

Multifaceted purinergic regulation of stimulus-secretion coupling in the neurohypophysis

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Submitted: July 12, 2002

Accepted: July 14, 2002

Key words: **neurohypophysis; ATP; adenosine; purinergic receptor; vasopressin; oxytocin; nerve terminals; neurohypophysial astrocytes; autocrine/paracrine feedback**

Neuroendocrinology Letters 2002; 23:273-280 pii: NEL230402R01 Copyright © Neuroendocrinology Letters 2002

Abstract

The neurohypophysis is an original model of the CNS secretory system releasing vasopressin (AVP) and oxytocin (OXT), two neuropeptides hormones synthesized by the magnocellular neurons of the hypothalamus. Specific patterns of action potentials originating from cellular bodies of magnocellular neurons control the release of AVP and OT, but intra-neurohypophysis regulations do modulate the neuropeptides release. There is now good evidence for the effects of extracellular purines in the control of neurohypophysial secretion. This paper brings together evidence for the multiple, intricate actions of purines in the extracellular space of the neurohypophysis. It covers four main points. First, the activity-dependent release of endogenous ATP in the neurohypophysis. Second, the action of ATP on both neuronal and non-neuronal compartments of the neural lobe. Third, the termination of ATP positive feedback by ecto-nucleotidases. And finally the possible involvement of adenosine in the regulation of neurohypophysial secretion and glial plasticity. The data suggest that ATP and adenosine are physiological modulators of the release of neurohypophysial peptides by acting directly on nerve terminals and indirectly on neurohypophysial astrocytes. Since purinergic receptors are widespread in nervous and endocrine systems, the neurohypophysis appears as an useful model for studying the role of purines in the regulation of stimulus-secretion coupling and neuron-glia interactions. The feedback mechanisms found in the neurohypophysis could be ubiquitous, occurring throughout the central nervous system and in other secretory systems.

Introduction

ATP, besides the function of intracellular energy source, appears as an extracellular signal in a wide variety of systems where it is involved in both physiological and physiopathological conditions [reviewed in 1]. ATP acts as a neurotransmitter in the central and peripheral nervous systems. The co-storage of ATP with acetylcholine and catecholamines has been demonstrated for a variety of peripheral and central synaptic vesicles [2]. ATP co-released with these neurotransmitters can act as fast excitatory agent on both cholinergic [3] and adrenergic [4] neurons. Furthermore, ATP influences endocrine systems, where it can modulate hormones release [5 & 6]. The extracellular actions of ATP are mediated by specific membrane located receptors referred as P2 receptors. Based on the cloning of P2 receptors, two subtypes were defined as P2X the ligand-gated ion channel and P2Y the G protein-coupled receptor [7]. Adenosine, which is produced by hydrolysis of ATP by ecto-nucleotidases, may also act as a neuromodulator within the nervous system. It modulates the release of neurotransmitters [8], post-synaptic responsiveness [9], and the action of other receptors systems [10]. Specific receptors referred as P1 receptors and subdivided in different subtypes (A1, A2A, A2B and A3) intercede in the different actions of adenosine. The actions of both ATP and adenosine in the nervous system are not restricted to neurons, as several glial cells including astrocytes, microglia, oligodendrocytes and Schwann cells bear functional P2 and P1 purinergic receptors [11]. Moreover, data obtained *in vitro* indicate that extracellular purines control astrocyte proliferation and the production of trophic factors by glial cells [12].

The neurohypophysis contains vasopressin (AVP) and oxytocin (OXT) releasing neurosecretory endings originating from magnocellular neurons of the hypothalamus [13]. This system has been used to demonstrate the direct relationship between intracellular calcium increase and neurohormone secretion [14] and it is considered as an unique model for studying the regulation of stimulus-secretion. Although the release of the neuropeptides is driven by specific patterns of action potentials initiated by hypothalamic neurons, intrinsic regulations can modulate the hormone release at the neurohypophysis level [15]. Moreover, several lines of evidence strongly indicate that glia participates in the control of neurohypophysial secretion [13]. Consequently, this organ appears therefore as an excellent model for studying the role of extracellular purines in the regulation of secretion and for examining the involvement of these compounds in the control of secretion by glia.

This paper summarizes our research data and results of the literature indicating the multiple, complicated feedback regulatory mechanisms by which purines control neuroendocrine stimulus-secretion coupling.

Release of endogenous ATP in the neurohypophysis

Since ATP is stored in secretory granules with neurohypophysial hormones [16; 2], its release into the extracellular space of the neural lobe throughout the stimulation of secretion could be envisaged. Accordingly, co-release of endogenous ATP with neurohypophysial peptides is observed in response to nerve activity stimulation achieved by high K⁺ depolarization (*Figure 1A* and 17) or electrical field stimulation [18]. In these experiments, the release of ATP was assayed by the luciferin-luciferase technique, and the neuropeptides were detected by radioimmunoassay in the same perfusate samples. With a 2 mM intragranular ATP concentration, a extracellular concentration of 4-40 μ M could probably be achieved locally within the diameter of 10 μ m around the site of release [19, 20]. These data point to the release of ATP with neuropeptides from the secretory granules. However, Sperl agh *et al* [18] state that inhibiting the action potential with TTX blocks the release of AVP and OXT, while the release of ATP is almost unaffected. This puzzling result suggests that cellular and subcellular sources of the ATP and neurohormones released by the neurohypophysis are not strictly identical. The afferent nerve terminals in the neurohypophysis, especially the noradrenergic inputs, may also be a source of the released ATP, as observed in the hypothalamus [21]. As electrical stimulation of glial cells can result in the release of neurotransmitters [22, 11], the release of ATP by the neurohypophysial astrocytes might also be investigated in the future. Thus, further studies are needed to confirm this and identify the source(s) and the relative contributions of neurones and glia to the release of ATP from the neural lobe. Such a study should also determine whether the release of ATP is affected differently by physiological stimuli regulating the release of AVP and OXT, such as dehydration, lactation, osmotic regulation or stress.

Although the exact source of ATP remains to be determined, the evidence showing the presence of ATP in the extracellular space of the neurohypophysis prompted us to determine whether nerve terminal activity is regulated by this nucleotide.

Regulation of neurohypophysis nerve terminal activity by ATP

The regulation of secretion in the neurohypophysis can be studied at the nerve terminals level by means of isolated neurohypophysial terminals, which consist almost exclusively in oxytocin- and vasopressin-releasing endings [14]. Applying ATP (1-100 μ M) to such isolated nerve terminals causes a sustained increase in their calcium content, as measured by fura-2 imaging (19 and *Figure 1B*). This response occurs in only about 40% of the neurohypophysial terminals. Since the calcium content of ATP-insensitive terminals increases in response to HK⁺ depolarization, the lack of response by a subpopulation of nerve terminals is unlikely to result

from nerve damage, but suggests that there are two populations of terminals with different sensitivities to extracellular ATP. We next examined the question of whether the ATP-induced increase in calcium in nerve terminals is sufficient to trigger the release of hormones. To address this point, we measured the AVP and OXT release in response to ATP by specific radioimmunoassay. Interestingly, a similar concentration of ATP stimulates basal AVP release (*Figure 1C*), and potentiates high K^+ -evoked AVP release, but it has only a slight effect on OXT secretion [19]. Both the increase in calcium and AVP release are strongly and reversibly inhibited by suramin, a P2 purinergic receptor antagonist. The dependence on extracellular calcium and pharmacological studies indicate that the ATP causes the increase in intracellular calcium by stimulating the entry of calcium through an ionotropic $P2X_2$ channel receptor. A report by Loesch *et al* supports the physiological relevance of our observations [23]. Their *in situ* studies demonstrated the $P2X_2$ receptor expression in the hypothalamo-neurohypophysial system. Interestingly, the $P2X_2$ receptor was found only on a subpopulation of neurosecretory axons [23], in agreement with our calcium imaging studies showing that only 40% of isolated nerve terminals responded to ATP. Finally, as ATP has no effect on OXT release, we assume that only vasopressinergic neurosecretory terminals bear the $P2X_2$ receptor. The same group [24] found the expression of the $P2X_6$ ionotropic receptor on a subpopulation of neurosecretory axons. Surprisingly, the $P2X_6$ receptor appeared to be associated with the membrane of some neurosecretory granules and microvesicles within the positive terminals. The exact role, if any, of the $P2X_6$ receptor in the regulation of neurohypophysial secretion remains to be elucidated. Regarding the granular localization of $P2X_6$ receptor, the authors speculate beyond an implication in hormone release, in relationship with the intragranular ATP pool. On the basis of the data briefly summarized here, it seems correct to infer that endogenous release of ATP during secretory phase in the neurohypophysis might potentiate AVP release via autocrine positive feedback loops. The released ATP could also elicit AVP secretion from nearby terminals that are electrically inactive by recruiting them to the response. Besides its action on ionotropic P2X receptor, ATP also regulates hormone release by modulating the permeability of ionic channels located on the terminal plasma membrane. Wang and Lemos used conventional outside-out patches from isolated neurohypophysial nerve terminals to show the dose dependent inhibition of the delayed, outward type II Kca current by ATP ($IC_{50} = 50\mu M$) [25]. This effect of ATP is most likely due to the inhibition of the Kca channels either directly or via activation of an unidentified P2 purinergic receptor [25]. Functionally, the ATP released during the depolarization of neurohypophysial terminals might locally and transiently block the Kca channel, resulting in prolonged action potentials and, therefore, increased hormone release.

Altogether these data indicate that ATP emerges as a stimulator of neurohypophysial secretion, acting directly on the neurohypophysial nerve terminals (*Figure 3A*).

Action of ATP on neurohypophysial astrocytes (pituicytes)

In addition to its action of neurohypophysial nerve terminals, we have considered a possible effect of ATP on neurohypophysial astrocytes. The vast majority of glial cells in the neurohypophysis are specialized astrocytes referred as pituicytes [26, 27]. Pituicytes are intimately associated with neurosecretory terminals and therefore perfectly located to receive any released compounds. The presence of synaptoid contacts on these astroglia [28] and their remarkable morphological plasticity during periods of intense neurohormone demand, such as dehydration, parturition and lactation, suggest that these cells are involved in the modulation of neurosecretion [13]. Hence, ATP may well act on pituicytes. Primary cultures of pituicytes can be obtained from adult rat neurohypophysial explants [29]. Cells migrate out of the explant to produce a monolayer of flattened cells that react positively to anti-GFAP antibodies [29]. The presence of GFAP on cultured cells is a good evidence to identify these cells as pituicytes, since the expression of GFAP in pituicytes has been abundantly described *in situ* [26, 27]. In most pituicytes in primary culture ATP triggers a fast, transient increase in intracellular calcium (*Figure 2A* and 30, 31). This increase in calcium is dose-dependent (ATP: 2.5-50 μM , EC_{50} : 4.8 μM) and involves the discharge of internal calcium stores. Pharmacological studies, particularly the inhibition of ATP action by the specific antagonist of the P2Y receptor, Reactive Blue-2 (RB-2) [32], strongly indicate that the purinergic receptor involved in ATP induced calcium increase belongs to the G-protein-linked P2Y receptor family [30]. Unfortunately, *in situ* studies have not looked for the P2Y receptor on pituicytes. Loesch *et al* [23, 24] report the expression of two ionotropic purinergic receptor ($P2X_2$, $P2X_6$) on a subpopulation of pituicytes, but their involvement in the ATP induced calcium increase remains to be determined. The physiological significances of these P2Y receptors and the increase in calcium resulting from their activation are essentially unknown. Nevertheless, we have shown that exogenous ATP modulates permeability of pituicytes to ions, especially K^+ . ATP stimulates the efflux of potassium from pituicytes by a mechanism that requires P2Y activation and an increase in intracellular calcium (*Figure 2B* and 33). Several Ca^{2+} -activated potassium channels subtypes (SK, IK and BK) are involved in the K^+ efflux that occurs in response to ATP. These experiments were performed in conditions where extracellular ionic concentrations mimicked those of extracellular fluid ($[K^+] \sim 4$ mM). Hence, ATP may contribute to the rise in extracellular K^+ that occurs following stimulation of the neurohypophysis *in vivo* [34, 35] by stimulating the efflux

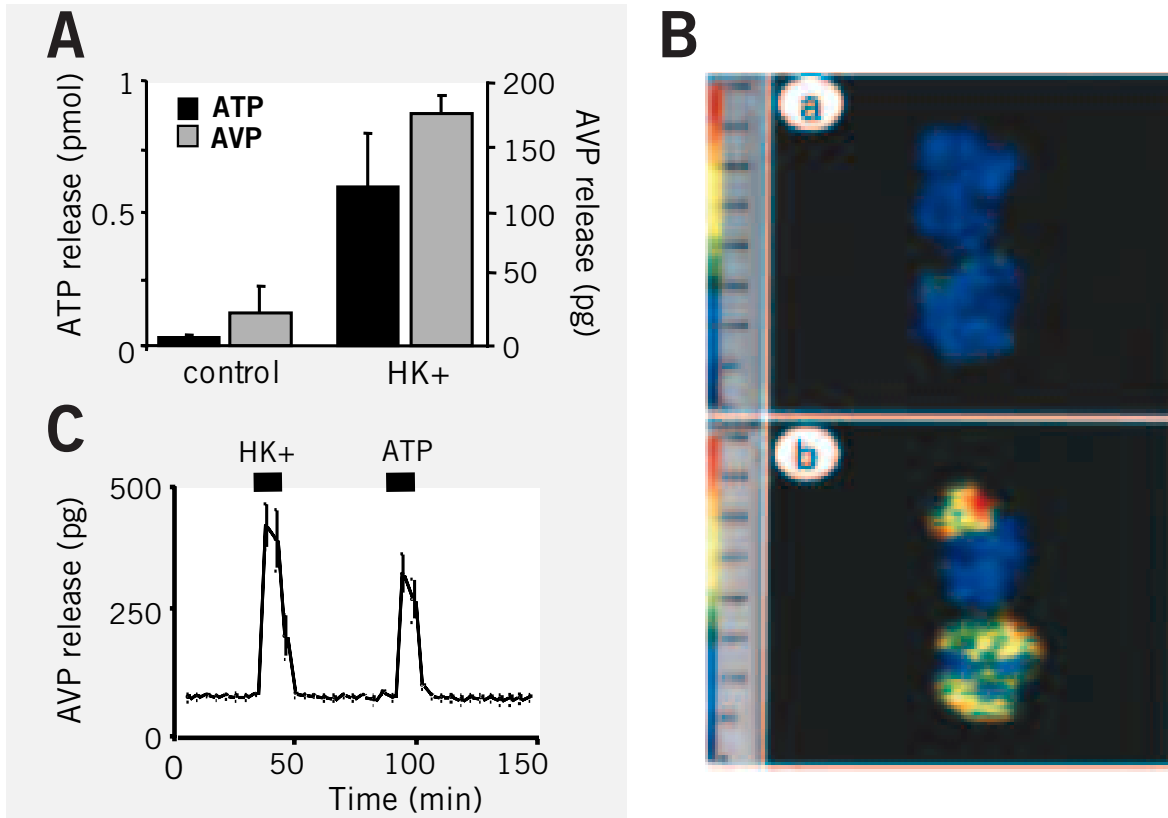


Figure 1: Action of released ATP on neurohypophysial nerve terminals

A. Depolarization by high K⁺ of isolated neurohypophysis, causing the release of ATP and neurohypophysial hormones. **B.** Calcium imaging of isolated nerve terminals obtained under resting conditions (a) and after perfusion with 100 μM ATP (b). Note that 3 of the 4 terminals increased their calcium content in response to ATP. **C.** Time course of AVP release from isolated nerve terminals exposed to a depolarizing stimulus (50 mM K⁺) and then to ATP (100 μM).

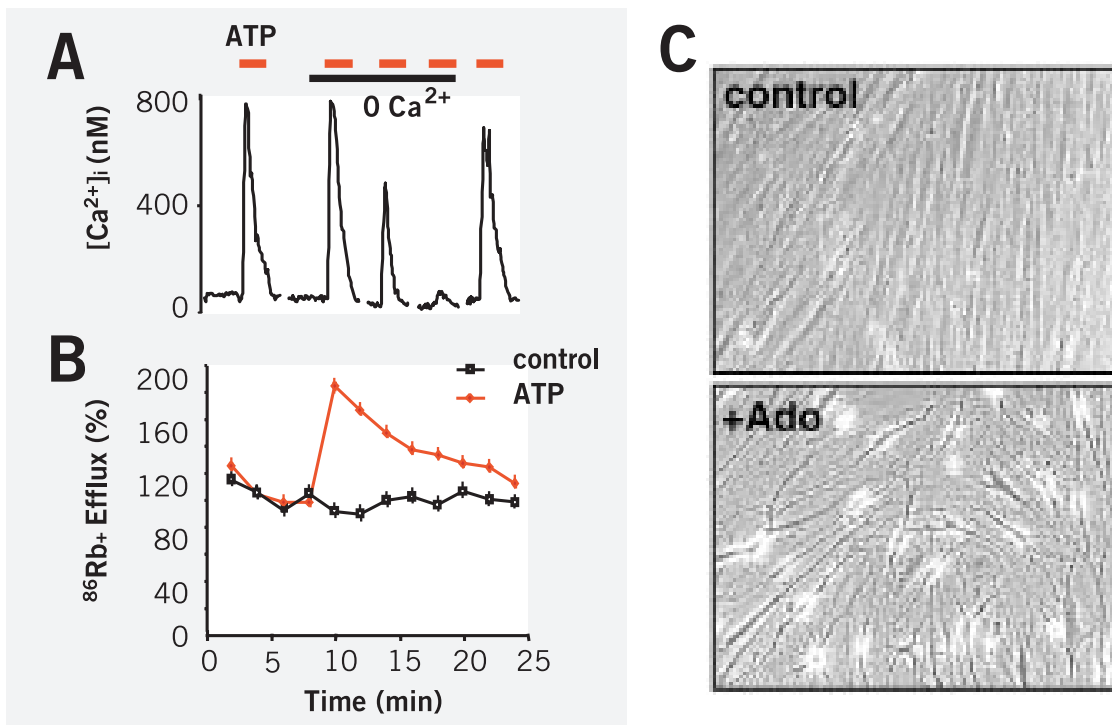
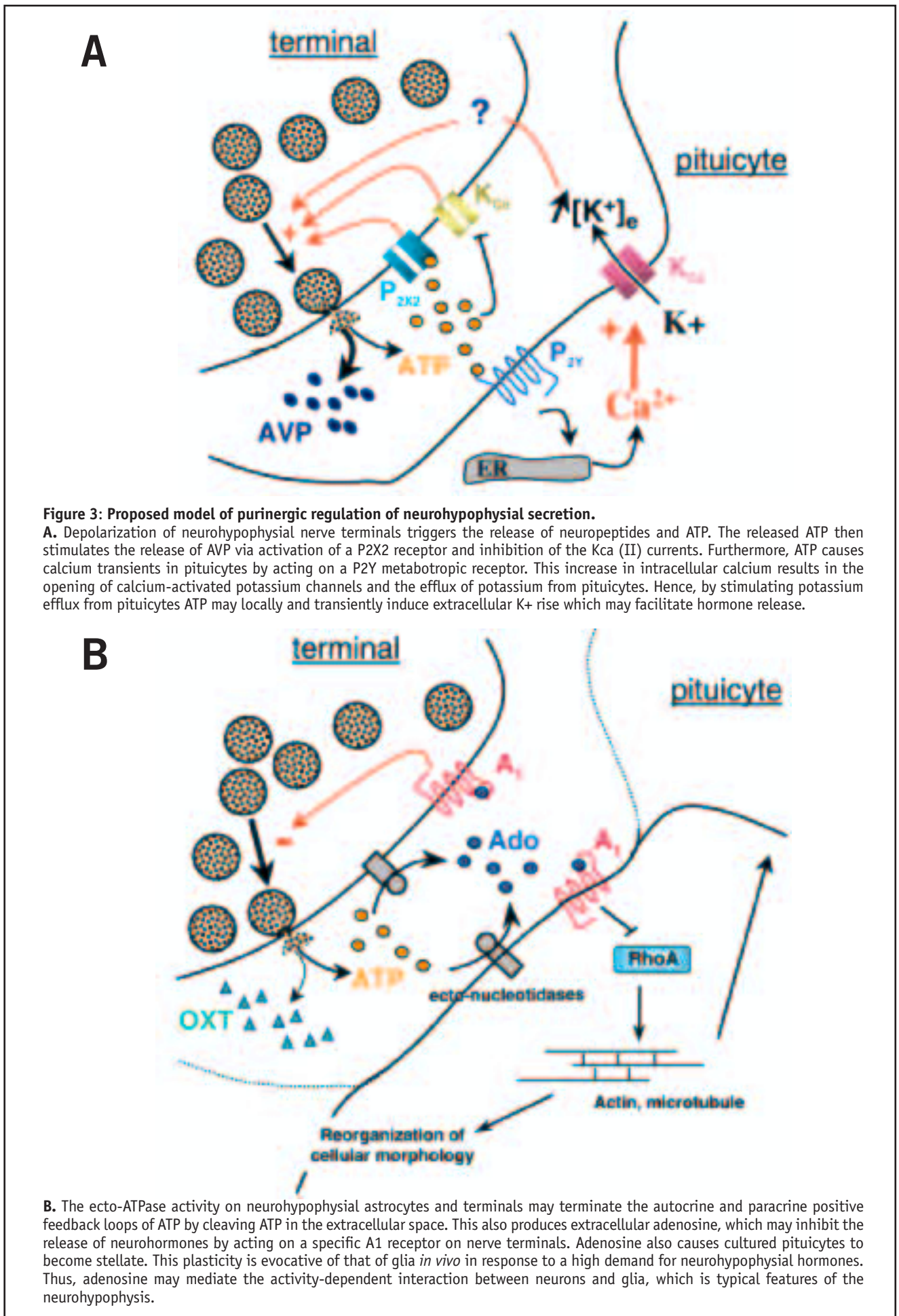


Figure 2: Effects of purines on neurohypophysial astrocytes

A. Change in intracellular calcium in response to 100 μM ATP – recorded in Fura-2 loaded pituicytes. ATP was applied in the presence and absence of extracellular calcium. Each exposure to ATP was separated by a ~20-min resting period. **B.** Time course of ⁸⁶Rb⁺ efflux from cultured adult pituicytes in response to ATP (100 μM). **C.** Effect of extracellular purines on pituicyte morphology. Micrographs of cultured pituicytes (fixed field) exposed to 10 μM adenosine.



of potassium from pituicytes, and, thus, participate in the control of secretion and facilitate hormone release. Indeed, the composition of the extracellular fluid has a direct influence on the cell membrane potential and can modulate its spontaneous firing frequency. Membrane depolarization resulting from increased extracellular K^+ ion concentration may bring it closer to its threshold, increasing the spontaneous firing of neurons and in turn increasing the probability that spikes cause the increase in calcium required for peptide release.

Termination of ATP positive feedback by ecto-nucleotidases

Numerous lines of evidence suggest that inactivation of nucleotides implies surface-located enzyme, i.e. ecto-nucleotidases [36], that can hydrolyze nucleotides in the external compartment. The final hydrolysis product of nucleotides is adenosine, which can act on specific receptors or replenish intracellular purine stores. Our cytochemical studies on ATPase activity in the neurohypophysis showed an ecto-ATPase activity on the external side of the plasma membrane of pituicytes and most neurosecretory terminals [37]. Furthermore, exogenous ATP, added to isolated neurohypophysis, is readily hydrolyzed to ADP and AMP, demonstrating that these ecto-nucleotidases are functional [18]. This ecto-ATPase activity could terminate the autocrine and paracrine positive feedback loops of ATP by cleaving the ATP in the extracellular space. Indeed, ADP is found less active than ATP in inducing calcium increase in neurohypophysial terminals and pituicytes, while AMP is totally ineffective [19, 30 and 31]. Sperl agh *et al* [18] also report the expression of an ecto5'-nucleotidase that is responsible for the hydrolysis of AMP to adenosine. They detected adenosine formation when AMP was added to isolated neurohypophysis. Hence, adenosine is the end product of ATP inactivation in the neurohypophysis. Since adenosine is a well-known neuromodulator by acting on specific P1 receptors [9], this result raises questions about the capacity of adenosine to act as a regulator in the neural lobe of the pituitary gland.

A role for adenosine in the regulation of neurohypophysial hormones release ?

The glial cells in the hypothalamus-neurohypophysis system show remarkable morphological plasticity during the output of large amounts of AVP or OXT [13]. For instance, the number of terminals surrounded or engulfed by pituicyte processes decreases during dehydration, promoting neuron-neuron interactions [38]. Pituicytes also retract from the perivascular spaces, facilitating access of the secreting terminals to the basal lamina and release of neurohormones into the blood [39, 40]. However, little is known about the mediators of this plasticity. Explant cultures are useful for studies designed to identify these mediators. The pituicytes can switch from a flat shape to a rounded, stellate morphology in response to appropriate stimuli, in a man-

ner evocative of that occurring *in vivo* during stimulation of the hypothalamus-neurohypophysis axis. Hatton *et al* [41] analyzed the shape changes of such a system to exogenous noradrenaline and suggested that β_2 -adrenergic receptors were involved. The evidence for the activity dependent release of ATP in the neurohypophysis strongly suggests that this nucleotide is involved in the activity dependent plasticity of pituicytes. We recently showed that purines influence the plasticity of pituicytes [42]. The pituicyte cell morphology was dramatically altered (from a flat shape to stellate morphology) by ATP. However the action of ATP requires its hydrolysis by ecto-nucleotidases and the formation of adenosine. Adenosine causes 60% of pituicytes *in vitro* to become stellate by a mechanism that requires activation of the A1 receptor, depolymerization of F-actin and microtubule reorganization. Adenosine acts rapidly (~ 30 min), and its effects can be reversed by removing the adenosine, which seems to involve the downregulation of activated RhoA (*Figure 2C*). We propose that purines act via adenosine to influence the activity-dependent plasticity of glia in the neurohypophysis and thus modulate neurohormone release (*Figure 3B*).

As observed for ATP, the action of adenosine in neurohypophysis seems not restricted on astroglial compartment of this organ. Indeed, experiments on isolated neurohypophysial terminals have shown that adenosine acts via A1 receptors to inhibit the terminal N-type Ca^{2+} channel in a dose-dependent and reversible manner [43 and our unpublished data]. This inhibition of calcium current causes a marked reduction in high K^+ depolarization triggered release of AVP or OT [43]. The two conflicting actions of ATP and adenosine on the excitability of neurohypophysial nerve terminals suggest that purines can help to explain how the release of AVP and OXT are differently facilitated in response to physiological stimuli *in vivo*.

Concluding remarks

The findings described above indicate that extracellular nucleotides and nucleosides are part of a complex system for regulating secretion and neurone-glia interplay in the neurohypophysis (*Figure 3*). An initial stimulus, the firing of hypothalamic neurons, which triggers primary hormone release from the neurohypophysis may be modulated by purines released within the neurohypophysis. But many questions remain unanswered. For instance, we need to identify all the purinergic receptors expressed in the neurohypophysis, together with the signaling pathways activated downstream of these receptors. We also need to know whether the expression of purinergic receptors is regulated during physiological stimuli that increase hormone release, i.e. dehydration and lactation, so as to assess the physiological relevance of purinergic regulation. Ecto-nucleotidases appear crucial for the balance between ATP versus adenosine action, their expression during different physiological conditions should be also carefully studied. Even though our understanding of

how neurohypophysial secretion is regulated by extracellular purines is still in its infancy, the neurohypophysis clearly emerges as a useful model for studying the action of purines on neurone-glia conversation and control of secretion.

Acknowledgements

We are most grateful to Dr. Dayanithi, Dr. Poujeol and Pr. Lemos for helpful and stimulating discussions. We also thank Mr. Julien Ducreux for constant support.

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