for the presence of P2X2 channels in somata of SCN neurons as ATP-induced current was observed in only 7% of SCN neurons, and P2X2 immunoreactivity colocalized with synapsin (Bhattacharya et al., 2013). Recent imunohistochemistry and PCR studies revealed that some P2X receptors in mouse SCN show time of day dependent variations in their expression (Lommen et al., 2017; Ali et al., 2020).

Purines are supposed to play an important role in controlling the activity of vasopressin but not oxytocin-secreting SON neurons. This conclusion was initially derived from experiments measuring the effects of extracellularly added ATP on vasopressin and oxytocin release from isolated magnocellular neurosecretory nerve terminals (Troadec et al., 1998). Immonohistochemistry showed that P2X are only present in AVP terminals (Knott et al., 2012). Another study showed that ATP potentiates AVP secretion in nerve terminals through P2X2, P2X3, P2X4 and possibly P2X7 (Lemos et al., 2012). Electrophysiological evidence for the existence of P2X currents in AVP nerve terminals but not in terminals labeled for oxytocin have also been obtained (Knott et al., 2005). Colocalization of P2X

receptors with vasopressin and oxytocin, studied using double-labeling fluorescence immunohistochemistry, showed that P2X are differentially expressed on vasopressin- and oxytocin-containing neurons in the SON and PVN (Guo et al., 2009). In agreement with this, ATP-induced somatic current was observed only in a subpopulation (62 %) of non-identified SON neurons (Vavra et al., 2011). Under physiological conditions, ATP has been shown to mediate an excitatory noradrenergic neuron input to supraoptic vasopressin cells (Day et al., 1993). ATP is co-released with noradrenaline from presynaptic terminal ending on magnocellular neurosecretory neurons and by activating P2X receptors increases intracellular calcium (Song et al., 2007) which can further affect secretion of hormones (Gomes et al., 2009).

Purinergic receptors are also involved in astrocytic modulation of magnocellular neurosecretory cells (Espallergues et al., 2007). ATP is released by hypothalamic astrocytes that are positioned in a close proximity of synapses to sense and modulate afferent synaptic activity. Glial-derived ATP contributes directly to the regulation of postsynaptic efficacy at glutamatergic synapses onto magnocellular neurosecretory neurons in the rat PVN (Gordon et al., 2005) and potentiates GABA release through presynaptic P2X at synapses in appetite-regulatory brain regions in dorsomedial hypothalamus (Crosby et al., 2018). In studies on rat brain slices, ghrelin was shown to affect vasopressin neurons of PVN through glial ATP (Haam et al., 2014). Interestingly, microglial P2X7 was shown to activate cytokines that in turn mediate secretion of oxytocin from PVN (Du et al., 2015). Astrocytic ATP modulate stress and reward response presumably mainly via P2X and it has been found that P2X2 in the medial prefrontal cortex mediated the antidepressant-like effects of ATP (Cao et al., 2013).

1.3.2. P2Y distribution and function in SON and SCN

Calcium imaging studies provided evidence for the functional expression of both P2X and P2Y in the SON, and showed that P2Y1 subtype is more prominent than P2Y2, P2Y4, or P2Y6 (Song et al., 2007). Significant expression level of P2Y1 and P2Y2 has been documented in SON and SCN tissue at the level of mRNA (Vavra et al., 2011; Bhattacharya et al., 2013). In fura-2 AM-loaded hypothalamic slices, the ATP-induced calcium response was reduced in the presence of MRS2179, specific P2Y1 blocker, and ADP stimulated an increase in intracellular calcium concentration ($[Ca^{2+}]_i$), indicating that the SON and SCN cells, most probably astrocytes, express functional P2Y receptors (Vavra et al., 2011; Bhattacharya et al., 2013).

1.3.3. Circadian ATP release from astrocytes

The SCN generates a circadian rhythm in intracellular and extracellular ATP accumulation, which negatively correlates with the electrical activity and AVP secretion rhythms (Yamazaki et al., 1994; Womac et al., 2009). Extracellular ATP levels fluctuate rhythmically within the rat SCN *in vivo* and SCN-derived SCN2.2 cell cultures containing 80% astrocytes (Womac et al., 2009; Burkeen et al., 2011). ATP release from cultured cortical astrocytes also follows circadian rhythm (Marpegan et al., 2011), indicating that oscillations in ATP release are intrinsic properties of astrocytes. However, the pathway by which ATP travels from the cytosol of astrocytic cells to the extracellular space is still unknown.

Astrocytes release ATP and glutamate (van den Pol et al., 1992; Bhattacharya et al., 2013) that both have positive feedback and evoke $[Ca^{2+}]_i$ signals that might trigger further release of ATP or glutamate (Haydon, 2001; Illes et al., 2012). Interestingly, the glutamate-evoked increase in astrocytic $[Ca^{2+}]_i$ in the intact optic nerve is significantly reduced in P2X7 knock-out mice (Hamilton et al., 2008) indicating the possible role of P2X7 in release of these gliotransmitters.

1.4. Experimental protocols used to stimulate synthesis and release of hormones

Experimental conditions that have been widely used to stimulate the synthesis and release of neurohypophyseal peptides are: lactation (de Kock et al., 2004; Panatier and Oliet, 2006), feeding with sweetened condensed milk (SCM) gavage (Verbalis et al., 1991) for oxytocin, and salt-loading (Leng et al., 2001) and water deprivation or refeeding after fasting for vasopressin and to smaller extend also for oxytocin (Carreno et al., 2011; Gottlieb et al., 2011; Yao et al., 2012; Yoshimura et al., 2013; Lucio-Oliveira et al., 2015).

1.4.1. Lactation

Lactating female rat, postpartuition days 7-9, and virgin females were used to study functional and morphological changes in synapses of the SON and PVN under conditions of intense secretion of neurohypophysial hormones. It has been shown that calcium influx through NMDA receptor channels, in the absence of postsynaptic cell firing, is able to induce vesicle fusion from non-synaptic sites in nucleated outside-out patches of SON neurons of female rats in particular during their reproductive stages, and that NMDA-induced oxytocin release is

thus up-regulated compared to that in non-reproductive rats (de Kock et al., 2004). Another study revealed that anatomical remodeling of synapses during lactation is characterized by a pronounced reduction in astrocytic coverage of neurons, which results in an increased number and extent of directly juxtaposed somatic and dendritic surfaces. As a consequence, astrocyte-mediated clearance of glutamate from the extracellular space is altered, which causes an increased concentration and range of action of glutamate in the extracellular space. This leads to a reduction of synaptic efficacy at excitatory and inhibitory inputs through the activation of presynaptic metabotropic glutamate receptors. By contrast, the action of gliotransmitters, including ATP, released from astrocytes and potentiating glutamatergic transmission on adjacent magnocellular neurons is supposed to be limited during such anatomical remodeling (Panatier and Oliet, 2006).

Interestingly, an increase in the density of GABA-containing synaptic boutons and a higher incidence of $GABA_A$ receptor-mediated spontaneous inhibitory postsynaptic currents has been found in the SON neurons of pregnant rats than in those of virgin rats (Brussaard and Kits, 1999), further indicating that reproductive stages are controlled by complex changes in synapses.

1.4.2. Sweetened condensed milk gavage

The magnocellular system releasing oxytocin can be specifically activated by SCM gavage. Firing rates in oxytocin cells began to increase soon after the onset of gavage and the increase peaks up to a maximum around 30 minutes after the onset of gavage (Verbalis et al., 1991). In the SON, the percentage of magnocellular oxytocin-immunoreactive neurons expressing c-Fos is significantly greater in rats voluntarily consuming SCM in comparison to controls, but there was no significant difference in the number of putative vasopressin cells expressing c-Fos when rats voluntarily ate SCM (Hume et al., 2017). This specific activation of the magnocellular system by SCM gavage is sufficient to stimulate both peripheral and central oxytocin release.

There are multiple mechanisms by which gut-initiated signaling could influence the activity of oxytocin neurons. For example, the increase in activity of oxytocin neurons in response to SCM gavage could be mediated by cholecystokinin, also called satiety mediator, a peptide signal released in response to nutrients entering the duodenum of the small intestine. Cholecystokinin acts via vagal afferents to activate noradrenergic projections from the nucleus tractus solitarius to magnocellular oxytocin neurons (Fig. 8) resulting in oxytocin release from the posterior pituitary (Leng et al., 1999). *In vivo* electrophysiology during SCM

gavage in anaesthetized rats revealed that oxytocin cells can be distinguished from vasopressin cells by their opposite response to intravenous cholecystokinin (Leng et al., 1999).

1.4.3. Salt-loading

Acute osmotic stimuli activates oxytocin and vasopressin cells to a similar extent, and chronic dehydration or salt loading produce similar depletion of the pituitary stores of both hormones (Leng et al., 1999). Systemic osmotic stimulation induced by injection of hypertonic sodium chloride solution intraperitoneally or intravenously increases vasopressin and oxytocin release within the SON (Ludwig and Leng, 2000). Slow intravenous infusion of hypertonic solution stimulates organum vasculosum of the lamina terminalis, which activates monosynaptic projections to the SON that are in part inhibitory and in part excitatory (Fig. 8). Recording of the electrical activity showed that both oxytocin and vasopressin cells in the SON of normal rats respond to intravenous infusions of hypertonic saline with gradual increases in discharge rate. In hyponatremic rats, oxytocin and vasopressin cells also responded linearly to intravenous infusions of hypertonic saline but with much lower slopes (Leng et al., 2001). It has been concluded that hypertonic infusions lead to coactivation of excitatory and inhibitory inputs and that this complex coactivation may confer appropriate characteristics on the output behavior of magnocellular neurosecretory neurons (Leng et al., 2001). In a study using microarrays to profile the transcriptome of the SON, oxytocin gene expression is reported to elevate after changes in plasma osmolality induced by salt loading or water deprivation in rats (Greenwood et al., 2015).

There are also several mechanisms by which salt-loading could influence the activity of vasopressin neurons. Changes in osmotic pressure induced by salt loading activates unique actin filaments in AVP producing neurons which in turn leads to increased hormone production (Barad et al., 2020). Another study showed that high salt loading upregulates brain derived neurotrophic factor (BDNF) indicating that BDNF produced in the SON might be necessary for increased vasopressin secretion during high salt loading (Balapattabi et al., 2018). High salt intake enhances the expression and activity of epithelial sodium channels (ENaCs) in oxytocin neurons, which could depolarize the basal membrane potential close to the action potential threshold. However, ENaCs appear to have only a minor role in the regulation of the firing activity of vasopressin neurons in the absence of synaptic inputs (Sharma et al, 2017), confirming that phasic activity in vasopressin secreting neurons is

controlled by complex glutamatergic synaptic inputs rather than by intrinsic neuronal properties (Israel et al., 2010).

1.4.4. Refeeding after fasting

Refeeding after fasting represents a complex stimulation to hormone secretion involving the volume/baroreceptors and peripheral/central osmoreceptors (Fig. 11). Food deprivation and refeeding for 2 – 6 h stimulate selectively synthesis and release of the AVP, although changes in the oxytocin might also be observed. Food deprivation for 48 h causes a reliable decline of AVP level in the SON, while little change in oxytocin concentration was detected (Burlet et al., 1992). Increased plasma osmolality by food intake (after 48 h of fasting) stimulates baroreceptors in neurons of the brain subfornical organ projecting to the SON (Fig. 8), and evokes augmentation of plasma AVP (Lucio-Oliveira et al., 2015) and oxytocin (Lucio-Oliveira and Franci, 2012). Refeeding after fasting increases immediate early gene c-fos expression in identified vasopressin and oxytocin cells indicating increased activation of these neurons (Johnstone et al., 2006; Lucio-Oliveira and Franci, 2012; Lucio-Oliveira et al., 2015).

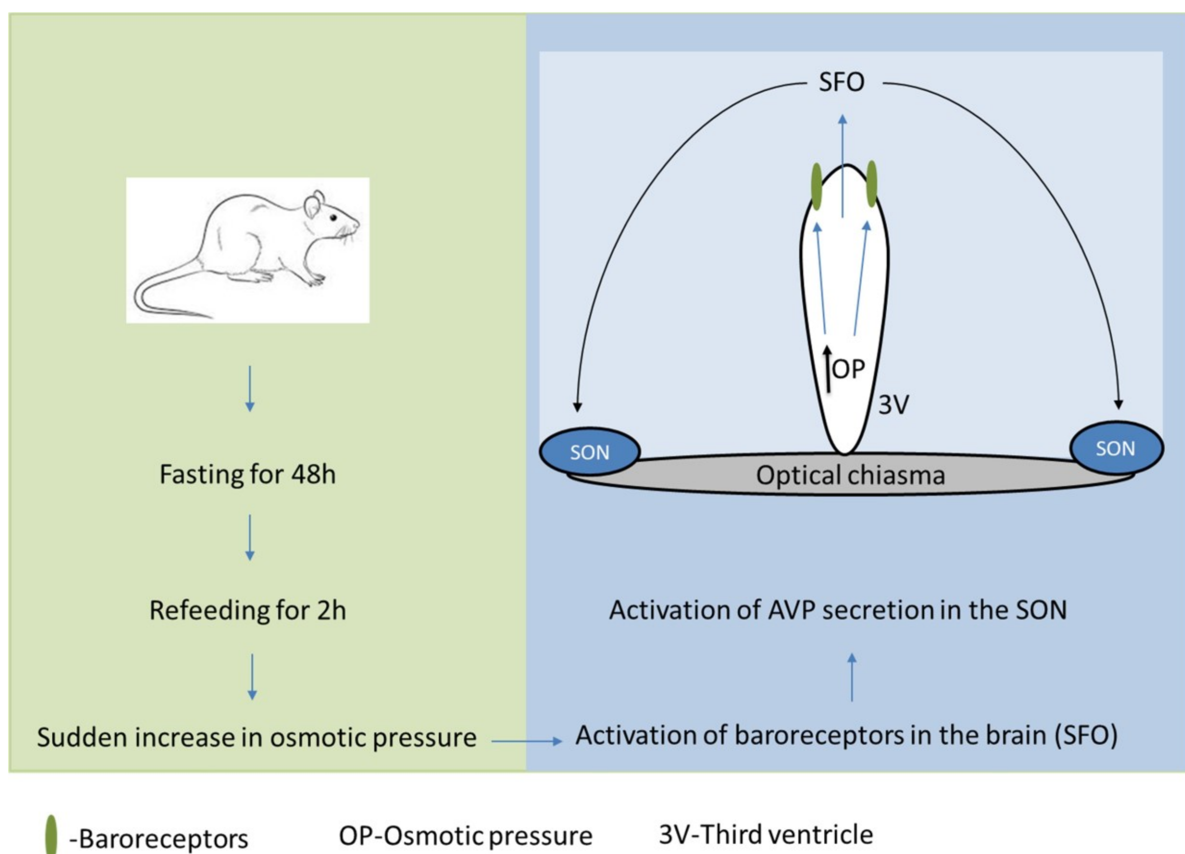

Figure 11. Scheme of the fasting/refeeding protocol. Refeeding after fasting represents a complex stimulation to hormone secretion from SON involving the volume/baroreceptors in subfornical organ (SFO) and peripheral/central osmoreceptors.

It has been shown that 2-4h of refeeding after 48h of fasting significantly increases mRNA levels of both oxytocin and AVP in the mice hypothalamus compared to normally fed mice (Poplawski et al., 2010). It also increases expression of c-Fos in vasopressinergic and to lesser extent oxytocinergic SON neurons (Kohno et al., 2008; Johnstone et al., 2006), confirming that AVP positive neurons in SON could show higher activity than oxytocin positive neurons after refeeding (Timofeeva et al., 2005). Interestingly, c-fos gene is activated as a consequence of receptor-mediated events, not directly or necessarily associated with electrical activation, although in general these are normally associated (Luckman et al., 1994). Increased number and percentage of Fos-positive oxytocin neurons in the SON, without significant alteration in oxytocin mRNA expression, was also found after refeeding (Uchoa et al., 2009).

2. Aims of the study

The main objectives of this thesis were to test a hypotheses that P2X contribute to enhanced function of SON neurons under conditions of intense secretion of neurohypophysial hormones, and that these receptors are involved in circadian ATP release from SCN astrocytes. To accomplish these objectives, two goals were established:

- To investigate the effect of 2h of refeeding after 48 h fasting, which is known to stimulate selectively synthesis and release of the AVP, on P2X mRNA expression and function of P2X in the rat SON neurons in slices.

- To explore the role of P2X7 and P2Y in circadian rhythm of extracellular ATP accumulation in SCN organotypic cultures.

3. Materials and methods

3.1. Animals, tissue slices and primary cultures

3.1.1. Animals

Animals were obtained from established breeding couples in the animal facility (Animal facility of the Institute of Physiology, Czech Academy of Sciences; approval number #56379/2015-MZE-17214). All animal procedures were approved by the Animal Care and Use Committee of the Czech Academy of Sciences (dissection protocol # 67985823). The animals were kept since their birth under conditions of stable temperature and humidity, 12 h light/dark cycles and food and water provided ad libitum (Animal Facility of the Institute of Physiology, Czech Academy of Sciences). We employed a previously reported protocol to challenge the peptide producing system of the SON, refeeding with standard chow for 2 h after two days of fasting (Fig. 11), which increases the activity of AVP neurons (Lucio-Oliveira et al., 2015). Experiments were performed in 30- to 32-day-old Wistar rats. A total of 64 Wistar rats (37 normally fed and 27 fasted/refed) of both sexes were investigated. Euthanasia was performed by decapitation after anaesthesia with isofuranum (Forane, AbbVie s.r.o. Czech Republic) that does not smell and does not damage epithelium in the respiratory system.

3.1.2. Slice preparation

Brains were removed and placed into ice-cold (4°C) oxygenated (95% O_2 + 5% CO_2) ACSF. Hypothalamic slices (200- to 300-μm-thick) containing SON were cut with a vibratome (DTK-1000, D.S.K., Dosaka, Japan). The slices were allowed to recover for at least 1 h in oxygenated ACSF at 32-33°C before being transferred into a recording chamber. During the experiments, slices were submerged in continuously flowing oxygenated ACSF at 1–2 ml min-1 at room temperature. Slices were viewed with an upright microscope (Olympus BX50WI, Melville, NY, USA) mounted on a Gibraltar X-Y table (Burleigh) using a water immersion lens (60x and 10x) and Dodt infrared gradient contrast (Luigs & Neumann, GmbH, Germany). The SON of rats was identified by the position relative to the chiasma opticum (Vavra et al., 2011).

3.1.3. Organotypic culture preparation

Coronal sections of the hypothalamus (~350 µm thick) were cut from approximately 1x1 mm tissue blocks containing the SCN using a vibratome. In some experiments, the SCN was punctured out from slices. Three slices cut from one animal were transferred onto one cell culture insert with a pore size of 0.001 mm (BD Falcon, Tewksbury, MA, USA). Culture inserts with 3 slices, further referred to as the organotypic cultures, were placed in 6-well plates (BD Falcon) and submerged in 1 ml of Neurobasal A-based medium saturated with a 95% air and 5% CO_2 mixture. Plates containing inserts with slices/cultures were incubated in a humidified 5% CO_2 atmosphere at 37°C. Slices were cultured in Neurobasal medium for 7 days before starting ATP accumulation assays to allow slices to stabilize (Svobodova et al., 2003). During this time, organotypic SCN cultures maintain the clear organization of SCN cells along the dorsoventral axis (Fig. 12).

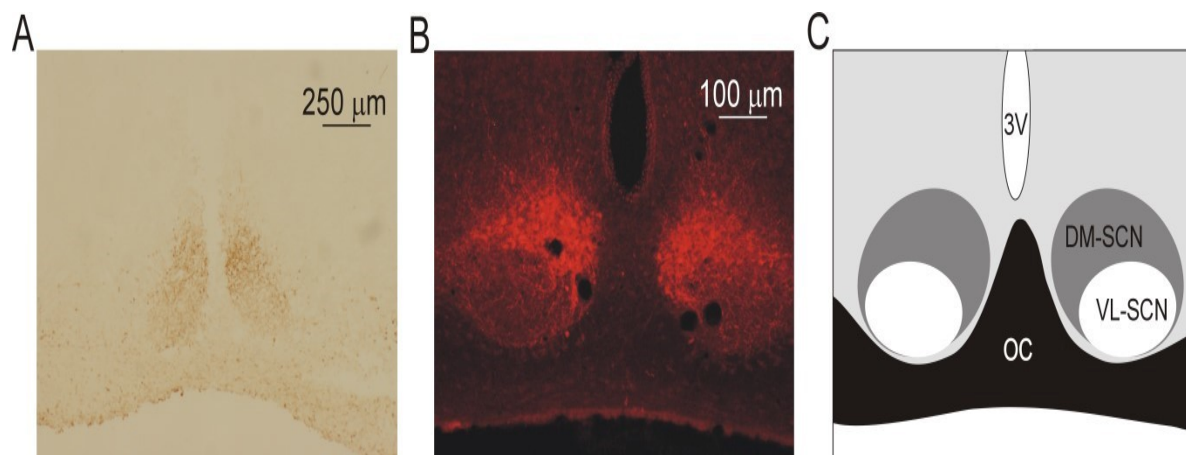

Figure 12. Organotypic cultures maintain structure of the SCN. (A) An example of immunohistochemistry experiments performed on the rat organotypic cultures containing the SCN. After three days in culture the sections were fixed in 2% paraformaldehyde for 15 min, washed in PBS, and cryoprotected in 20% sucrose in PBS overnight at 4°C. The slices were then frozen on dry ice, sectioned into a 12-lm thick serial sections using a cryostat, and levels of SCN-specific phospho-p44/42 mitogen-activated protein kinase (ERK1/2; antibody purchased from Cell Signaling Technology, Inc., Danvers, MA), were assessed by immunohistochemistry. The immunopositive signal predominates in the dorsomedial subdivision of the SCN (experiments performed by Zdena Bendova, Faculty of Sciences, Charles University). (B) Acutely isolated hypothalamic slice containing SCN stained with anti-vasopressin antibody (Abcam - ab39363). The AVP immunoreactivity (red) is present on dorsomedial subdivision of the SON. (C) Illusory boundaries showing dorsomedial SCN (DM-SCN), ventrolateral SCN (VL-SCN), optic chiasm (OC) and the third ventricle (3V).

3.1.4. Primary cultures of SCN astrocytes

Postnatal day 2-5 newborn rats were euthanized by decapitation. SCN regions were dissected from ~600 µm thick hypothalamic slices and cells were dissociated after treatment with trypsin, according to published methods (Watanabe et al., 1993; Svobodova et al., 2003).

Next, cells were purified on a discontinuous protein gradient, and approximately 100,000 cells were placed on coverslips coated with a 1% poly-L-lysine solution (Sigma) in 35 mm culture dishes (BD Falcon) and cultured in Neurobasal A medium with 2% B27 supplement and 0.5 mM L-Glutamine in a humidified CO_2-containing atmosphere at 37°C until use (14-21 days).

3.2. Experimental assays and techniques

3.2.1. Patch-clamp recordings

ATP-induced currents and membrane potentials were recorded from SON slices using standard whole-cell patch-clamp techniques with an Axopatch-200B amplifier (Axon Instruments, Union City, CA, USA). Patch pipettes were pulled on the horizontal Flaming Brown P-97 model puller (Sutter Instruments, Novato, CA, USA) from borosilicate glass (World Precision Instruments, Sarasota, FL), polished by heat to a tip resistance of 6-8 MΩ, and backfilled with intracelllar solution (see Solutions). The access resistance (average 14.2 ± 1.2 MΩ, n=18) was monitored throughout each experiment. The mean capacitance of the cells was 6-8 pF, 50-80% series resistance compensation was used, and liquid junction potential (~4 mV), calculated using the program CLAMPEX 9, was corrected offline when determining the resting membrane potential of SON cells. Data were captured and stored using the pClamp 9 software package in conjunction with the Digidata 1322A A/D converter (Axon Instruments). Signals were filtered at 1 kHz and sampled at 10 kHz. The ATP-induced currents and spontaneous miniature postsynaptic currents (mPSCs) were recorded from cells voltage-clamped in the presence of 0.5 µM tetrodotoxin (TTX) that was used to block action potentials. The cell membrane potential was held at -60 mV.

3.2.2. ATP luminescence assay

ATP secretion by SCN cells into the medium from the above mentioned organotypic cultures was measured every 4 h over a 24 - 48 h incubation period. Extracellular ATP concentrations in the medium were determined using an ATP bioluminescent assay. Before assay, at 8:00, cultures were washed with ATP-free Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA) supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin, and then incubated with fresh ATP-free DMEM-based medium in a humidified 5% CO_2 atmosphere at 37°C for 4 h. In our experimental protocol spanning 24 - 48 h, media (1 ml) above the slices were collected every 4 h starting at 12:00. Samples were collected as a full volume of medium (1 ml) and were replaced with fresh ATP-free DMEM. Media samples

were then stored at -20°C for 3 days, and the ATP concentration was then measured using an ATP Bioluminescence Assay Kit CLS II (Hoffmann-La Roche AG, Basel, Switzerland). Treatments were performed by completely replacing the medium with fresh drug-containing culture medium every 4 h. Each tested drug was added at 8:00, and the cultures were exposed to these drugs over a 24 - 48 h incubation period. In control experiments, the protocol was identical and the medium was replaced with fresh drug-free culture medium every 4 h.

3.2.3. Calcium imaging

For intracellular Ca^{2+} fluorescence imaging, primary cultures of SCN astrocytes on coverslips were incubated in 2 ml ACSF containing 1 μM of membrane-permeant ester form of Fura-2 (Fura-2AM, Invitrogen, Molecular Probes) and 0.15% dispersing agent Pluronic F-127 for 30 – 45 min in carbogen atmosphere (95% O2 and 5% CO2). After 15 min of washing in fresh ASCF, Fura-2 fluorescence from single cells was measured using a MicroMAX CCD camera (Princenton Instruments, USA) and an Olympus BX50WI epifluorescent microscope coupled to a monochromatic illumination system (T.I.L.L., Photonics). Hardware control and image analysis were performed using MetaFluor software (Molecular Devices). Cells were examined under a water immersion objective during exposure to alternating 340- and 380-nm light beams. The emitted light images at 515 nm were acquired through a d 40 x 0.9 NA objective, and the intensity of light emission was measured. The ratio of light intensity (F340/F380) reflects changes in $[Ca^{2+}]_i$ and was followed in ~20 single cells simultaneously at the rate of one point per second.

3.2.4. Quantitative Real-Time PCR

Coronal hypothalamic slices (1000 mm) containing SON(s) were dissected from rat brains using a Vibratome slicer, and SON(s) were punched out of the slices under visual control (magnification 20x) using a needle punch with an internal diameter of approximately 1 mm. Samples were either used immediately or frozen in RNAlater™ (Sigma-Aldrich) at -80 C. Total RNA was extracted from the tissues using the mirVana™ miRNA Isolation Kit (Ambion™, Thermo Fisher Scientific, United States). Briefly, samples were homogenized in lysis buffer provided with the kit using ceramic balls (MagNA Lyser Green Beads) and a MagNA Lyser homogenizer (Roche Diagnostics GmbH, Germany). RNA was then extracted from the tissue homogenate using acid-phenol:chloroform extraction and subsequent column purification with glass-fiber filter containing columns according to the manufacturer's

protocol. The concentration of total RNA in each sample was measured using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific,Wilmington, DE, United States). Samples were volume adjusted with DNAase and RNAase free DEPC treated water provided with the RT kit and normalized for their RNA content. The first strand cDNA was synthesized from up to 1 mg of isolated RNA using SuperScript™ VILOTM cDNA Synthesis Kit (Invitrogen, Thermo Fisher Scientific, United States) in a 20-mL reaction volume using random primers provided with the kit according to the manufacturer's protocol. The expression levels of specific mRNA(s) for the AVP, oxytocin, P2X 2, 4, and 7 (P2X2, P2X4, and P2X7), P2Y1 and P2Y2 genes were measured using a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, United States). The probes and primers (TaqMan® probes) used for these experiments were developed as TaqMan Gene Expression Assay by Applied Biosystems. Specifically, AVP (Rn00566449_m1), oxytocin (Rn00564446_g1), P2X2 (Rn00586491_m1), P2X4 (Rn00580949_m1), P2X7 (Rn00570451_m1), P2Y1 (Rn00562996_m1), and P2Y2 (Rn00568476_m1) were used. Eukaryotic 18 s rRNA endogenous control (VIC R /MGB Probe, Primer Limited; Hs99999901_s1) was used as a housekeeping gene/endogenous control. Realtime PCR amplification was performed in 30 ml aliquots on a 96-well fast optical plate in a duplex reaction format. Each reaction contained TaqMan target gene probes labeled with FAM/TAMRA, 18 s RNA probes (VIC/MGB), TaqMan Universal Master Mix II, no UNG (Applied Biosystems) and cDNA. The efficiency of different probes was found to be very similar, and therefore, the $2^{-\Delta\Delta}C_T$ method was used to calculate the relative mRNA levels of all genes of interest normalized to the endogenous control (18 s RNA). Final results were expressed as fold changes in relative mRNA expression with the treatment of the animals (controls vs. 2 h fasted/refed groups).

3.2.5. Immunohistochemistry

Adult male rats were deeply anesthetized with thiopental (50 mg/kg) and perfused through the aorta with heparinized saline followed by phosphate-buffered saline (PBS; 0.01 M sodium phosphate/0.15 M NaCl, pH 7.2) and 4% paraformaldehyde in PBS. Brains were removed, postfixed for 12 hrs at 4°C, cryoprotected in 20% sucrose in PBS overnight at 4°C, and stored at -80°C. Brains were sectioned into series of 30-µm-thick free-floating coronal slices throughout the rostral-caudal extent of the SCN. Levels of P2X7 receptor protein in astrocytes were assessed using anti-GFAP conjugated with Cy3 (ab49874, Abcam, Cambridge, United Kingdom) mouse monoclonal, 1:1000, anti-P2X7 (APR-004, Alomone Labs, Israel) rabbit,

1:1000. The P2X7 labeling was visualized using Alexa Fluor® 488-conjugated secondary anti-rabbit antibody (Invitrogen, Carlsbad, CA; dilution 1:500). The images were acquired with confocal microscope Leica TCS SP2.

3.2.6. Drug application by rapid solution changer

ATP (100 µM) was applied in HEPES-buffered extracellular solution (see Solutions). The solutions were delivered to the recorded cells by a gravity-driven microperfusion system containing nine glass tubes with a common outlet approximately 300 µM in diameter (RSC-200 Rapid Solution Changer, Biologic, Claix, France). The application tip was routinely positioned approximately 500 mm away from the recorded cell and 50 µm above the surface of the slice. The time of each ATP application (5–20 s) was controlled.

3.2.7. Solutions

Acutely isolated slices were preincubated at 32–33 °C in oxygenated ACSF that contained (in mM): 130 NaCl, 3 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 19 $NaHCO_3$, 1.25 NaH_2PO_4 and 10 glucose (pH 7.3–7.4; osmolality 300–315 mOsm). ATP was diluted and applied in a N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES)-buffered extracellular solution (ECS) containing (in mM): 142 NaCl, 3 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES, the osmolality was 300–315 mOsm and the pH was adjusted to 7.3 with 1 M NaOH. While testing the effect of ATP application, neurons were exposed to HEPES-buffered ECS for no more than 2 min. The patch electrodes used for whole-cell recording were filled with an intracellular solution containing (in mM): 140 KCl, 3 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES and 5 EGTA, and the pH was adjusted to 7.2 with KOH. The osmolality of the intracellular solutions was 285–295 mOsm. Organotypic cultures were incubated in Neurobasal A medium supplemented with 2% serum-free B-27, 50 U/ml penicillin, 50 µg/ml streptomycin and 0.5 mM L-glutamine (all from Thermo Fisher Scientific, Waltham, MA)

3.2.8. Chemicals

Fura-2 (Fura-2AM, Invitrogen, Molecular Probes), Pluronic F-127, AVP (Rn00566449_m1), OT (Rn00564446_g1), P2X2 (Rn00586491_m1), P2X4 (Rn00580949_m1), P2X7 (Rn00570451_m1), P2Y1 (Rn00562996_m1), and P2Y2 (Rn00568476_m1), Eukaryotic 18 s rRNA endogenous control (VIC R/MGB Probe, Primer Limited; Hs99999901_s1), FAM/TAMRA, 18 s RNA probes (VIC/MGB), TaqMan Universal Master Mix II, no UNG (Applied Biosystems) and cDNA. TTX, 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]

pyridine hydrochloride (A438079); Apyrase; D-2-Amino-5-phosphonopentanoic acid (AP5); N-Cyano-N"-[(1S)-1-phenylethyl]- N'-5-quinolinyl- uanidine (A804598); N-[2-[[2-[(2–Hydroxyethyl)amino]ethyl]amino]-5- quinolinyl]-2-tricyclo[3.3.1.13,7]dec-1-ylacetamide dihydrochloride (AZ10606120); 5-(3- Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one (5-BDBD); 7-Chloro-5- (2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157); 2-[(3,4- Difluorophenyl)amino]-N-[2-methyl-5-(1-piperazinylmethyl)phenyl]-acetamide trihydrochloride (GW791343); pyridoxalphosphate-6-azophenyl-2', 4'-disulfonic acid (PPADS); 2-deoxy-N6-methyladenosine 3,5-bisphosphate tetrasodium salt (MRS2179); [[(1R,2R,3S,4R,5S)-4-[6-Amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt (MRS2365) and Uridine-5'-tetraphosphate δ-phenyl ester tetrasodium salt (MRS2768) were purchased from Tocris-Cookson (Bristol, UK). ATP, Brilliant Blue G (BBG); 2'-3'-O-(4-benzoylbenzoyl)-ATP (BzATP); ATP, N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES), carbenoxolone (CBX); nifedipine and all other drugs and chemicals were from Sigma (St. Louis, MO). The A438079, AZ10606120, 5-BDBD, CGP37157, nifedipine and PPADS were used at 1nM - 10 μM from a 10 mM working stocks in DMSO; hence, 0.00001 - 0.1% (v/v) DMSO vehicle controls were applied in parallel to these incubations.

3.3 Statistics and data analysis

3.3.1. Electrophysiological data analysis

The frequencies and amplitudes of miniature GABAergic inhibitory postsynaptic currents, mIPSCs, were manually analyzed off-line using pClamp 10 software (Molecular Devices, United States). The currents were detected using a threshold based event search and visually evaluated by the experimenters. Only events exceeding 10 pA and lasting 15–20 ms were used in subsequent analysis. Miniature glutamatergic excitatory postsynaptic currents, mEPSCs, were identified as smaller and shorter (~5 ms) events (Vavra et al., 2011), The amplitudes of individual events were determined by the detection program, and the frequency was calculated by dividing number of events by the recording time. The average time of recordings used for frequency calculation was 19.6±0.4 s and 8.6±1.0 s (n = 10) before and after ATP application, respectively. The kinetics of mEPSC and mIPSC current decay were fitted by a single exponential function [$y = A \exp(-t/\tau)$] and by the sum of two exponentials [$y = A1 \exp(-t/\tau 1) + A2 \exp(-t/\tau 2)$], respectively, using the program CLAMPFIT 9, where A1 and A2 are relative amplitudes of the first and second exponential, and $\tau 1$ and $\tau 2$ are time

constants. The derived time constant for current decay was labeled as t. All values are reported as the mean ±SEM.

3.3.2. Statistical analysis

All numerical values in the text are reported as the mean±SEM. Comparisons between two groups were performed by Student's unpaired t-test, and for comparison of multiple groups, significant differences were determined by two way analysis of variance (ANOVA) and Tukey's post hoc test using SigmaPlot v10.01 with $p < 0.01$ (**) and $p < 0.05$ (*). Graphing was performed using SigmaPlot and CorelDraw software. The 'n' indicates number of cells throughout the study, if not otherwise stated.

4. Results

4.1. The effect of refeeding after fasting on P2X expression and function in the rat SON (Study I)

Although activation of presynaptic P2X have been described in many parts of the brain to facilitate neurotransmitter release (Vavra et al., 2011; Khakh and North, 2012), a satisfactory understanding and the precise physiological function of this form of modulation of synaptic transmission is still lacking. In the present study, we tested a hypothesis that P2X play a role in the release of hormones from magnocellular neurons in the SON stimulated through fasting/refeeding. We examined the effect of 2 h of food intake after 48 h of starvation (Lucio-Oliveira et al., 2015) on AVP, oxytocin, P2X and P2Y mRNA expression in SON tissue and ATP-induced electrophysiological responses in the SON neurons of rat brain slices. Normally fed rats of the same age were used as controls for this condition.

4.1.1. Upregulation of AVP and P2X2 mRNA and decrease of P2Y1 mRNA in fasted/refed rats

Previously it has been shown that the mRNA expression of seven P2X receptors (P2X1–P2X7) and three P2Y receptors (P2Y1, P2Y2 and P2Y12) exhibit significantly differential expression in rat SON tissue; the most expressed are P2X2>P2X7>P2X4>P2Y1=P2Y2, while the mRNA expression for the other P2 receptors was minimal (Vavra et al., 2011). Therefore, to address whether the increased synthesizing and releasing activity of AVP neurons induced

by the fasting/refeeding protocol (Fig. 11 and 13A) is accompanied by changes in P2X gene expression in the SON, we performed quantitative PCR analysis with reverse transcription (RT-qPCR) for mRNA expression of AVP, oxytocin, P2X2, P2X4, P2X7, P2Y1 and P2Y2. Analysis was performed in SON tissue from 30-day-old rats, refed for 2 h after 48 h of starvation, and SON tissue isolated from controls, i.e. normally fed rats. These experiments showed a significant increase in the expression of AVP (ratio = 4.93 ± 1.17, n = 3, unpaired t-test, $P<0.01$) and P2X2 (ratio = 1.61 ± 0.17, n = 3, $P<0.05$) mRNA, a decrease in P2Y1 mRNA (ratio = 0.78 ± 0.04, n = 3, $P<0.01$) in fasted/refed animals compared to controls (Fig. 13B).

Figure 13. Effect of fasting/refeeding on purinergic P2 receptor expression. (A) Schematic diagram of the fasting/refeeding experiment. One group of 30-day-old rats was fed food *ad libitum* for the entire experiment (Control), and the second group of 30-day-old rats was fasted for 48 h and subsequently refed with standard chow for 2 h (Refed). Both groups were allowed to drink water *ad libitum*. At the end of this procedure, hypothalamic slices were prepared from both groups. (B) Effect of fasting/refeeding on the relative mRNA expression (calculated as probe/18sRNA ratio) of the P2X2, P2X4, P2X7, P2Y1 and P2Y2 receptors as well as AVP and oxytocin (OT) in the SON normalized to the control group. Data represent the mean ± S.E.M with a scatterplot of the individual data points (n=6 tissue samples from 3 animals). Significant differences between control and fasted/refed animals were estimated using the unpaired t-test, $p<0.01$ (**) and $p<0.05$ (*).

However, changes in the mRNA expression of oxytocin, P2X4, P2X7 and P2Y2 were nonsignificant. These results showed that fasting/refeeding increases the expression of P2X2 and this effect correlates with the upregulation of AVP in the SON.

4.1.2. Basal electrophysiological properties of SON neurons in slices

We used acutely isolated hypothalamic slices to determine the basic electrophysiological properties of SON neurons from fasted/refeed and normally fed rats. Whole-cell current clamp recordings performed 1–6 h after isolation of slices showed that the resting membrane potential of SON neurons was -49.1 ± 2.3 mV (n=8 cells) in control rats and -50.7 ± 2.3 mV (n=8 cells) in fasted/refed rats (Fig. 14A and B). The average frequency of action potentials was 6.5 ± 1.8 mV (n=8) and 7.9 ± 1.9 mV (n=8, p= 0,571) in control and fasted/refed rats, respectively (Fig. 14A and C). Thus, no significant change in the basal membrane properties and spontaneous neuronal activity was observed.

In the presence of 0.5 μM TTX, voltage-dependent sodium channel blocker, whole-cell voltage clamp recordings showed that under our experimental conditions (intracellular [Cl^-], 144 mM; extracellular [Cl^-], 151 mM; holding potential, -60 mV; the theoretical equilibrium potential for Cl calculated by the Nernst equation is about 0 mV), both mEPSCs and mIPSCs could be recorded as small inward currents (Fig. 14D and E). Using bicuculline (BIC; 20 μM) to block GABAergic currents, we found that mEPSCs and mIPSCs differ in their amplitudes and decay time constants. The amplitude of BIC-sensitive currents (mIPSCs) was 72 ± 4 pA, and the decay phase was well fitted by a sum of two exponentials with time constants of 6.1 ± 0.6 ms (contribution 41±16 %) and 33±6 ms (n=8 cells; Fig. 14D). The amplitude of BIC-insensitive currents (mEPSCs) was much smaller, 22 ± 4 pA, and the decay phase was well fitted with a single exponential with a time constant of 1.5 ± 0.3 ms (n=5 cells; Fig. 14E). Vavra et al. have shown previously that these events are inhibited with a mixture of 6,7-dinitroquinoxaline-2, 3-dione (DNQX; 20 μM) that blocks AMPA receptors and 2-amino-5-phosphonopentanoic acid (AP5; 50 μM) that blocks NMDA receptors (Vavra et al., 2011), indicating that these are mEPSCs. The frequency of mEPSCs was 0.24 ± 0.10 Hz (n = 6) and 0.32 ± 0.08 Hz (n = 8; p=0,4556) in controls and fasted/refed rats, respectively.

In the presence of BIC, the frequency of mEPSCs could be stimulated by ATP application (Fig. 14E). Since the mEPSCs were observed in only about 30% of SON neurons from both animal groups, these events were not studied further. The differences in the amplitude and time course allowed us to examine the effects of ATP on mIPSCs in the

absence of specific glutamatergic blockers (see Methods for mEPCs discrimination). The GABAergic mIPSCs could be recorded in all SON neurons, and the average basal frequency was not significantly different between the two animal groups (control, 2.9 ± 0.3 Hz, n=60; fasted/refed, 2.2 ± 0.2 Hz; n=52; p=0.5745).

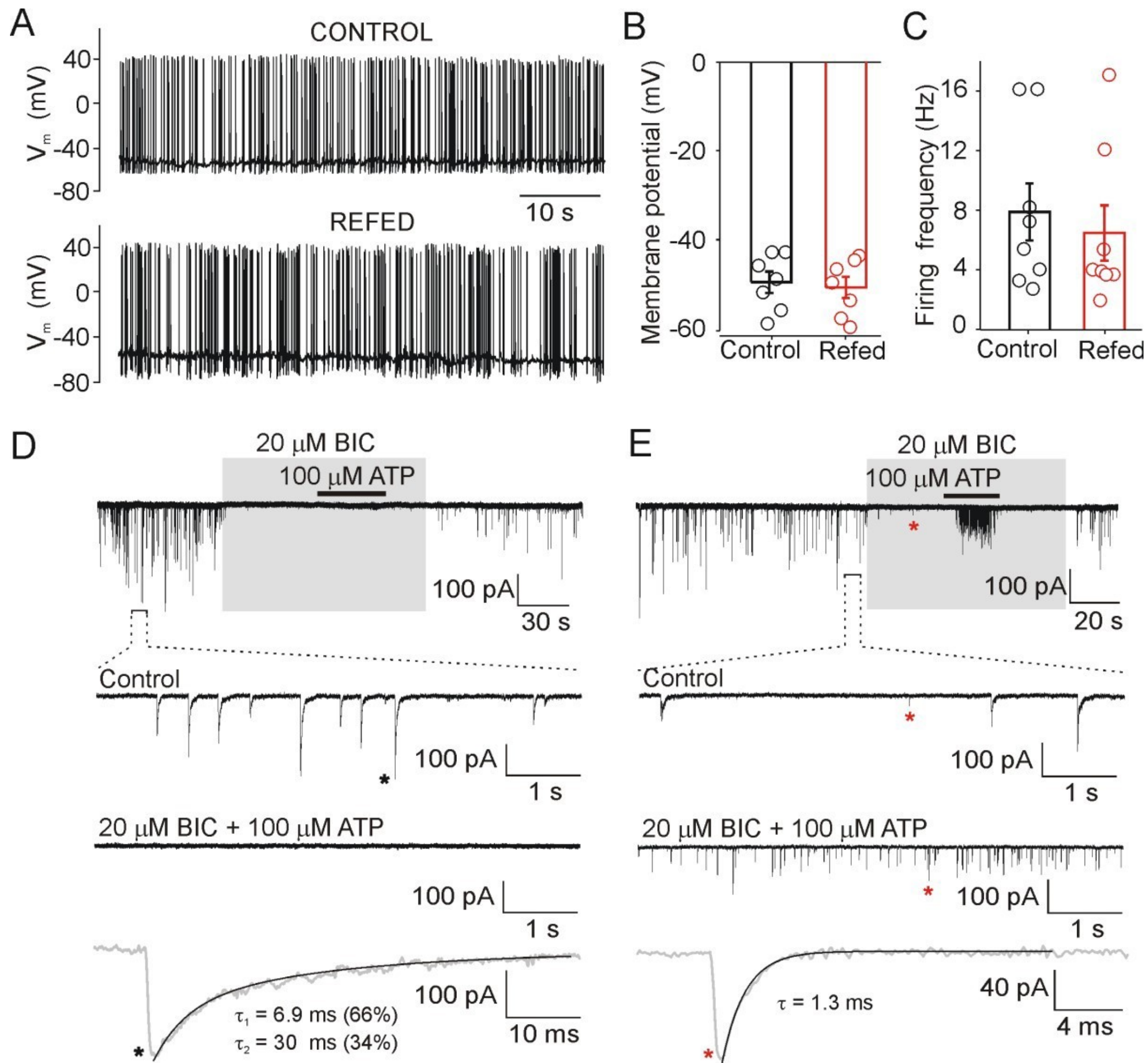

Figure 14. Biophysical properties of SON neurons. (A) Action potentials recorded from SON neurons in slices from normally fed (Control) and fasted/refed (Refed) animals using current clamp patch-clamp configuration. Resting membrane potential (B) and frequency of action potentials (C) were comparable in both animal groups. Data represent the mean ± S.E.M of 6-8 cells from three independent experiments. (D, E) Miniature postsynaptic currents recorded from SON neurons voltage-clamped at -60 mV in the presence of TTX (0.5 μM). (D) Example record from cell exhibiting only mIPSCs. Application of bicuculline (BIC, 20 μM) inhibited all GABAergic synaptic currents both in the presence and absence of ATP (100 μM). Lower trace shows mIPSC (black asterisk) on expanded time scale. (E) Example record from cell exhibiting both mIPSCs and mEPSCs. Application of bicuculline (BIC, 20 μM) inhibited GABAergic synaptic currents and glutamatergic excitatory postsynaptic currents persisted. Lower trace shows mEPSC (red asterisk, in the presence of ATP and BIC) on expanded time scale. The time constant (τ) was measured by fitting the curve to a monoexponential (mEPSCs.) or biexponential (mIPSCs) functions; time constants derived from biexponential fitting were weight.

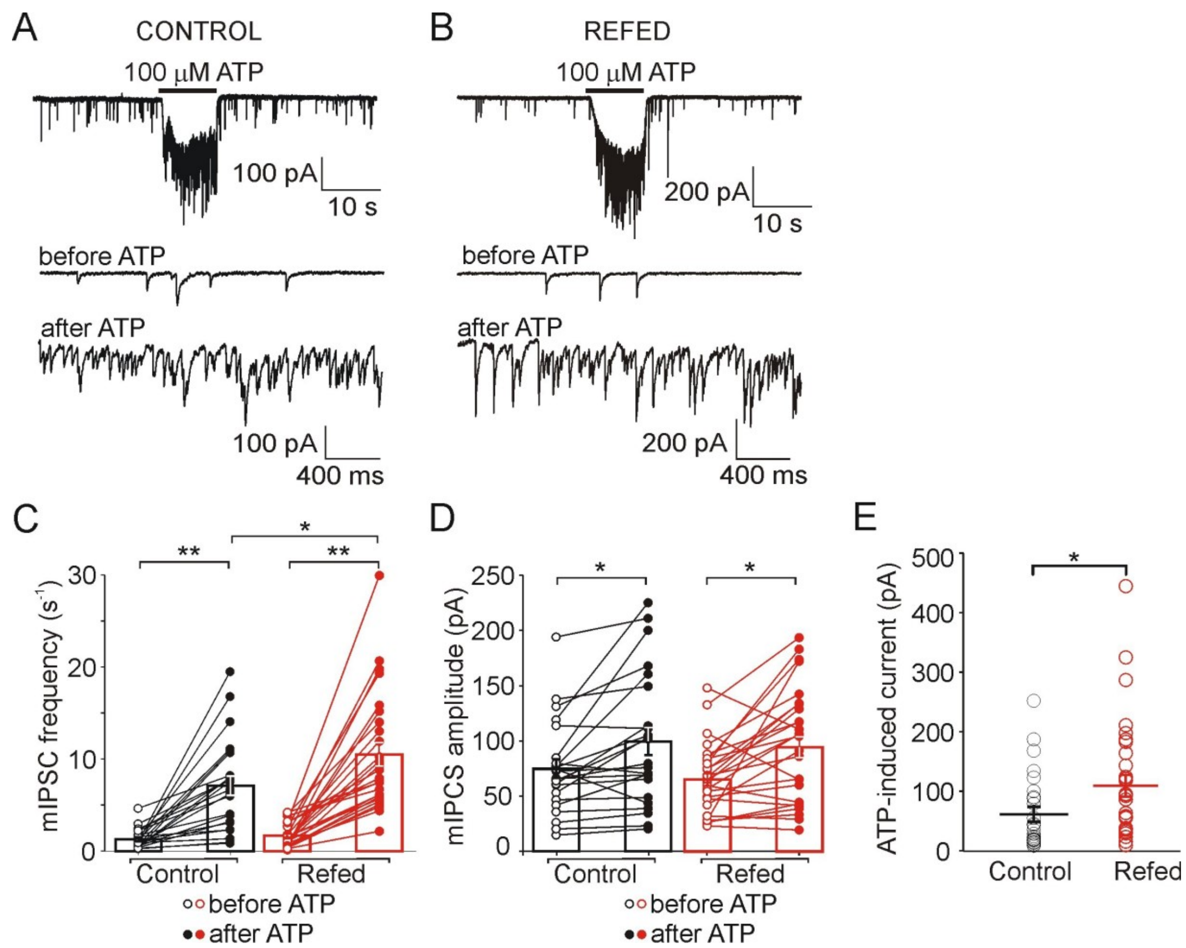

Figure 15. ATP-induced somatic current and increase in mIPSC frequency in P2X-expressing SON neurons. (A, B) ATP application evoked somatic current and increased the frequency of mIPSC in SON neurons from control (A) and fasted/refed (B) animals. Traces on an expanded time scale show spontaneous inhibitory synaptic currents before and after ATP application. (C, D) The frequency (C) and amplitude (D) of mIPSCs in control (black) and fasted/refed (red) animals in the presence (closed symbols) and absence (open symbols) of ATP. Data represent the mean ± S.E.M with a scatterplot of the individual data points (control, n=17 cells; refed, n=26 cells). (E) The amplitude of current induced by 5 -10 s ATP application. Data represent the mean ± S.E.M with a scatterplot of the individual data points (control, n=26 cells; refed, n=34 cells). Analysis was performed by two-way ANOVA and Tukey's post hoc test. (**) P < 0.01 (*) P < 0.05. The ATP-induced current and ATP-induced frequency increase were significantly higher in fasted/refed rats than control rats.

4.1.3. Increased amplitude of ATP-induced somatic current in fasted/refed rats

Application of 100 μM ATP to some of the SON neurons voltage-clamped at -60 mV induced inward somatic current. This concentration of ATP is close to the effective concentration producing a half-maximal effect of ATP in SON slices from control rats (EC_{50} = 70 ± 10 μM; (Vavra et al., 2011), and was used throughout the study. Voltage clamp whole cell recording revealed that the brief application (5-10 s) of ATP evoked inward currents in 26 out of 60 control neurons (43%; 57 slices from 37 rats; Fig. 15A and 16A) and in 30 out of 52 neurons from fasted/refed rats (58%; 43 slices from 27 rats; Fig. 15B and 16B). According to the literature, the P2X-expressing SON neurons are magnocellular AVP neurons (Troadec et al.,

1998; Song et al., 2007; Shibuya et al., 1999a). The mean amplitude of ATP-induced somatic current was significantly higher in fasted/refed rats than controls (control, 75 ±18 pA; fasted/refed, 132 ± 25 pA; $p<0.05$; Fig. 15E), indicating that refeeding after fasting upregulates the P2X protein level on the cell somata of SON neurons compared to SON neurons from normally fed rats.

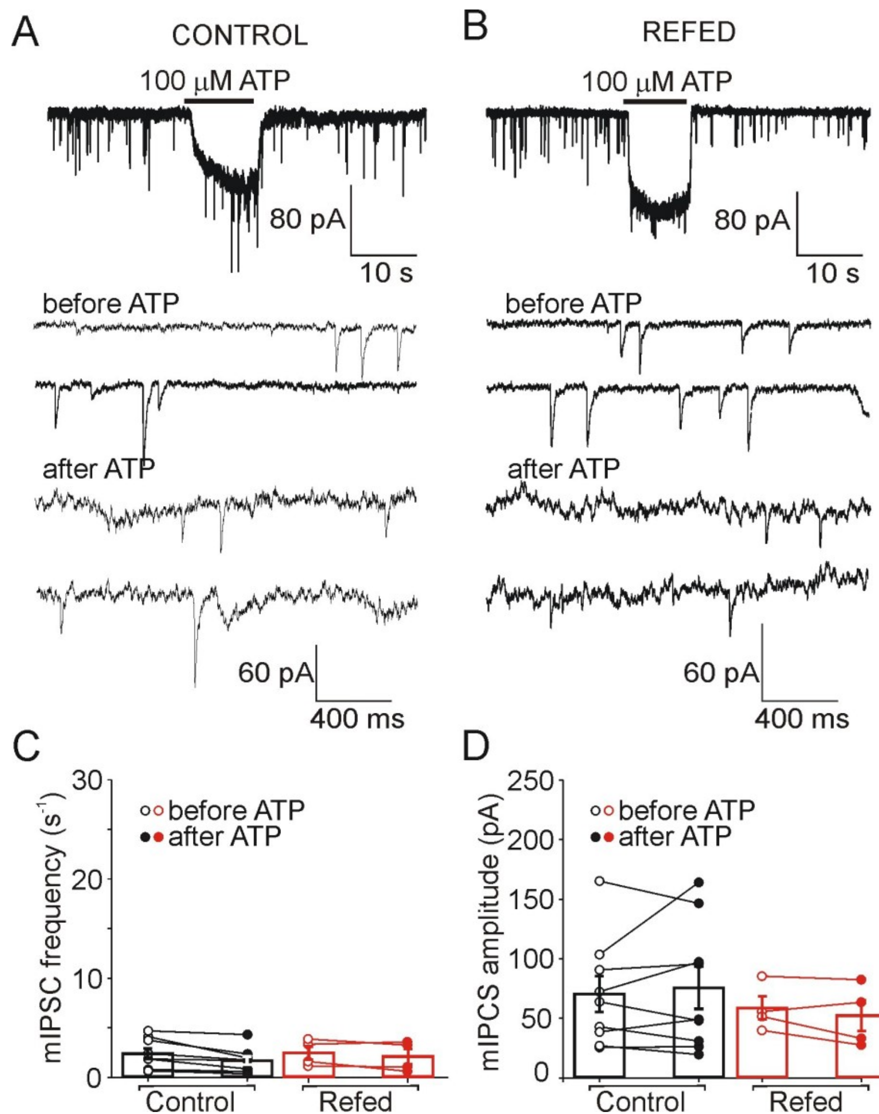

Figure 16. The absence of a presynaptic effect of ATP in a subpopulation of P2X-expressing SON neurons. (A, B) ATP-induced current without increases in the frequency of mIPSCs in control (A) and refed (B) animals. Traces on an expanded time scale show spontaneous inhibitory synaptic currents before and after ATP application (C, D) The frequency (C) and amplitude (D) of mIPSCs in control (black) and refed (red) animals in the presence (closed symbols) and absence (open symbols) of ATP. Data represent the mean ± S.E.M with a scatterplot of the individual data points (control, n=9 cells; refed, n=4 cells).

4.1.4 Increased ATP-induced potentiation of GABA release in the P2X-expressing SON neurons

In addition to the amplitude of ATP-evoked somatic current, we studied the potentiating effect of ATP application on the frequency and amplitude mIPSC which was due to

presynaptic P2X receptor activation and stimulation of vesicular GABA release. We first examined the effect of ATP on mIPSC frequency and amplitude in SON neurons exhibiting ATP-evoked somatic current, most probably AVP neurons (Fig. 15C and D). In 65% (17/26) of control P2X-expressing neurons, the application of ATP increased the mIPSC frequency by 1198 ± 202% (before ATP, 1.29 ± 0.23 Hz; after ATP, 8.60 ± 1.26 Hz; n=17; p<0.01; Fig. 15C, left) and the amplitude from 66.60 ± 7.38 pA to 99.71 ± 15.33 pA (p<0.05; Fig. 15D, left). The remaining 35% (9/26) of neurons showed ATP-evoked somatic current without ATP-induced increase in mIPSC frequency (before ATP, 2.35 ± 0.52 Hz; after ATP, 1.95 ± 0.42 Fig. 16C, left) and amplitude (before ATP, 68.98 ± 15.01 pA; after ATP, 76.30 ± 17.78 pA; n=9, Fig. 16D, left).

In fasted/refed rats, ATP-induced increase in mIPSC frequency and amplitude was similar but was observed more often, increasing to 87% (26/30) of P2X-expressing neurons. The application of ATP increased the mIPSC frequency by 1102±241% (before ATP, 1.45 ± 0.23; after ATP, 10.79 ± 1.35 Hz; p<0.01; Fig. 15C, right) and increased the amplitude from 64.01 ± 5.35 pA to 98.16 ± 9.81 pA (p<0.05, Fig. 15D, right). The difference between control and fasted/refed rats in the ATP-induced increase in mIPSC frequency was significant (two-way ANOVA, p<0.05; Fig. 15C), but the difference in the ATP-induced increase in mIPSC amplitude was not (Fig.15D). The remaining 13% (4/30) of cells from fasted/refed animals exhibited ATP-evoked somatic current without ATP-induced increase in mIPSC frequency (before ATP, 2.25 ± 0.85 Hz; after ATP, 2.07 ± 0.75 Hz; Fig. 16C, right) and amplitude (before ATP, 58.94 ± 9.67 pA; after ATP, 53.46 ± 10.15 pA; n=4 Fig. 16D, right).

These data revealed that refeeding after fasting upregulates the expression of P2X also in presynaptic neurons terminating on presumptive AVP neurons compared to presynaptic neurons in normally fed rats.

4.1.5. Effect of ATP in a subpopulation of SON neurons not expressing somatic P2X

Next, we investigated the effect of ATP on GABAergic synaptic transmission in SON neurons not exhibiting ATP-evoked somatic current (Fig. 17 and 18), most probably oxytocin neurons (Troadec et al., 1998; Song et al., 2007; Shibuya et al., 1999a). In 18% (6/34) of control neurons, ATP application increased mIPSC frequency by 207±22% (before ATP, 2.06 ± 0.62 Hz; after ATP, 4.27 ± 1.26 Hz; p<0.05; Fig. 17A and C, left), without impacting the amplitude (before ATP, 67.30 ± 20.56 pA; after ATP, 63.01 ± 22.51 pA; p=0.509; Fig. 17D, left). These data also show that the amplitude of postsynaptic current did not increase when

ATP application induces moderate increase in mIPSC frequency, confirming the presynaptic effect of ATP.

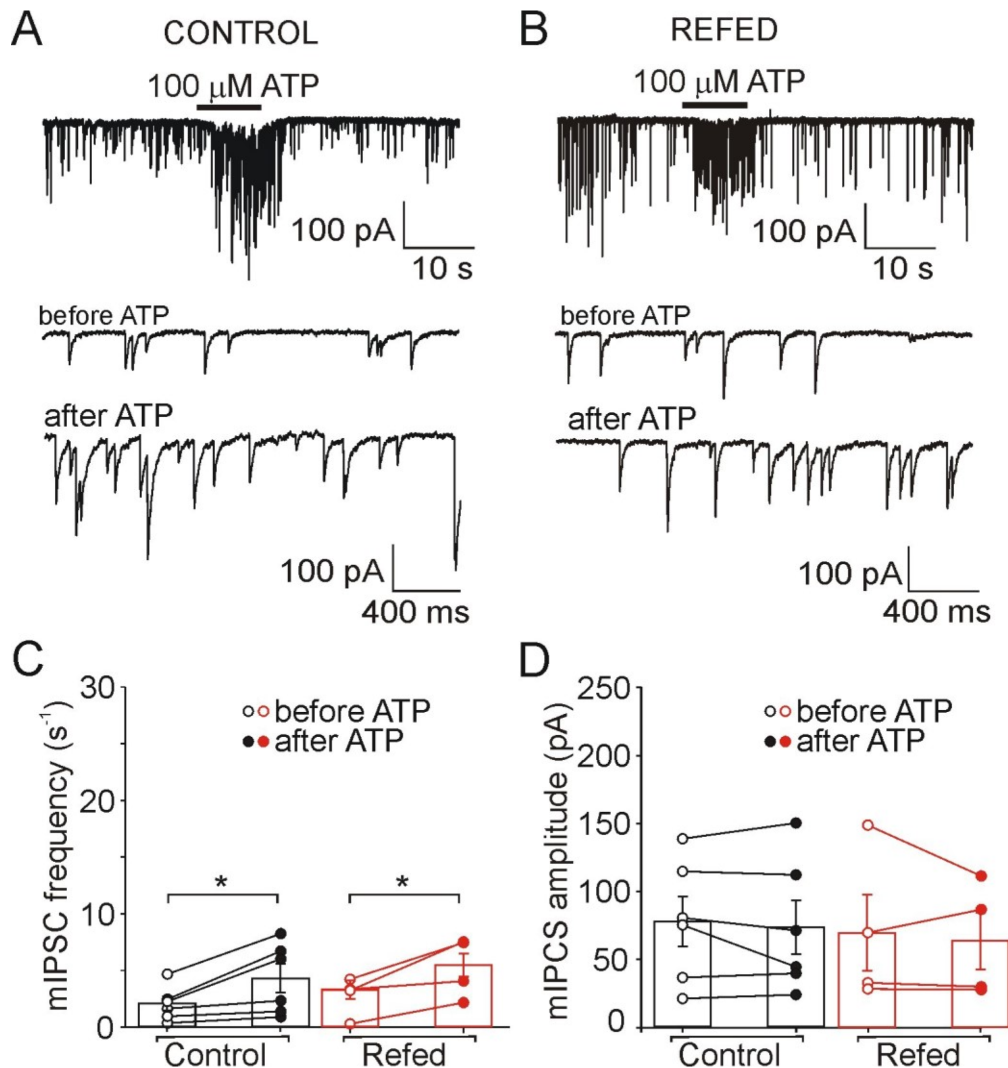

Figure 17. Presynaptic effect of ATP in SON neurons not expressing P2X, presumptive oxytocin neurons. (A, B) ATP-induced increases in the frequency of mIPSCs in control (A) and refed (B) animals. Traces on an expanded time scale show spontaneous inhibitory synaptic currents before and after ATP application (C, D) The frequency (C) and amplitude (D) of mIPSCs in control (black) and refed (red) animals in the presence (closed symbols) and absence (open symbols) of ATP. Data represent the mean ± S.E.M with a scatterplot of the individual data points (control, n=6 cells; refed, n=4 cells). Analysis was performed by two-way ANOVA and Tukey's post hoc test. (**) $P < 0.01$ (*) $P < 0.05$.

The remaining 82% (28/34) of these neurons exhibited no effect of ATP on mIPSC frequency (before ATP, 4.05 ± 0.44 Hz; after ATP, 3.59 ± 0.45 Hz; Fig. 18A and C, left) and amplitude (before ATP, 72.61 ± 6.49 pA; after ATP, 60.78 ± 6.51 pA; n=28; Fig.18D, left).

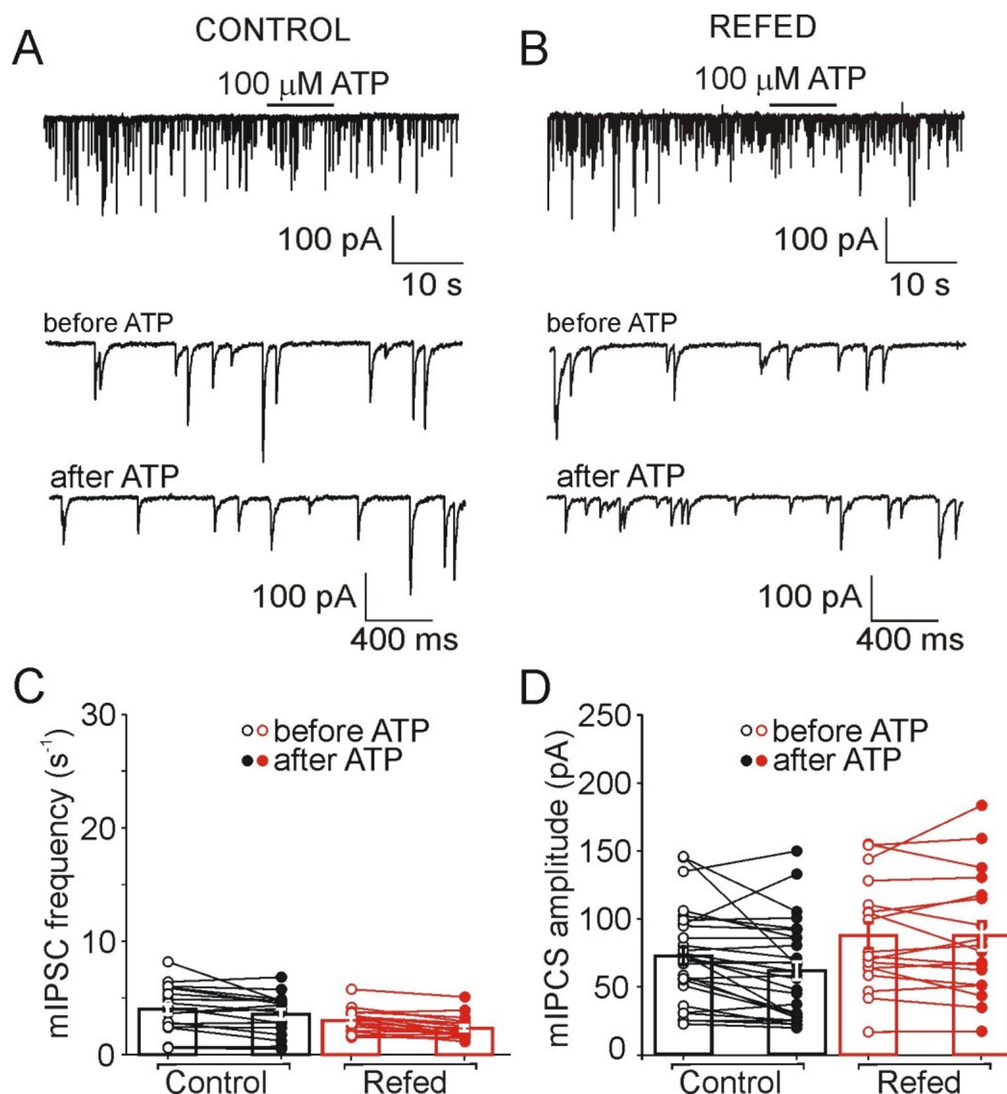

Figure 18. The absence of both somatic and presynaptic effects of ATP in a subpopulation of SON neurons. (A, B) Lack of effect of ATP on the frequency of mIPSCs in control (A) and refed (B) animals. Traces on an expanded time scale show spontaneous inhibitory synaptic currents before and after ATP application (C, D) The frequency (C) and amplitude (D) of mIPSCs in control (black) and refed (red) animals in the presence (closed symbols) and absence (open symbols) of ATP. Data represent the mean ± S.E.M with a scatterplot of the individual data points (control, n=28 cells; refed, n=18 cells).

Similarly, in 18% (4/22) of presumably oxytocin SON neurons from fasted/refed animals, ATP application increased mIPSC frequency by 296±140% (before ATP, 3.02 ± 1.00 Hz; after ATP, 4.95 ± 1.26 Hz; $p<0.05$; 17B and C, right), without impacting sIPSC amplitude (before, 69.61 ± 27.87 pA; after ATP, 63.68 ± 20.88 pA; p=0,638; Fig. 17D, right). The difference between control and fasted/refed rats in the ATP-induced increase in mIPSC frequency was not significant (two-way ANOVA, p = 0.6660). The remaining 82% (18/22) of cells displayed no ATP-induced increase in mIPSC frequency (before ATP, 3.02 ± 0.25 Hz; after ATP, 2.35 ± 0.24 Hz; Fig. 18B and C, right) and amplitude (ECS, 85.36 ± 9.61 pA; ATP, 84.97 ± 10.91 pA; n=18; Fig. 18D, right).

These data reveal that fasting/refeeding has no effect on presynaptic ATP-induced responses in SON neurons not expressing somatic P2X receptors, presumably oxytocin neurons.

4.2. Involement of P2X7 and P2Y in circadian ATP release in organotypic SCN cultures (Study II)

The second part of the thesis was carried out to address a possible role of P2X7 and P2Y in circadian ATP release from SCN astrocytes. We have investigated this hypothesis using organotypic cultures of rat brain slices containing the SCN, primary cultures of SCN astrocytes, ATP bioluminescent assays, immunohistochemistry, patch-clamping, and calcium imaging.

4.2.1. Characterization of the circadian ATP release in SCN organotypic cultures

First, we examined circadian rhythm in extracellular ATP accumulation in medium with SCN organotypic cultures. Extracellular ATP concentrations in the medium were determined every 4 h over a 24 - 48 h incubation period (Fig. 19) using an ATP bioluminescent assay. Organotypic SCN cultures exhibited circadian rhythm in ATP release with the peak between 24:00 – 04:00, and the trough at approximately 12:00 (Fig. 19 A-D, open circles). The time points of the peak and trough were stable in 62 of 66 independent control cultures (94%). In the remaining 6% of cultures (n=4), either no rhythm was observed or the peak did not occur between 24:00 – 04:00; these cultures were discarded. These data showed that circadian rhythm of extracellular ATP accumulation peaks at an opposite phase to neuronal activity and AVP secretory rhythms that peake at approximately 12:00 in similar organotypic SCN cultures (Svobodova et al., 2003), indicating that ATP is released by astrocytes.

We also monitored extracellular ATP accumulation in organotypic cultures from which the SCN was removed to determine the role of the SCN. In non-SCN cultures, the circadian rhythm of extracellular ATP accumulation was still present (Fig. 19A), but exhibited much lower amplitude, and cumulated ATP release was significantly reduced compared to control SCN-containing cultures (66 ± 11%, n=3, $P<0.05$; Fig. 19E). Thus, in addition to the SCN, other hypothalamic nuclei located near the SCN that also express components of the molecular clock, such as the lateral hypothalamus and supraoptic nucleus (Abe et al., 2002; Guilding et al., 2009), contribute to circadian ATP release rhythms but with a lower efficacy as compared to the SCN.

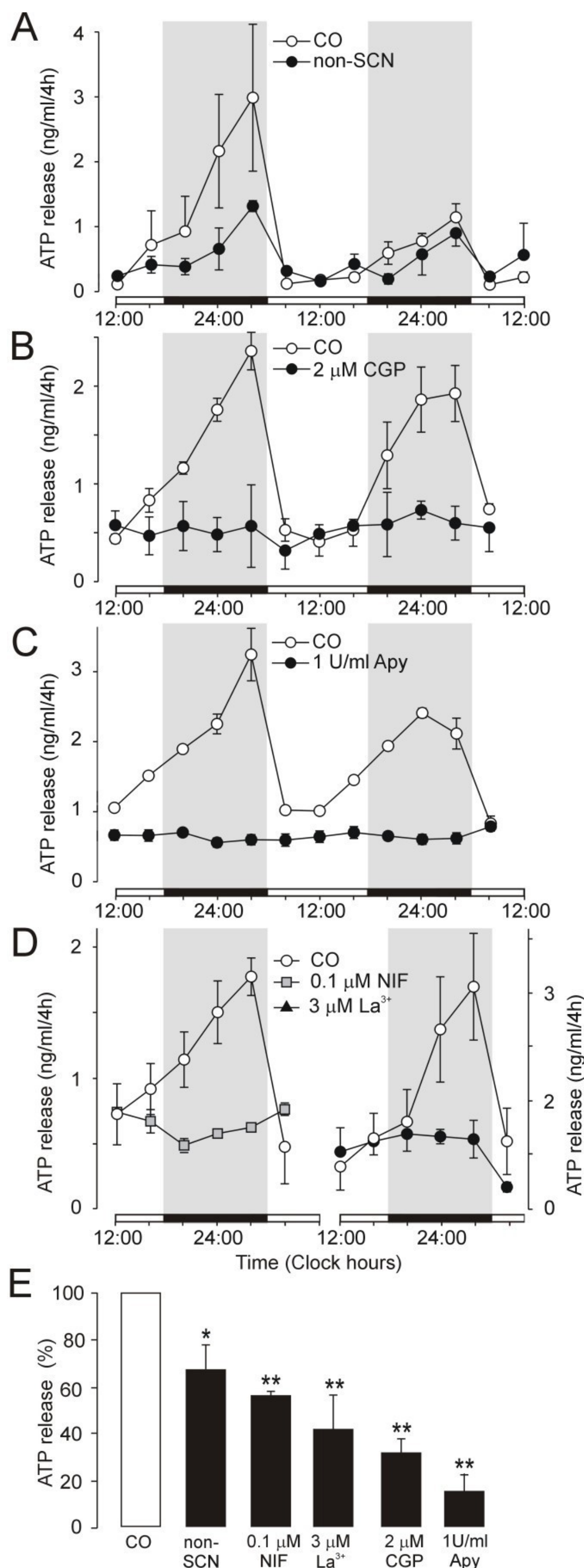

Figure 19. Endogenous circadian oscillations of ATP release in organotypic cultures of the rat SCN. (A - C) Examples of an ATP rhythm measured during 2 days in control cultures (open symbols) and experimental cultures (closed symbols). Comparison of ATP rhythm in control organotypic cultures containing the SCN (CO) and organotypic cultures lacking the SCN (non-SCN) (A) and inhibition of ATP release by a selective inhibitor of Na^+-Ca^{2+} exchange in mitochondria, the benzothiazepine CGP37157 (CGP) (B). Basal ATP release in the presence of ecto-ATPase, apyrase (Apy) (C). Inhibition of ATP release by the L-type Ca^{2+} channel inhibitor nifedipine (D, left panel) and a non-selective voltage-gated Ca^{2+} channel blocker La^{3+} (D, right panel). Summary histogram showing cumulated ATP release in control cultures (open column) and experimental cultures (black columns) (E). Medium was sampled every 4 h and the ATP content was quantified (in ng/ml). ATP release was spontaneous, with a circadian rhythm that is a continuation of the endogenous rhythm in the SCN in vivo. Open horizontal bars on X axes thus indicate the light periods experienced by donor animals (from 12:00 to 24:00 and from 24:00 to 12:00) and solid bars indicate the dark periods (12 h). Treatments were performed by completely replacing the medium with fresh drug-containing culture medium every 4 h throughout the whole incubation period. Tested drugs were applied at 08:00 and measurements started at 12:00. Parallel cultures from the same experiments are shown. Data are presented as the means ± SEM, each study was repeated on cultures derived from 3 to 6 different dissections (experiments). Statistical significance of differences between control and experimental groups: (*) $P < 0.05$ and (**) $P < 0.01$.

Next, we performed a bath application of selective inhibitor of the mitochondrial Na^+-Ca^{2+} exchange transporter CGP37157 (White and Reynolds, 1997) to examine a possible link between extracellular ATP accumulation and mitochondrial function (Burkeen et al., 2011). The effective threshold concentration of CGP37157 that inhibited ATP release was 200 nM (43 ± 8% of control, n=3, P<0.01; not shown), and a 2 μM treatment inhibited ATP release to

31 ± 6% of control (n=4; Fig. 19B). Incomplete inhibition of extracellular ATP accumulation after treatment with CGP37157 indicated that ATP is released as a transmitter, not as a metabolite.

We also wanted to determine basal ATP release that persists after ATP hydrolysis. *In vivo*, the ecto-nucleotide triphosphate diphosphohydrolase family of enzymes (eNTPDases) hydrolyze extracellular ATP and/or ADP to AMP and adenosine (Zimmermann, 2000). The addition of apyrase (1 U/ml), a soluble ecto-nucleotidase, completely abolished the ATP secretory rhythm (Fig. 19C). However, apyrase did not remove all ATP, as basal ATP concentrations were observed in the bath (14 ± 8% of control, n=3; Fig. 19E), indicating that most probably ATP was also contained in extracellular compartment not accessible to apyrase such as exosomes.

To examine the dependence on extracellular Ca^{2+}, we tested the effect of voltage-gated Ca^{2+} channel blockers. Consistent with a hypothesis that astrocytes release ATP by Ca^{2+}-dependent mechanism (Fumagalli et al., 2003) and express voltage-gated calcium channels (Yan et al., 2013), nifedipine (0.1 μM), the L-type Ca^{2+} channel inhibitor, and La^{3+} (3 μM), a non-selective voltage-gated Ca^{2+} channel blocker, abolished ATP rhythm and significantly inhibited cumulated ATP release (nifedipine: 56 ± 3% of the control, n=3, $P<0.05$; La^{3+}: 41 ± 15% of the control, n=3, $P<0.01$, Fig. 19D and E). Since Ca^{2+} channel blockers significantly, but not completely inhibited ATP release, these results indicate that Ca^{2+} -independent mechanism(s) might also play a role in evoking ATP release.

4.2.2. Dependence of extracellular ATP accumulation on P2X7 activity

We examined the effects of several selective P2X7 antagonists to investigate the role of P2X7 in ATP release from SCN organotypic cultures (Fig. 20). At low concentrations (1-10 nM), negative allosteric modulator AZ10606120 (Michel et al., 2007) reduced the amplitude of the circadian ATP rhythm (Fig. 20A and B) and partially inhibited cumulated ATP release (10 nM: 44 ± 16% of the control, n=3, $P<0.01$; Fig. 20E). At higher concentrations (100 nM -1 μM), AZ10606120 completely inhibited extracellular ATP rhythm (Fig. 20C and D) to approximately basal levels (100 nM: 24 ± 7% of the control, n=4, $P<0.01$; 1 μM: 16 ± 3% of the control, n=3, $P<0.01$; Fig. 20E), similar to levels observed in the presence of apyrase (Fig. 19C).

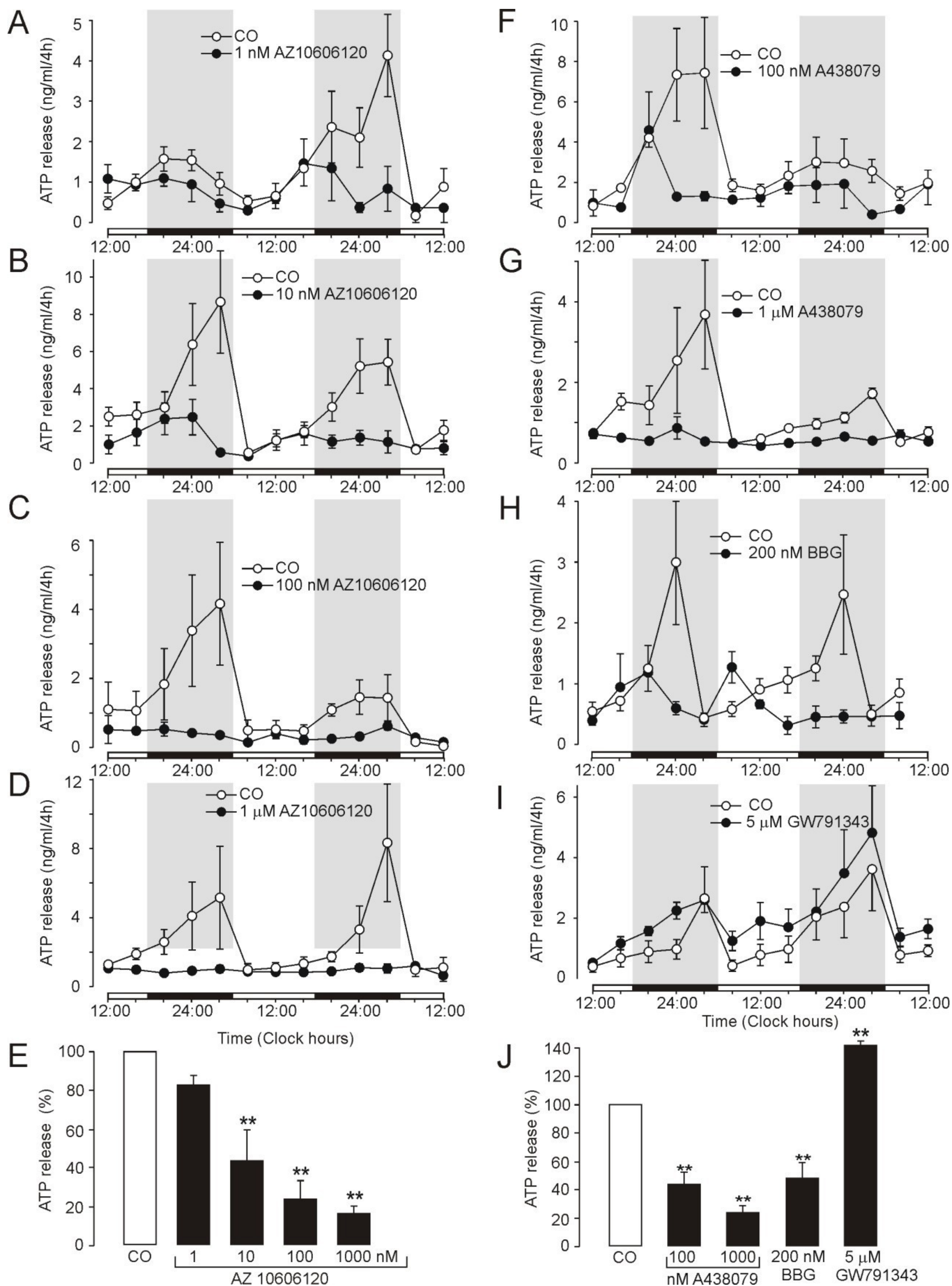

Figure 20. Effect of selective P2X7 inhibitors, AZ10606120, A438079 and BBG and a positive allosteric modulator, GW791343 on circadian ATP release. (A – J) Examples of the ATP release rhythm in control cultures (open symbols) and cultures incubated with the P2X7 modulators (closed symbols): 1 nM AZ10606120 (A), 10 nM AZ10606120 (B), 100 AZ10606120 nM (C) and 1 μM AZ10606120 (D). Inhibitory effects of 100 nM A438079 (A), 1 μM A438079 (B) and 200 nM BBG (C), and potentiating effect of the positive allosteric modulator GW791343 at 5 μM concentration (D). Summary histogram comparing the effects of various concentrations of AZ10606120 (E) and positive and negative modulators (J) on cumulated ATP release. Data are presented as the means ± SEM of 3 to 6 (7) experiments. (**) $P < 0.01$ compared to the control.

Another selective P2X7 antagonist, the structurally unrelated negative allosteric modulator A438079 (Donnelly-Roberts et al., 2009), also inhibited ATP release in a concentration-dependent manner (Fig. 20F and G). The effective threshold concentration was 10 nM (data not shown), and at concentrations of 100 nM and 1 μM, ATP release was inhibited to 44 ± 7% (n=4, $P<0.01$) and 30 ± 5% (n=3, $P<0.01$) of the control level, respectively (Fig. 20J). Treatment with the classical P2X7 antagonist BBG (200 nM) (Jiang et al., 2000a) abolished the ATP rhythm (Fig. 20H) and inhibited cumulative ATP release to 48 ± 11% of control levels (n=3, $P<0.01$; Fig. 20J). In contrast GW791343 (100 nM), a positive allosteric modulator of rat P2X7 (Michel et al., 2008), enhanced the amplitude of ATP release rhythm (Fig. 20I) and extracellular ATP accumulation to 144 ± 6% of control levels (n=3, $P<0.01$; Fig. 20J).

Next, we used double-label immunohistochemistry to examine the expression of P2X7 in combination with the astrocytic marker GFAP in acutely isolated rat brain slices. As shown in Fig. 21, a relatively high signal for the P2X7 protein colocalized with GFAP was observed throughout the SCN region.

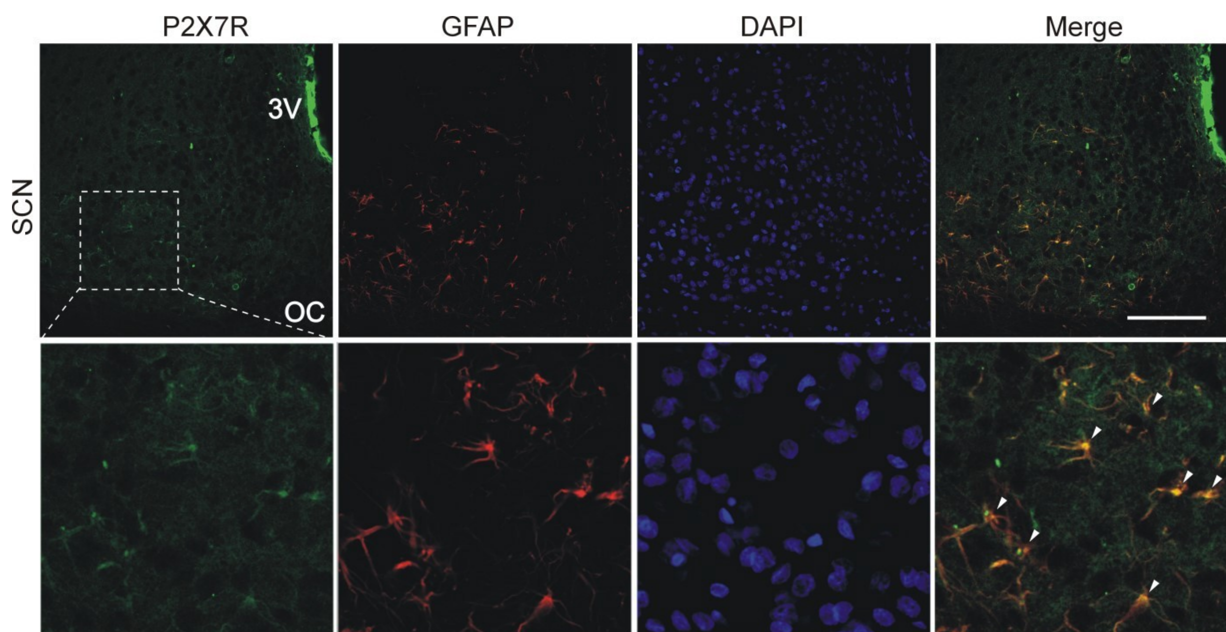

Figure 21. Cellular localization of P2X7 receptors in the SCN. Immunohistochemical staining of rat hypothalamic slices containing the SCN, optic chiasm (OC) and the third ventricle (3V). P2X7 receptor (P2X7R) immunoreactivity (green) is present on SCN cell somata. DAPI (blue) stains cell nuclei and astrocytes were identified using anti-GFAP antibodies (red). Scale bar: 100 μm (upper row). Colocalization of P2X7 with the astrocyte marker. Arrowheads show representative structures that are double-labeled with anti-P2X7 and anti-GFAP antibodies (lower row). Figure represents example of experiment that was performed on 3 animals.

These results showed that P2X7 receptors play an important role in the circadian accumulation of extracellular ATP in SCN organotypic cultures, and astrocytes represent the source of P2X7-dependent circadian ATP release.

4.2.3. Role of pannexin-1 hemichannel in ATP release

We also considered whether the pannexin-1 hemichannel might contribute to the extracellular ATP accumulation. Inhibition of astrocytic panneixin-1 hemichannels by carbenoxolone (CBX, 10 μM), which might have also a minor inhibitory effect on connexin hemichannles and gap junction channels (Li et al., 2011a), reduced the amplitude of the circadian rhythm of ATP release (Fig. 22A) and partially inhibited cumulated ATP release (44 ± 9% of the control, n=4, *P*<0.01; Fig, 22E). This data indicated that pannexin-1 hemichannels, alone or in complex with P2X7, play a role in ATP release.

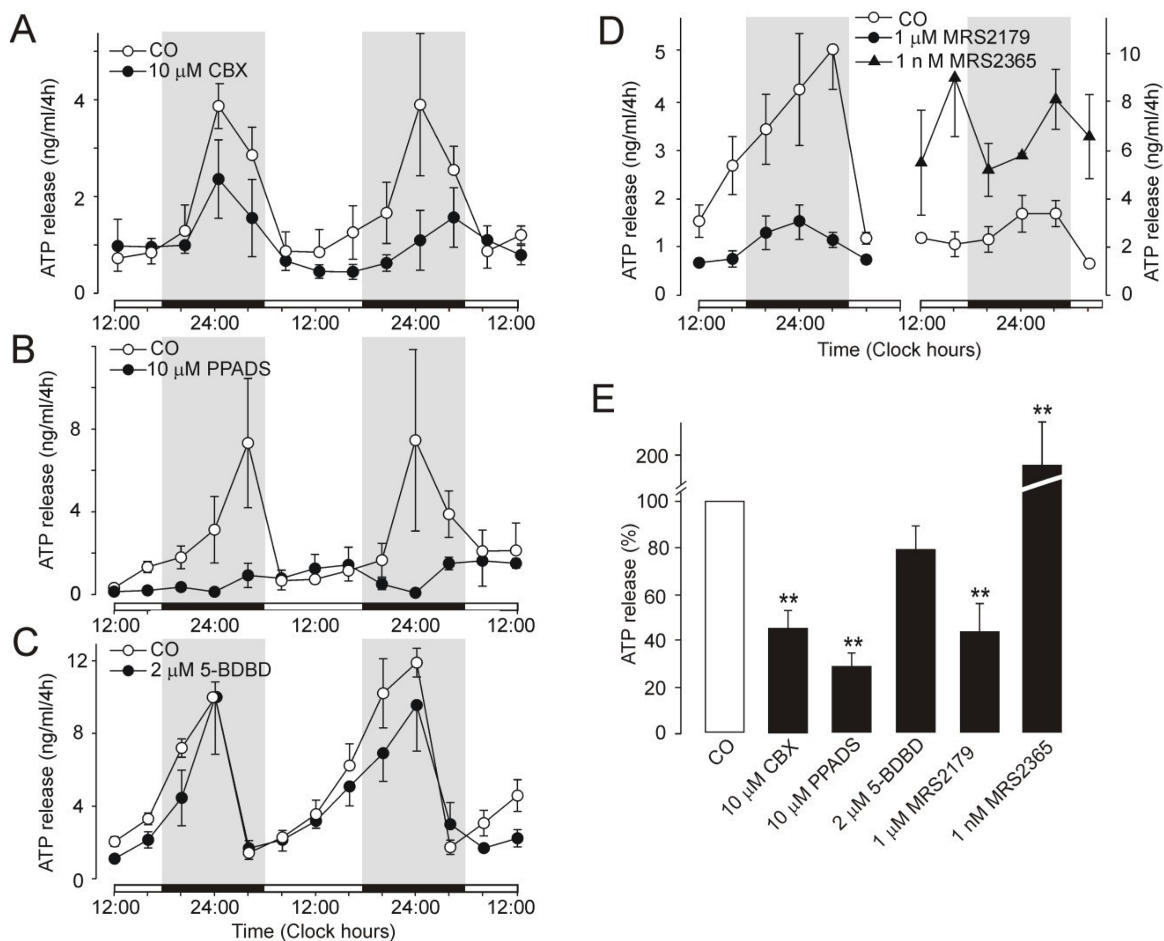

Figure 22. Effects of carbenoxolone and other P2 receptor blockers on the circadian rhythmicity of ATP release. (A) Partial inhibitory effect of the pannexin-1 hemichannel blocker carbenoxolone (CBX). (B) Inhibition by the non-specific P2 receptor blocker PPADS. (C) Lack of an effect of the P2X4-selective antagonist 5-BDBD. (D) Circadian ATP release was significantly decreased by the P2Y1-selective inhibitor MRS2179 (left panel) and potentiated by the P2Y1-selective agonist MRS2365 (right panel). (E) Summary graph showing the effects of the tested drugs on cumulative ATP release. Data are presented as the means ± SEM of 3 to 4 experiments. (**) P < 0.01 compared to the control.

4.2.4. Dependence of extracellular ATP accumulation on P2Y1 and P2Y2

In addition to P2X7, transcripts for P2X1-5 and several P2Y receptors (P2Y1, P2Y2 and P2Y12) have also been detected in the SCN tissue (Bhattacharya et al., 2013). For this reason, we investigated blockers of other P2 receptors. The non-selective P2 receptor antagonist PPADS (10 µM) (North, 2002) (Coddou et al., 2011b) almost completely inhibited the ATP rhythm (Fig. 22B) and extracellular ATP accumulation (28 ± 6% of the control, n=3, $P<0.01$; Fig. 22E), whereas the P2X4-selective negative allosteric modulator 5-BDBD (2 µM) (Balazs et al., 2013) did not exert a significant effect (79 ± 11%, $P>0.05$; n=3; Fig. 22C and E). Treatment with the P2Y1-selective antagonist MRS2179 (1 µM) reduced the amplitude of the ATP rhythm (Fig. 22D, left panel) and inhibited ATP secretion to 44 ± 12% of control levels (n=5, $P<0.01$; Fig. 22E). In contrast, the P2Y1-selective agonist MRS2365 (1 nM) potentiated extracellular ATP accumulation (Fig. 22D, right panel) to 186 ± 25% of control levels (n=3, $P<0.01$; Fig. 22E). Similar effect was observed with the P2Y2-selective agonist MRS2768 (0.5 µM; 231 ± 32%, $P>0.05$; n=2; data not shown). These results showed that, in addition to P2X7 and pannexin-1 hemichannels, metabotropic P2Y receptors also participate in regulation of circadian ATP release.

4.2.5. Ca^{2+} signals mediated by P2X7 and P2Y in primary cultured SCN astrocytes

P2Y1 and P2Y2 receptors are coupled to Gq/11 and may thereby contribute to extracellular ATP accumulation by stimulating phospholipase C (PLC) and mobilizing intracellular Ca^{2+} (Abbracchio and Burnstock, 1994). We examined the effects of the P2Y1 agonist MRS2365, the P2Y2 agonist MRS2768, ATP, and the prototypic P2X7 agonist BzATP on the intracellular Ca^{2+} concentration in primary cultures of SCN astrocytes preincubated with Fura-2AM (n = 16 cultures, ~20 cells per culture; Fig. 23). Single cell calcium measurements showed that both ATP and BzATP induced increases in $[Ca^{2+}]_i$, and the amplitude of the BzATP-induced response was the same as or greater than the ATP-induced responses (Fig. 23A), indicating the presence of functional P2X7 receptor. Consistent with this finding, both ATP- and BzATP-induced increases in $[Ca^{2+}]_i$ were depressed by treatment with the P2X7-selective blockers AZ10606120 (1 µM, 60 ± 9% inhibition, n=3; Fig. 23A
and G) and A804598 (1 µM, 25 ± 6% inhibition, n=4; Fig. 23B, C and H). ATP-induced responses were also partially reduced by MRS2179 (10 µM, 57 ± 9% inhibition, n= 3; Fig. 23D and I), indicating involvement of P2Y1. Application of the P2Y1-selective agonist

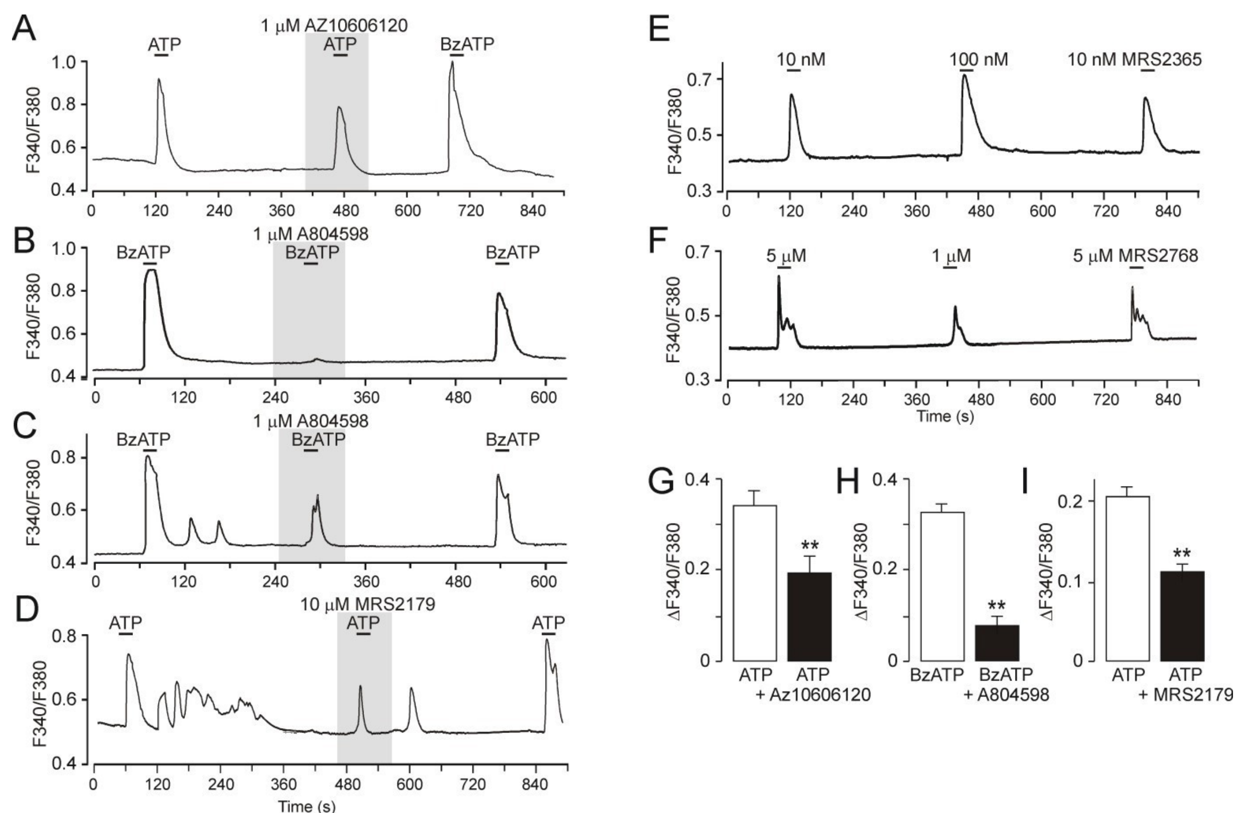

Figure 23. Calcium signaling mediated by P2X7 and P2Y1 receptors in primary cultures of SCN astrocytes. (A) Increases in intracellular calcium in cultured SCN astrocytes in response to ATP and BzATP (both 50 μM) and partial inhibition of the ATP-induced $[Ca^{2+}]_i$ increase by the P2X7 blocker AZ 10606120. The ratio of light intensity (F_{340}/F_{380}) reflects changes in $[Ca^{2+}]_i$. (B-C) BzATP-induced $[Ca^{2+}]_i$ increases completely (B) or partially (C) inhibited by the P2X7 blocker A438079. (D) Inhibition of ATP-induced responses in the presence of the P2Y1-selective blocker MRS2179. (E) Concentration-dependent elevation of astrocytic calcium levels by the P2Y1 agonist MRS2365. (F) Concentration-dependent effect of the P2Y2 agonist MRS2768. (G) Summary graph showing the effects of P2 receptor agonists and antagonists on astrocytic $[Ca^{2+}]_i$. Data are presented as the means ± SEM of 3 to 4 experiments. (*) $P < 0.05$; (**) $P < 0.01$ compared with the control.

MRS2365 elevated $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 23E), and a similar effect was observed with the P2Y2-selective agonist MRS2768 (Fig. 23F). Based on these results, both P2X7 and P2Y receptors may stimulate intracellular calcium signals in SCN astrocytes and thereby participate in the Ca^{2+} dependent ATP release.

4.2.6. Lack of an effect of P2X7 inhibitors and mitochondrial blocker on SCN neuronal activity

We examined the effect of A438079, A 804598, AZ10606120 and CGP 37157 on firing of action potentials by SCN neurons in acutely isolated hypothalamic slices (Fig. 24) to test the possibility that the P2X7 antagonists and blocker of the mitochondrial Na^+/H^+ exchanger might have an effect on astrocytic ATP release due to their influence on the electrical activity

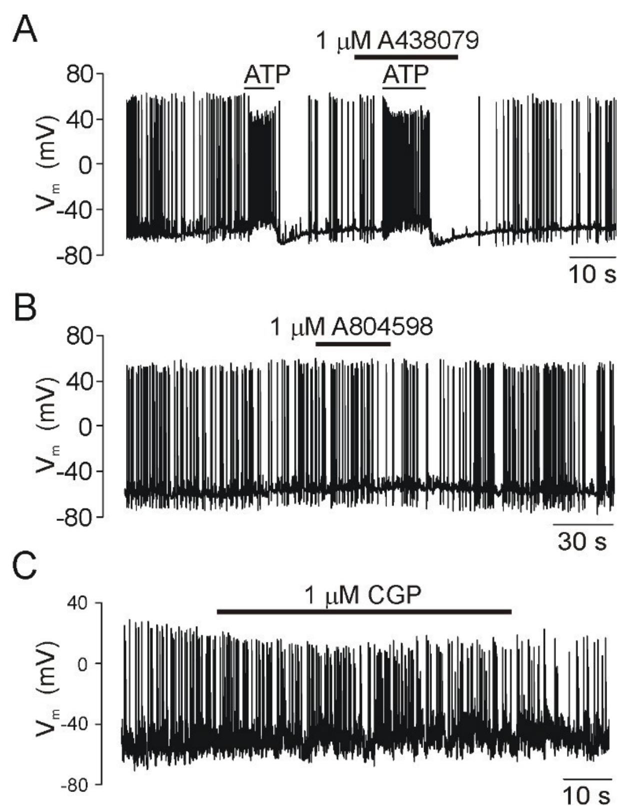

Figure 24. The electrical activity of SCN neurons in slices is not modulated by P2X7 and mitochondria blockers. (A) The exogenous ATP application induced an increase in the frequency of action potentials as a result of the increased frequency of depolarizing GABAergic currents caused by the high concentration of chlorides in our intracellular solution (see the Materials and Methods). (B) The P2X7 receptor-selective blocker A 4804598 had no effect on the electrical activity of SCN neurons. (C) A selective inhibitor of Na^+-Ca^{2+} exchanger in the mitochondria CGP37157 (CGP), gradually reduced the amplitude of action potentials with no effect on frequency. Traces shown in this figure are representative of 5-6 similar recordings.

of SCN neurons. Although 100 μM ATP induced an increase in the frequency of action potentials, the preapplication of P2X7 antagonist A438079 had no effect on ATP-induced response (Fig. 24A). Application of P2X7 antagonists A438079 (Fig. 24B) or AZ10606120 (not shown) was also without any effect. Application of CGP37157 slightly reduced the amplitude of action potentials without any effect on the firing frequency (Fig. 24C). These results provide evidence that the inhibitory effects of P2X7 and mitochondrial blockers on ATP release from astrocytes are not associated with the inhibition of neuronal activity.

5. Discussion

In the present work, we elucidated the role of P2X in the SON neurons under conditions which require adjustment in hormone secretion. The specificity of P2X2 receptor subtype was confirmed in experiments with quantitative RT-PCR analysis and electrophysiologically measured effects of ATP in acutely isolated rat brain slices (Study I). Additionally, we

demonstrated that P2X7 and P2Y receptors are involved in circadian ATP release from SCN astrocytes which was investigated in organotypic cultures (Study II).

5.1. Increased expression and function of P2X2 in SON of fasted/refed rats (Study I)

We found significantly increased AVP and P2X2 expression and decreased P2Y1 expression, but no changes in oxytocin, P2X4, P2X7 and P2Y2 mRNA expression in the rat SON after 48 h of starvation and 2 h after food intake. Using acutely isolated rat brain slices, we showed that the changes in P2X2 mRNA expression are accompanied by functional effects such as higher amplitude of ATP-stimulated somatic current and increased incidence of P2X-mediated presynaptic facilitation of GABA release onto P2X-expressing SON neurons. These data suggest that the recruitment of P2X2 receptors to both postsynaptic and presynaptic sites could be associated with the increased synthesis and release of AVP in the SON of fasted/refed rats.

It is well established that food deprivation for 48 h causes a decline in the AVP level in the SON, while little change in the oxytocin concentration was detected (Burlet et al., 1992). Food intake after 48 h of fasting evokes increases in plasma AVP (Lucio-Oliveira et al., 2015) and oxytocin (Lucio-Oliveira and Franci, 2012) levels. In another study, 2-4 h of refeeding after 48 h of fasting significantly increased the mRNA levels of both oxytocin and AVP in the mouse hypothalamus compared to the oxytocin and AVP mRNA expression in normally fed mice (Poplawski et al., 2010). Refeeding after fasting increases the expression of the immediate early gene c-FOS in identified AVP and, to a lesser extent, oxytocin cells, indicating that AVP-positive neurons in the SON could show higher activity than oxytocin-positive neurons under these experimental conditions (Timofeeva et al., 2005; Johnstone et al., 2006; Kohno et al., 2008; Lucio-Oliveira and Franci, 2012; Lucio-Oliveira et al., 2015). In agreement with this, we found that refeeding after fasting significantly increases AVP mRNA expression, while changes in oxytocin mRNA expression were not significant.

Previous studies on the hypothalamic SON and/or PVN showed the presence of mRNA transcripts not only for P2X2 but also for the P2X3, P2X4, P2X6 and P2X7 (Bo et al., 1995; Collo et al., 1996; Vulchanova et al., 1996; Xiang et al., 1998; Shibuya et al., 1999a; Vavra et al., 2011). Experiments with specific P2X knockout mice revealed that endogenously released ATP acts on P2X2 but not P2X3 or P2X7 in posterior pituitary nerve terminals (Custer et al., 2012). Functional and pharmacological studies on SON neurons also identified P2X2 as a dominant subtype of the P2X (Gomes et al., 2009; Troadec et al., 1998; Song and

Sladek, 2006; Vavra et al., 2011). P2X2 activation increases the release of AVP from hypothalamo-neurohypophyseal system explants (Gomes et al., 2009; Troadec et al., 1998; Song and Sladek, 2006) and evokes somatic current in the SON neurons of hypothalamic slices (Vavra et al., 2011).

There are numerous lines of evidence indicating that P2X2-expressing neurons are magnocellular AVP neurons. First, locally applied ATP increases cytosolic free Ca^{2+} concentrations in identified somata of dissociated AVP neurons from the SON (Troadec et al., 1998; Song et al., 2007; Shibuya et al., 1999a) and evokes AVP release from isolated posterior pituitary nerve terminals, but no significant oxytocin release (Troadec et al., 1998; Song and Sladek, 2006; Gomes et al., 2009; Lemos et al., 2018). Second, ATP endogenously released from the posterior pituitary during electrical stimulation depolarizes the nerve terminals and potentiates AVP secretion (Knott et al., 2008). Finally, the data presented here show that the increased expression of P2X2 mRNA and enhanced amplitude of ATP-induced current in experimental refed animals corresponds with the increased expression of AVP mRNA. These results altogether support the idea that the upregulation of P2X2 expression and function during fasting/refeeding is selectively associated with increased synthesizing and releasing activity of AVP neurons.

P2X2 has also been shown to be expressed on presynaptic nerve terminals in hypothalamic slices and its activation facilitates glutamate and GABA release in a subpopulation of SON neurons (Vavra et al., 2011). The ATP-induced increase in mIPSC amplitude observed here in P2X-expressing neurons might represent multiquantal release of GABA that was due to a dramatic influx of calcium through a high number of presynaptic P2X2 channels. This idea is supported by the fact that the amplitude of postsynaptic current did not increase when ATP-induced increase in frequency was moderate, such as in neurons not-expressing somatic P2X (Fig. 17D).

Quantitative analysis of GABA-synthesizing enzyme glutamate decarboxylase or GABA immunostaining combined with oxytocin and AVP immunolocalization revealed that GABAergic innervation in the SON is very extensive and uniformly distributed within the nucleus and that GABAergic nerve terminals contact oxytocin and AVP neurons to a similar extent (Theodosis et al., 1986; Meeker et al., 1993). On the other hand, electron microscopy observations has shown that not all axons in the rat SON display immunoreactivity to P2X2 (Loesch et al., 1999). Our electrophysiology results revealed that most of P2X-expressing GABAergic inputs terminate on P2X-expressing neurons (probably AVP neurons), while most GABAergic inputs without presynaptic P2X terminate on neurons not expressing P2X

(probably oxytocin neurons). These results support idea that upregulation of presynaptic P2X2 during fasting/refeeding is selectively associated with activity of AVP neurons.

Both P2X and P2Y participate in the ATP-induced increase in $[Ca^{2+}]_i$ in the SON cells (Song et al., 2007) and P2Y have been reported in rat SON astrocytes (Espallergues et al., 2007). P2Y receptors couple with the phospholipase C (PLC) pathway, the activation of which results in $[Ca^{2+}]_i$ increase due to Ca^{2+} release from intracellular stores and the stimulation of a Ca^{2+}-dependent K^+ current (Schicker et al., 2010). This indicates that P2Y activation might hyperpolarize the membrane and inhibit neuronal firing. In this scenario, reduced expression of P2Y1 mRNA, observed in our study, might contribute to increased excitatory effect of ATP on neuronal somata.

SON neurons receive GABAergic afferent inputs from osmosensitive neurons of the circumventricular subfornical organ (Weiss and Hatton, 1990), locus coeruleus (Jones and Moore, 1977; Leng et al., 1999) or interneurons within the perinuclear zone (Tappaz et al., 1983). The perinuclear zone presumably mediates the projections from other regions (Leng et al., 1999; Wang et al., 2015) and could account for the large number of intact synapses remaining in the SON after its surgical isolation by slicing (Leranth et al., 1975). GABA innervation appears to play a role in the patterning of the pulsatile discharge of oxytocin neurons (Voisin et al., 1995; Moos, 1995; Brussaard and Kits, 1999). We suggest that the mechanism by which stronger GABAergic inhibitory input observed here contributes to the increased synthesizing and releasing activity of AVP neurons might be associated with changes in the patterning of the discharge of AVP neurons *in vivo*.

In conclusion, the results of this study support the idea that there are differences in P2X2 receptor expression between oxytocin and vasopressin neurons, and that the increase in expression and function of purinergic P2X2 are significantly linked with the complex stimulation of AVP neurons and corresponding hormone secretion induced by refeeding after fasting. These findigs are summarized in Fig. 25.

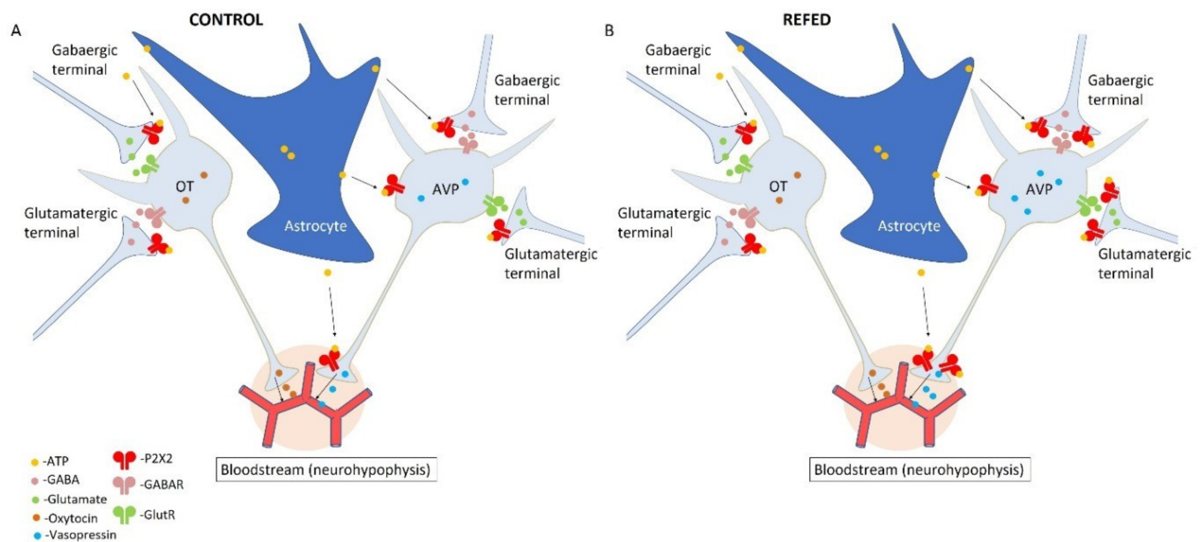

Figure 25. Differences in P2X2 receptor expression between oxytocin and vosopressin neurons, and between control and fasted/refed rats. (A) Under normal conditions (CONTROL), P2X2 receptors are expressed on somata of AVP neurons and their nerve terminals in neurohypophysis, but not on oxytocin (OT) neurons and their projections in neurohypophysis. Another P2X2 recepors are expressed also presynaptically, i.e. in nerve terminals releasing GABA or glutamate on both oxytoxin and AVP neurons. (B) Under conditions of refeeding after fasting (REFED), the expression of the P2X2 recepors increases in somata and nerve terminals of AVP neurons, as well as in presynaptic nerve terminals releasing GABA on AVP neurons. We can speculate that the expression of presynaptic P2X2 recepors increases also in nerve terminals releasing glutamate, but evidences are currently unknown.

5.2. Role of P2X7 and P2Y in extracelllar ATP rhythm in the SCN (Study II)

In study II, we elucidated the mechanism of astrocytic ATP release in the SCN, which can be measured as circadian accumulation of extracellular ATP in medium with SCN organotypic cultures. The main finding of this study is that specific P2X7 and P2Y1 antagonists inhibited circadian extracellular ATP accumulation in organotypic SCN cultures, and specific P2X7, P2Y1 and P2Y2 agonists or positive allosteric modulators that elevated intracellular calcium levels in primary cultures of SCN astrocytes increased extracellular ATP accumulation, suggesting that both P2X and P2Y contribute to the circadian rhythmicity of ATP release that might occur via a vesicular or a non-vesicular pathway.

Non-vesicular pathways for ATP release from intact cells (Fig. 26, *left*) may include gap junction proteins like connexins (Stout et al., 2002) or pannexin-1 hemichannels (Schenk et al., 2008; Suadicani et al., 2012) and ATP-gated P2X7 channels (Pellegatti et al., 2005; Hamilton et al., 2008; Norenberg et al., 2011a). In the brain, both neurons and astrocytes express connexins and form gap junctions, primarily with their own cell type (Contreras et al., 2004). Gap junctional networks of astrocytes are more common and generally more extensive, whereas little evidence is available for gap junctions among SCN neurons (Welsh and Reppert, 1996; Colwell, 2000). The P2X7 protein is also well-documented to be expressed on

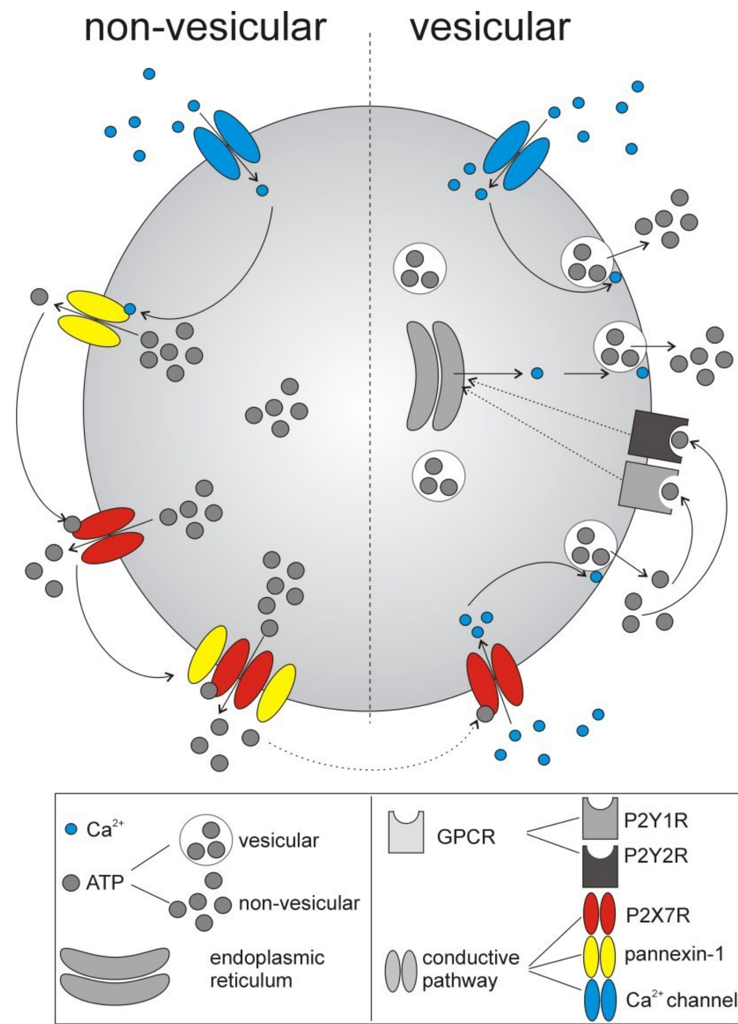

Figure 26. Schematic summary of identified mechanisms underlying ATP release from SCN astrocytes. The non-vesicular release (predominantly Ca^{2+}-independent) is potentially associated with P2X7 receptor and pannexin-1 hemichannel activation, while the vesicular release (Ca^{2+}-dependent) is associated with activity of Ca^{2+}-channels, G-protein coupled receptors (GPCRs), P2Y and P2Y2, and migh involve also P2X7.

astrocytes (Narcisse et al., 2005; Sperlagh et al., 2006; Hamilton et al., 2008; Kamatsuka et al., 2014; Zhao et al., 2016), whereas its expression in neurons is still questionable (Illes et al., 2017).

Our present study also shows the expression of the P2X7 protein in SCN astrocytes. Together with the ability of the P2X7 and pannexin-1 hemichannel blockers to reduce extracellular ATP accumulation, these data suggest that P2X7 alone or in complex with pannexin may function as a permeation channel for Ca^{2+}-independent astrocytic ATP release. Similar functions of P2X7 receptors have been proposed for the Ca^{2+}-independent release of gliotransmitters, such as purines (Ballerini et al., 1996), GABA (Wang et al., 2002), L-glutamate and D-aspartate (Duan et al., 2003; Cervetto et al., 2013; Di Cesare Mannelli et al., 2015), D-serine (Pan et al., 2015) and ATP (Anderson et al., 2004; Suadicani et al., 2012),

from cultured astrocytes. These results may also explain why circadian ATP release in SCN2.2 cells is independent of changes in intracellular Ca^{2+} concentrations (Burkeen et al., 2011) and occurs in cultured mouse cortical astrocytes even after disruption of the vesicular release mechanism (Marpegan et al., 2011).

However, astrocytic $[Ca^{2+}]_i$ is high in SCN slices during the night (Brancaccio et al., 2017), indicating that Ca^{2+}-dependent release of vesicular ATP could also contribute to circadian ATP release (Fig. 26, *right*). P2X7 activation increases $[Ca^{2+}]_i$ in rat cerebellar astrocytes (Carrasquero et al., 2009; Norenberg et al., 2011b; Illes et al., 2012), primary human fetal astrocytes in culture (Narcisse et al., 2005) and rat SCN astrocytes (Bhattacharya et al., 2013). This effect has been also demonstrated to be associated with the P2X7-dependent and Ca^{2+}-dependent release of vesicular ATP (Ballerini et al., 1996; Suadicani et al., 2012) and glutamate (Cervetto et al., 2013) in astrocytes. Our finding that ATP release rhythm in SCN organotypic cultures is significantly inhibited by the voltage-gated Ca^{2+} channel blockers is consistent with the possibility that activation of P2X7 also produces membrane depolarization and subsequent activation of Ca^{2} entry via voltage-gated Ca^{2+} channels. Finally, increases in $[Ca^{2+}]_i$ might activate pannexin-1 hemichannels (Stout et al., 2002) which is relevant for the ATP release through conductive mechanisms.

The involvement of P2Y1 in mediating ATP-evoked Ca^{2+} signals and Ca^{2+}-dependent release of vesicular gliotransmitters is also well established in astrocytes (Fumagalli et al., 2003; Verkhratsky et al., 2009). A P2Y1 agonist (MRS2365), and P2Y2 agonist (MRS2768) increased $[Ca^{2+}]_i$ and potentiated astrocytic ATP release in our study. However, it is noteworthily that P2Y11 is another possible target for ATP (von Kugelgen and Wetter, 2000). This receptor is also coupled to Gq/11 and has been recently identified in cultured foetal human cortical astrocytes (Muller and Taylor, 2017). However, no selective agonists or antagonists are available yet, and thus the role of P2Y11 in the SCN could not be tested.

Metabotropic P2Y1 and ionotropic P2X7 receptors have been demonstrated to be involved in autocrine stimulation of astrocytic Ca^{2+} signals in intact optic nerves (Hamilton et al., 2008) and astrocyte-to-astrocyte Ca^{2+}-mediated communication in culture (Fumagalli et al., 2003). Our data suggest that both P2X7 and P2Y are involved in ATP-induced ATP release from SCN astrocytes, and that the P2X7-stimulated ATP release is mediated by both Ca^{2+}-dependent and Ca^{2+}-independent pathway (summarized in Fig. 26).

Our observations raised two important questions. First, we do not know whether the rhythmic ATP secretion from astrocytes normally plays a role in controlling circadian rhythms. Genetically manipulated cortical astrocytes in culture in which IP_3 signaling is

inhibited display normal circadian rhythms in clock gene expression despite the presence of arrhythmic extracellular ATP accumulation (Marpegan et al., 2011). Thus, rhythmicity in ATP release is not required for the molecular clockwork. On the other hand, extracellular ATP and its metabolites may function as paracrine modulators in the SCN. We found previously that ATP application modulates the synaptic activity of SCN neurons and via presynaptic P2X2 receptors potentiates GABA release (Bhattacharya et al., 2013). Enhanced ATP release could potentially contribute to the low excitability of SCN neurons during the night. Second, further studies are needed to clarify the molecular mechanism by which P2X7 channels are rhythmically activated. Currently, no evidence has been reported suggesting day-night variability in the expression of the P2X7 mRNA or protein in the SCN. Astrocytes respond to many neurotransmitters, but ATP and glutamate are the most prominent (van den Pol et al., 1992; Bhattacharya et al., 2013) and both evoke $[Ca^{2+}]_i$ signals that might trigger the further release of ATP or glutamate (Haydon, 2001; Illes et al., 2012). Glutamate is released from astrocytes in SCN slices during the night period (Schousboe et al., 2013; Brancaccio et al., 2017), similar to ATP (Yamazaki et al., 1994; Womac et al., 2009) Provided that the glutamate induces ATP release which stimulates the P2X7 and the P2Y, this mechanism could initiate ATP-induced ATP release. Surprisingly, the glutamate-evoked increase in astrocytic $[Ca^{2+}]_i$ in the intact optic nerve is significantly reduced in P2X7 knock-out mice (Hamilton et al., 2008).

In conclusion, study II revealed one intriguing property of circadian ATP release mechanism in the SCN: accumulation of extracellular ATP is dramatically dependent on activity of P2X7 and as well as on the level of intracellular calcium controlled by P2Y, suggesting that ATP release might occur via a vesicular as well as a non-vesicular pathways involving multiple purinergic P2 receptors (Fig. 26). The non-vesicular release (predominantly Ca^{2+}-independent) is associated with P2X7 receptor and pannexin-1 hemichannel activation. Released ATP further stimulates G-protein coupled receptors (GPCRs), P2Y and P2Y2, that contribute to Ca^{2+}-dependent vesicular release of ATP. Thus, multiple purinergic P2 receptor systems, with different intracellular mechanisms, control rhytmic release of ATP from SCN astrocytes.

6. Conclusions

In this doctoral thesis, we elucidated the physiological impact of modulation of neuronal activity and synaptic transmission by purinergic P2X receptors in hypothalamus, particularly in magnocellular SON neurons which synthesize and release AVP and oxytocin. Additionally, we identified multiple purinergic receptors in SCN astrocytes that control circadian ATP release. We used electrophysiology, biochemistry, immunohistochemistry, molecular biology, calcium imaging and experimental protocol that stimulates synthesis and release of neurohypophyseal hormones in rats. The main conclusions are:

- Under physiological conditions of intense secretion of hormones, increased expression of AVP mRNA correlates with the increase of P2X2 and decrease of P2Y1 mRNA expression in SON tissue.
- Refeeding after fasting upregulates the P2X protein level on the cell somata and presynaptic nerve terminals releasing GABA on presumptive AVP neurons in the SON. No effect was observed on somatic and presynaptic ATP-induced responses in presumptive oxytocin neurons.
- In the SCN, circadian rhythm in extracellular ATP accumulation, that peaks between 24:00 – 04:00, is inhibited by specific blockers of P2X7, and potentiated by a positive allosteric modulator of this receptor. Double-immunohistochemical staining revealed high expression of the P2X7 protein in SCN astrocytes. These data strongly indicate that P2X7 receptors play an important role in circadian ATP release from astrocytes..
- The pannexin 1 hemichannel blocker carbenoxolone and specific P2Y1 antagonist partially abolished ATP rhythm suggesting that pannexin-1 hemichannels and metabotropic P2Y contribute to circadian ATP release.

The results of this thesis indicate that the unique properties of P2X receptors could facilitate various physiological functions the receptors are involved in. Particularly, nondesensitizing P2X2 receptor represents the mechanism through which neuroendocrine output can be enhanced, and a large pore of P2X7 represents the pathway through which ATP upregulates its own release from astrocytes.

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