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ATP appears to act via different receptors in terminals vs. somata of the Hypothalamic Neurohypophysial System

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Abstract

ATP-induced ionic currents were investigated in isolated terminals and somata of the Hypothalamic Neurohypophysial System (HNS). Both terminals and somata showed inward rectification of the ATP-induced currents and reversal near 0 mV. In terminals, ATP dose-dependently evoked an inactivating, inward current. However, in hypothalamic somata ATP evoked a very slowly inactivating, inward current with a higher density, and different dose dependence; EC₅₀ of 50 μ M in somata vs. 9.6 μ M in terminals. The ATP induced currents, in both the HNS terminals and somata, were highly and reversibly inhibited by suramin, suggesting the involvement of a P2X receptor. However, the suramin inhibition was significantly different in the two HNS compartments: IC₅₀ of 3.6 μ M in somata vs 11.6 μ M in terminals. Also, both HNS compartments show significantly different responses to the purinergic receptor agonists ATP- γ -S and Benzoyl-benzoyl-ATP. Finally, there was an initial desensitization to ATP upon successive stimulations in the terminals which was not observed in the somata. These differences in EC₅₀, inactivation, desensitization, and agonist sensitivity in terminals vs. somata indicate that different P2X receptors mediate the responses in these two compartments of HNS neurons.

Previous work has revealed mRNA transcripts for multiple purinergic receptors in micropunches of the hypothalamus. In the HNS terminals, the P2X purinergic receptor types P2X2, 3, 4, and 7 but not 6 have been shown to exist in AVP terminals. Immunohistochemistry now indicates that P2X4R is only present in AVP terminals and that the P2X7R is found in both AVP and OT terminals and somata. We speculate that these differences in receptor types reflects the specific function of endogenous ATP in the terminals vs. somata of these CNS neurons.

Keywords

neuropeptides; oxytocin; vasopressin; P2X receptors

Introduction

Extracellular Adenosine Triphosphate (ATP) is a purine known to exert effects on a variety of tissues through specific purine (P1 and P2) receptors. Some of these actions involve initiation of second messenger systems or direct activation of ion channels (1–3). Nearly two decades ago ATP was first shown to affect sensory neurons (4) and, since then, evidence has revealed that extracellular ATP modulates a family of channels that are permeable to Na⁺, K⁺, and Ca²⁺ (5). All of these play a role in regulating the release of neurotransmitter, either directly, as in the case of calcium ion influx, or by inducing or modulating action potentials.

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Functionally, in endocrine cells, ATP can act as an agonist of hormone release. For example, ATP stimulates the release of catecholamines from PC12 cells (6) and of luteinizing hormone from gonadotrophs (7). On the other hand, ATP has been shown to inhibit calcium currents in chromaffin cells (8). Finally, ATP has been shown to activate a ligand-gated ion channel in cholinergic terminals (3), and to directly increase intraterminal calcium levels, which is coupled with release from neurohypophysial (NH) terminals (NHT) (9). However, it has only recently been shown to directly gate ion channels in CNS terminals (10).

The Hypothalamic Neurohypophysial System (HNS) has allowed us to begin to unravel the complicated purinergic roles in the CNS by allowing us to compare their effects on the different HNS compartments, *i.e.*, the somata and terminals of the same CNS neurons. In addition, the mRNAs for multiple types of ATP purinergic receptors have been identified in micropunches of the rat hypothalamus (11). RT-PCR studies show that multiple subtypes of Purinergic receptors (P2xR) are expressed in the HNS (11) and immunoreactivity (IR) studies have localized them to the NHT (12). Arginine-vasopressin (AVP) containing NHTs display IR to P2X2R, P2X3R, P2X4R and P2X7R subtypes. P2X2R and P2X3R expressed in AVP-NHT play a role in AVP release, but are not involved in oxytocin (OT) release (9, 12). The P2X7R subtype has been identified in the brain, based on the presence of mRNA transcripts, IR, and pharmacological intervention of putative downstream functions (13–15). However, neuronal specific expression of P2X7R is controversial (16, 17).

We have found that ATP acts through different types of receptors in these two HNS compartments (10). We now demonstrate, using both electrophysiology and immunocytochemistry, that ATP can exert its effects through a mixture of P2X2, P2X3, P2X4, and P2X7 receptor types in isolated vasopressinergic terminals (12), whereas P2X3 and P2X7 do not appear to play a role in the isolated MCN. Thus, the HNS is an ideal system to use as a tool to begin unraveling the complicated purinergic effects in the CNS by comparing such effects on its different components.

Materials & Methods

NH Terminals

Dissociated isolated neurohypophysial terminals, from male Sprague-Dawley rats (Taconic Farms, Germantown, NY), were obtained as previously described (18, 19). The ATP receptor mediated inward cation currents were obtained using the perforated patch whole-cell recording technique (18) performed with 2–4 M Ω resistance pipettes. Perforation of the terminals' membranes was obtained by adding 30 μ M amphotericin B to the pipette solution (see *Solutions*). After perforation the terminals were voltage-clamped at –90 mV and perfused continually with the control Normal Locke's (see *Solutions*). Selected agonists and antagonists were applied for 2 seconds (solution switch controlled by an SF-77B Perfusion Fast-Step, Warner Instruments), using a gravity driven sewer-pipe perfusion system, followed by a 60 second rinse interval. Successive ATP stimulations revealed that there was a one-time desensitization (reduced response to the next agonist challenge) following the first ATP exposure which stabilized afterwards (Figure 1A), therefore all analog and antagonist comparisons were done using subsequent ATP exposures. All experiments were performed at room temperature and currents were recorded using a Dagan amplifier, filtered at 1 kHz and stored on computer for later analysis (pClamp5).

Hypothalamic Somata

Dissociated SON somatas from male Wistar rats were obtained as previously published (20). Whole cell recordings were performed with 4–6 M Ω resistance pipettes (see pipette *Solution* below). Large cells were selected (>12 μ m in diameter) and held at –60 mV. Cells were

continuously perfused with control solution (see *Solutions*). ATP and agonists were applied via a U-tube controlled by computer-operated valves. Antagonists were applied in the bath (about 1 min) prior to co-application with the agonist. Currents were recorded with an Axopatch 2A amplifier, filtered at 50 Hz (Frequency Devices Inc.), digitized at 133 Hz and stored on computer (pClamp6). Series resistance was compensated. All experiments were performed at room temperature.

Immunocytochemistry in isolated terminals

The ATP receptor and neuropeptide precursor (neurophysin I or II, for OT and AVP, respectively) labeling was done using the immunofluorescent technique as previously described (12). Briefly, male Sprague-Dawley rats (Taconic Farms, Germantown, NY), were sacrificed and isolated terminals were prepared from the neurohypophysis of the brain. Terminals were plated onto coverslips, fixed in 4% formaldehyde, and then rinsed. Terminals were subsequently permeabilized with standard PBS containing 1.0 % Triton X-100 and in the presence of 10 % normal donkey serum to block non-specific antibody binding. Then, terminals were incubated for either 1 h at room temperature with neurophysin antibodies, (1:200) or overnight at 4°C with anti-P2X receptor antibodies (1:200), as indicated, with 10% donkey serum. P2X4 or P2X7 receptors were labeled with rabbit polyclonal antibodies directed against the carboxyl terminus of indicated receptor. Mouse monoclonal anti-neurophysin I antibody and goat polyclonal anti-neurophysin II antibody were used to label oxytocin and vasopressin, respectively. The terminals were rinsed, then incubated with Alexa Fluor 350 conjugated donkey anti-goat, 488 anti-rabbit, or 594 anti-mouse fluorescently tagged secondary IgGs, diluted at 1:200 in PBS with 1% Triton X-100 for 1 h in the dark at room temperature. Afterwards, the coverslips were rinsed and then mounted onto slides with Prolong antifade reagent (Invitrogen). The images were acquired on a Zeiss Microscope, and fitted with a Hamamatsu digital camera, using MetaMorph software.)

Immunocytochemistry in hypothalamus

Immunolabeling in the SON was performed as previously described, with the following modifications (Shan et. al, 2010). Adult male Sprague Dawley rats were sacrificed and brains were immediately removed, cut in coronal sections to isolate the hypothalamus and embedded in OCT. Fresh frozen sections (10 µm) were cut, set on slides and air dried overnight at room temperature. Hypothalamic brain sections were subjected to Zamboni's fixative (American Mastertech, Lodi, CA) for 10 minutes (21), washed in standard PBS, air dried then stored at 4°C until ready to use. Sections were rehydrated in PBS, then blocked and permeabilized with PBS containing 5% donkey serum and 1% triton X-100, for 30 minutes. Slides were then incubated overnight at 4°C in a cocktail consisting of rabbit anti-P2X7 (1:200); mouse anti-Neurophysin I (1:200); and goat anti-neurophysin II (1:200) antibodies. Afterwards, they were washed and incubated with biotinylated sheep anti-mouse secondary IgG for 2 hours. Subsequently, sections were exposed to a mixture including Alexa Fluor 488 goat anti-rabbit IgG; Alexa Fluor 647 donkey anti-goat IgG; and Alexa Fluor 405 conjugated to avidin, for 2 hours at room temperature. Slides were given a final wash and coverslips were mounted using Fluormount-G (Southern Biotech, Birmingham, Alabama). Images were taken with a Leica TCS SP5, laser-disk scanning, confocal microscope and analyzed with NIH-ImageJ (22) using Deconvolution Lab plugin (23).

Materials

The following primary antibodies were used in this study: Neurophysin I (OT) mouse monoclonal antibody (PS-38) kindly provided by Harold Gainer, (NIH, Bethesda, MD) and Neurophysin II (AVP) goat polyclonal antibody obtained from Santa Cruz Biotechnology (Santa Cruz, CA). P2X4R and P2X7R rabbit polyclonal antibodies were obtained from

Millipore (Billerica, MA). The following antibody- or avidin-conjugates were used: Sheep anti-mouse-biotinylated antibody was obtained from GE Healthcare Biosciences Corp (Piscataway, NJ); Alexa Fluor-488 donkey anti-rabbit; -594 donkey anti-mouse; -647 donkey anti-goat, -350 Donkey anti-goat, and Alexa Fluor-405 avidin conjugate obtained from Invitrogen (Carlsbad, CA).

Data Analysis

The dose-response relationship was constructed for individual terminals or cells and normalized to the maximum current within each experiment. The different data from each terminal or cell was then combined to obtain the dose response curves. EC_{50} and IC_{50} were determined using Origin (version 7, Northampton, MA) software. Data are given as the mean $\pm$ SEM. Mann-Whitney tests (for unpaired data) and Student t-tests (for paired data) were used to examine the statistical significance.

Solutions

NH Terminals

Normal Locke's solution consists of; 130 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 2.2 mM $CaCl_2$, 10 mM HEPES, pH 7.2, ~300 mOsm. Pipette solution consists of; 130 mM CsGlutamate, 10 mM HEPES, 20 mM Tetraethylammonium Chloride, 1 mM $MgCl_2$, pH 7.2, ~ 295 mOsm. All ATP analogs were used at 100 μ M concentrations.

Hypothalamic Somata

Control rinse solution consists of; 132 mM NaCl, 4 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.4, ~300 mOsm. Pipette solution consists of; 140 mM CsCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.1 mM EGTA, 10 mM HEPES, 2 mM ATP, pH 7.2, ~ 300 mOsm. All ATP analogs were used at 50 μ M concentrations.

Results

Extracellular ATP effects on HNS somata vs. terminals

Recordings were obtained from large (6–12 μ m diameter) HNS terminals (n=38) having a mean capacitance of 1.4 ± 0.1 pF of which 70% responded to low ATP concentrations (10 μ M). These were compared to recordings obtained from large HNS magnocellular somata (n=124), having a mean capacitance of 18.7 ± 0.5 pF and input resistance of 1.4 ± 0.4 G Ω , of which only 52% responded to the application of 50 or 100 μ M ATP.

An examination of the ATP concentration-response traces in the terminals (Figure 1A) show that all concentrations of ATP elicit a steady inactivation (*i.e.*, relaxation in current) during each stimulus (10.6 ± 2.3 pA s⁻¹, n=9), although at the lowest dosage such inactivation is minimal (Figure 1C). Therefore, all current measurements were taken as the mean of the entire 2 second-long stimulation with points collected every 60 ms. However, inactivation was not present (Figure 1B) at any ATP concentration in the magnocellular somata current traces (Figure 1D). Two other phenomena in the terminals have been observed that have not been noticed in the cell bodies: 1) "Desensitization" (*i.e.*, decreased maximal current in subsequent application) between successive stimulations can be induced by decreasing the interval duration (<60 s; (12)) between successive ATP stimulations, and 2) a change in inactivation of the ATP-induced current to a 20 second exposure (2.5 ± 0.1 pA s⁻¹) as opposed to a 2 second exposure (10.6 ± 2.3 pA s⁻¹). These correspond to the two rate constants of inactivation previously described in NHT (12), but are not evident in somata. Overall there was a 21.2 ± 4.6 % decrease in the overall current during the short exposures

as compared to a $50.8 \pm 1.8\%$ during the longer exposures, which are equivalent to an AVP-burst duration (19).

The concentration-response curves (Figure 1E) for ATP yielded an EC_{50} for the terminals ($n=5$) of $9.6 \pm 1.0\ \mu\text{M}$ with a Hill coefficient of 1.6 ± 0.2 (see also (12)) and an EC_{50} for the magnocellular somatas ($n=6$) of $53.0 \pm 7.0\ \mu\text{M}$ with a Hill coefficient of 1.8 ± 0.2 (Figure 1E). The EC_{50} values were significantly different ($p=0.002$) which suggests that the isolated terminals could have a receptor population different from that in the cell bodies, with a higher sensitivity to ATP, while the similarity in Hill coefficients indicate that their binding cooperativity is the same.

The current-voltage relationship using the near maximum ATP activation concentration demonstrates a stronger inward rectification at positive potentials in terminals (Figure 2A) than in magnocellular somatas (Figure 2B) with a reversal potential of $\sim 0\ \text{mV}$ in both (Figure 2C). In addition, current amplitudes measured at maximum ATP concentrations and at optimum (for ATP effects) holding potentials showed a significant ($p<0.001$) difference in current density between terminals ($70.8 \pm 18.6\ \text{pA pF}^{-1}$, $n=6$) and magnocellular somatas ($11.1 \pm 2.6\ \text{pA pF}^{-1}$, $n=62$). This indicates that either the ATP receptor population is different (*e.g.*, greater conductance) or that the number of channels per unit area is greater.

P2X receptor antagonist and agonist effects

The currents activated by ATP at its EC_{50} were reversibly and concentration-dependently inhibited by the P2X receptor antagonist suramin in terminals (Figure 3A) and magnocellular somata (Figure 3B). The inhibition-response of suramin on ATP yielded an IC_{50} for the terminals ($n=5$) of $11.6 \pm 1.7\ \mu\text{M}$ (see also (12)) and for the magnocellular somata ($n=4$) of $3.6 \pm 0.1\ \mu\text{M}$ (Figure 3C). The IC_{50} values are significantly different ($p=0.004$) suggesting, as with the data from the EC_{50} analysis, that the receptor populations in the terminals and cell bodies are different and that those in the terminals are less sensitive to suramin.

Purinergic receptor agonist sensitivity was investigated by comparing evoked currents using the maximally effective concentration ($100\ \mu\text{M}$) of ATP and its analogs in terminals (Figure 4A) and in magnocellular somata (Figure 4B). Previously, a statistical analysis comparing the agonist sensitivity of the more efficacious agonists at $10\ \mu\text{M}$ (terminal EC_{50}) vs $100\ \mu\text{M}$ (see (12)) showed no significant differences. Thus, the maximum concentration was used in the terminal comparisons because of the 10-fold lower amplitude currents in the terminals. All analog comparisons immediately followed a control ATP challenge at the same concentrations (Figures 4A and B). The apparent rank order of potency compared to the preceding ATP response in both terminals and cell bodies (Figure 4C) was 2-methylthio-ATP (2-Met-S-ATP) > ATP- γ -S > Benzoyl-benzoyl-ATP (Bz-Bz-ATP). In the terminals there was also a minor response to α,β , methylene-ATP (α,β -Met) and β,γ , methylene-ATP (β,γ -Met) not seen in cell bodies. Although, there was variability in the effectiveness of the analogs when compared between somata and terminals, and significant ($p<0.01$) differences in the effect of both ATP- γ -S and Bz-Bz-ATP suggesting different P2X receptor populations, the rank order of the major agonists was not different.

Immunohistochemistry of ATP receptors in HNS somata vs. terminals

RT-PCR studies show that multiple subtypes of purinergic receptors (P2X_{2,3,4,6} & 7) are expressed in the HNS (11) and IR studies for all but the last receptor have been conducted on the hypothalamus (6). Thus, in SON and PVN slices, antibodies specific to the P2X₇R, AVP, and OT were used to determine if this receptor is in the HNS somata. Figure 5 show

the existence of P2X7 not only on AVP-containing somata, but also on OT-containing somata. This does not exclude the possibility that it is in glia, as well.

In isolated OT terminals, ATP induces only a sustained release response (24), suggesting that P2X2 and P2X3 receptors are not likely to be involved in regulating OT release. It has therefore been hypothesized that P2X4R and P2X7R may be involved in the regulation of OT. In order to establish this, the first step is to determine if these receptors are on OT terminals. Immunocytochemistry was utilized for this purpose. Antibodies specific to P2X4R, P2X7R, AVP, and OT were used in order to determine if these receptors are actually on HNS terminals. Figure 6 show the existence of P2X7R not only on AVP-containing terminals, but also on OT-containing terminals; but that P2X4R is found on terminals that contain AVP but not OT.

Discussion

The HNS has allowed us to determine that ATP acts through different P2X receptors in the somata and terminals of these CNS neurons. In both terminals and somata, ATP-activated currents are definitely of the P2XR type: rapid activation, reversal potential around 0 mV (in agreement with a non-discriminative cationic selectivity), and characteristic inward rectification. However, in the NHT ATP-activated currents show pronounced inactivation during short (2 s) applications and desensitization between successive applications. This is in contrast to our previous work (9) that did not reveal inactivation of ATP stimulated $[Ca^{2+}]_{in}$ responses. This discrepancy could be explained by the different Ca^{2+} permeabilities of purinergic receptors (25), or the increase in $[Ca^{2+}]_{in}$ could mainly result from entry of Ca^{2+} through voltage-activated Ca^{2+} channels subsequent to the ATP-induced depolarization (but, see (9)). In contrast, somatic ATP-activated currents show little or no desensitization/inactivation. Although Shibuya et al., (11) reported strong desensitization/inactivation in a minority of dissociated SON somata, we did not detect this in our cell preparations. It is possible that the apparent discrepancy could result from either their recording of some SON somata from a younger group of animals or that we did not select a somata from the inactivating/desensitizing group.

The ATP response also differed pharmacologically in the two HNS compartments; the receptors in the terminals showing a 5-fold higher sensitivity to ATP and a 4 fold lower sensitivity to suramin, as well as differences between the actions of ATP- γ -S and Bz-Bz-ATP. This suggests that the isolated terminals have a receptor population different from that in the cell bodies, with a higher sensitivity to ATP, while the similarity in Hill coefficients indicate that their binding cooperativity is the same. Lower sensitivity to ATP in HNS somata has also been recently reported by Vavra et al. (26). RT-PCR analysis in the hypothalamus (SON) reveal mRNA transcripts for P2X2, P2X3, P2X4, P2X6 and P2X7 receptors (11). Rank order of agonist potency, as well as suramin sensitivity, may point to a receptor formed by a P2X2 subunit. Interestingly, P2X2 receptors have been localized in both the somata of the SON (6) and in the neurosecretory endings of the neurohypophysis (12). However, P2X2 homomeric receptors are unlikely to constitute the only form of P2X receptors in HNS terminals since P2X2 receptors, when expressed in heterologous expression systems (25), exhibit little to no inactivation and have a much lower sensitivity to Bz-Bz-ATP (equivalent to that of ATP- γ -S). However, the above pharmacological profile could indicate the existence of P2X7R in terminals (27). Similarly, receptors formed by the heteromeric assembly of P2X2 and P2X3 subunits seem unlikely because they also show little or no inactivation (25). Thus, one possibility would be a mixture of P2X2 and P2X3 homomeric receptors in individual terminals. The high sensitivity for ATP ($EC_{50} = 9.6 \mu M$) in the terminals would also argue for the presence of P2X3 receptors since they have a higher sensitivity for ATP than P2X2 receptors (25). In contrast, for the SON somata, one

may envision the presence of homomeric P2X₂ as well as of receptors formed by the heteromeric assembly of P2X₆ with either P2X₄ or P2X₂ (25), all of which show a high expression in SON magnocellular neurons (6, 11). Small responses to Bz-Bz-ATP make it unlikely that there are functional P2X₇R in somata (see below and also Shibuya et al., (11)).

Whatever the molecular correlate to our observations in SON somata and terminals, the main functional difference is the pronounced inactivation and desensitization displayed by terminal receptors. NHT also show stronger inward rectification than in somata (Fig. 2). We speculate that these differences in kinetics and in sensitivity reflect the specific functions of the ATP receptors in these two CNS compartments (see below).

Previously, we have shown that ATP in the HNS acts in a positive feedback manner on neuropeptide release; by activation of P2X receptors and by inhibiting calcium-activated K⁺ channels (28). Activation of a P2X receptor leads to increases in intraterminal [Ca²⁺] and subsequent AVP release (9). We have hypothesized (28) that ATP and its metabolite, adenosine, have autocrine and paracrine modulatory effects on the release of AVP vs. OT during physiological bursting patterns of stimulation. In fact, these feedback mechanisms could explain the efficacy of such patterns in facilitating AVP vs. OT release (see below). The significantly more sensitive (EC₅₀ = 9.6 vs. 53 μM) ATP receptor in the terminals would make such endogenous feedback effects more likely. In fact, extracellular ATP concentrations have been measured in the whole, intact NH (9, 29), and reach high enough levels (>8 μM) for such physiological effects. The P2X₇R is also distinguished from other P2XR types by its very low sensitivity to ATP (EC₅₀ ≈ 120 μM, in rat). Thus, a high enough concentration of ATP to activate P2X₇ receptors at the somata may be less likely than even at terminals.

The lower sensitivity of the somatic receptor could be linked to their activation by ATP co-released from noradrenergic afferent fibers, where the concentration could reach higher levels (30). Moreover, since only about half of the cell somata responded, these could correspond to AVP somata, since only AVP neurons seem to be activated during moderate traumatic hemorrhage, which involves input from catecholaminergic neurons of the ventral medulla (31) and is mediated by ATP co-released with noradrenaline (30). This action requires astrocyte involvement, as they express α₁ adrenergic receptors, which is shown to be absent from most postsynapses (32–34).

ATP facilitates the process of secretion by stimulation of P2XR in secretory cells and neurons. While the expression and function of the P2X₇R-subtype in neurons is controversial, studies show that P2X₇R mRNA is present in the brain, including in the hypothalamic neurohypophyseal system (HNS). Our laboratory has reported that HNS AVP terminals exhibit P2X₇R IR (12) along with other P2XR subtypes, while here we report that oxytocin (OT) terminals display only P2X₇ IR. P2X₄ receptors are found in AVP-containing terminals, but not on terminals containing only OT. P2X₇ receptors are found on both AVP- and OT-containing terminals and somata. These results are consistent with previous work showing P2X₇R IR in the AVP-NHT (12) and further demonstrating IR in OT-NHT and in SON. AVP- and OT-NHT and somata demonstrate P2X₇R IR suggesting that this receptor is present. It is not surprising that this antibody (against the intracellular domain) would recognize this receptor in somata since its mRNA is there and functional P2X₇R appears to be in terminals (27). However, we see no obvious P2X₇R current in HNS somata, confirming Shibuya et al. (11) and, more recently, Vavra et al. (26). Furthermore, our preliminary data (27) indicate that the P2X₇ receptor is not appreciably localized to magnocellular somatic membranes, but it is observed in glial membranes (also, see (11)). Thus, even though P2X₇R is observed in the SON, our data does not support a functional role for this receptor in somata of magnocellular neurons.

OT neurons are characterized by a high frequency (up to 120 Hz) discharge during suckling which leads to the pulsatile release of OT into the blood and to subsequent milk-ejection (35). AVP neurons are characterized by their asynchronous (10–60 Hz) phasic activity (bursting) during maintained AVP 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, which facilitates hormone release (36–38).

Considering such stimulation patterns, the most intriguing difference between somata and terminal ATP receptors is the significant inactivation and desensitization in the latter HNS compartment. This could explain why burst durations and interburst intervals are critical for AVP release, in particular (39). The inactivation in terminals is >50% during 20 s exposures to high ATP concentrations. Therefore, we speculate that the positive feedback of ATP co-released with AVP would inactivate/desensitize during the typical 20–30 second long bursts (35) that are most efficient in causing this peptide's release (36–38). Furthermore, recovery from such desensitization might dictate the minimum of 21 seconds for AVP interburst intervals (37). Further analysis of this phenomenon, however, is necessary.

During bursts of action potential stimulation, like those found in peptide-releasing neurons, intracellular calcium levels reach a high enough concentration to induce neurotransmitter release. Interestingly, ATP is stored in all secretory vesicles and is co-released with neuropeptides such as AVP and OT. Thus, during the beginning of trains of action potentials, Ca^{2+} entry through P2X receptors could be an additional mechanism by which calcium can enter terminals and potentiate release (39). It is therefore postulated that purinergic feedback systems may be important to many, if not all, peptide releasing systems.

In conclusion, these differences in electrophysiological properties of purinergic receptor types reflect the specific function of endogenous ATP in the terminals vs. somata of these HNS neurons. Furthermore, these results lead to the hypothesis that ATP could be co-released to act at P2X receptors in a positive-feedback mechanism differentially modulating release initiated by bursts of action potentials in these and other CNS terminals (39).

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Figure 1. Comparison of the ATP concentration-dependent responses in isolated HNS terminals and cell bodies

An initial desensitization (compare 1st and 2nd traces) and repeated inactivation of ATP-induced inward currents in isolated terminals (A) that was not seen in cell bodies (B). Sample current traces of different ATP concentrations on (C) isolated terminals and (D) cell bodies. Again note inactivation of current for all but lowest dose of ATP in terminals only. (E) ATP concentration-response curve for isolated terminals (hollow circles) and cell bodies (filled circles). Currents were generated by 2 second exposures to ATP and normalized to a preceding maximum ATP stimulation (100 μ M in terminals and 300 μ M in cell bodies). All points are means of 5–6 experiments and vertical lines indicate S.E.M.; when no lines are visible, the error was within the size of the symbol. (— application of ATP)

Figure 2. Inward rectification of extracellular ATP-induced currents in isolated HNS terminals and cell bodies

Sample current traces of the maximum ATP response at indicated concentrations at different voltage-clamped holding potentials ranging from -90 to $+40$ mV on (A) isolated terminals and (B) cell bodies. (C) ATP current-voltage curves of isolated terminals (hollow circles) and cell bodies (filled circles). Both showed inward rectification. All points are means of 3 experiments and vertical lines indicate S.E.M.; when no lines are visible, the error was within the size of the symbol.

Figure 3. Comparison of suramin inhibition in isolated HNS terminals and cell bodies
 Sample traces of different suramin concentrations on the EC_{50} ATP concentration of (A) isolated terminals and (B) cell bodies. (C) Suramin concentration-response curves of isolated terminals (hollow circles) and cell bodies (filled circles). ATP and suramin exposures were normalized to a preceding EC_{50} ATP (10 μ M in terminals and 50 μ M in cell bodies) stimulation response. All points are means of 4–5 experiments and vertical lines indicate S.E.M.; when no lines are visible, the error was within the size of the symbol.

Figure 4. Comparison of ATP analog responses in isolated HNS terminals and cell bodies
 Sample traces of ATP analogs and preceding ATP normalizing traces in (A) isolated terminals at 100 μM (scale on right for each pair of traces) and (B) cell bodies at 50 μM (scale on left applies to first 3 traces and the scale on the right applies to the last 2 traces). (C) Histogram of analog effects compared between the isolated terminals and cell bodies (*p<0.001; **p=0.008). All bars are means of 3–7 experiments and vertical lines indicate S.E.M.; when no lines are visible, the error was within the size of the symbol. Although, the EC₅₀ values were used to make comparisons in the cell bodies, the maximum concentration on terminals (100 μM) was used to insure that there would be enough current to make a proper comparison (see text).

Figure 5. Immunoreactivity for P2X7R, AVP and OT in rat SON

P2X7R distribution was examined in the PVN (top row) and SON (bottom row) of adult rats using an antibody directed against the carboxyl-terminus of the receptor, as described in Methods. Panel figure shows IR of P2X7R (blue), oxytocin (green), vasopressin (red) and merged image, as indicated. Figures show P2X7R IR in the somata of both oxytocin and vasopressin containing neurons (arrowheads indicates co-localization between P2X7R and oxytocin IR; arrows indicates co-localization between P2X7R and vasopressin IR; scale bar on merged images is 50 μ m).

Figure 6. P2X4R vs. P2X7R immunoreactivity in NH Terminals

We examined P2X4R and P2X7R IR in isolated NHT using antibodies directed to the carboxyl terminus to evaluate its expression in OT-containing and AVP-containing NHT. P2X4R (above, green) is observed in only AVP-NHT (blue; arrow) not OT-NHT (red; arrowhead) as seen in the merged image (yellow and cyan). P2X7R IR (below green) can be observed in both AVP-NHT (blue; arrow) and OT-NHT (red; arrowhead) as seen in the merged image (yellow and cyan). These results are consistent with previous work showing P2X7R IR in the AVP-NHT (12) and further demonstrating IR in OT-NHT.