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Modulation/physiology of calcium channel sub-types in neurosecretory terminals

José R. Lemos^{a,*}, Sonia I. Ortiz-Miranda^a, Adolfo E. Cuadra^a, Cristina Velázquez-Marrero^a, Edward E. Custer^a, Taimur Dad^a, and Govindan Dayanithi^{b,c,d}

^aDepartment of Microbiology and Physiological Systems & Program in Neuroscience, University of Massachusetts Medical School, Worcester, MA 01655, USA

^bDepartment of Molecular Signaling, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, EU Centre of Excellence, Videnska 1083, 142 20 Prague 4, Czech Republic

^cInstitut des Sciences Biologiques, Centre National de la Recherche Scientifique, F-75794 Paris Cedex 16, France

^dInstitut National de la Santé et de la Recherche Médicale, Unité de recherche U710, Université Montpellier 2, F-34095 Montpellier Cedex 05, France

Abstract

The hypothalamic-neurohypophysial system (HNS) controls diuresis and parturition through the release of arginine-vasopressin (AVP) and oxytocin (OT). These neuropeptides are chiefly synthesized in hypothalamic magnocellular somata in the supraoptic and paraventricular nuclei and are released into the blood stream from terminals in the neurohypophysis. These HNS neurons develop specific electrical activity (bursts) in response to various physiological stimuli. The release of AVP and OT at the level of neurohypophysis is directly linked not only to their different burst patterns, but is also regulated by the activity of a number of voltage-dependent channels present in the HNS nerve terminals and by feedback modulators. We found that there is a different complement of voltage-gated Ca^{2+} channels (VGCC) in the two types of HNS terminals: L, N, and Q in vasopressinergic terminals vs. L, N, and R in oxytocinergic terminals. These channels, however, do not have sufficiently distinct properties to explain the differences in release efficacy of the specific burst patterns. However, feedback by both opioids and ATP specifically modulate different types of VGCC and hence the amount of AVP and/or OT being released. Opioid receptors have been identified in both AVP and OT terminals. In OT terminals, μ -receptor agonists inhibit all VGCC (particularly R-type), whereas, they induce a limited block of L-, and P/Q-type channels, coupled to an unusual potentiation of the N-type Ca^{2+} current in the AVP terminals. In contrast, the N-type Ca^{2+} current can be inhibited by adenosine *via* A_1 receptors leading to the decreased release of both AVP and OT. Furthermore, ATP evokes an inactivating $\text{Ca}^{2+}/\text{Na}^{+}$ -current in HNS terminals able to potentiate AVP release through the activation of P2X2, P2X3, P2X4 and P2X7 receptors. In OT terminals, however, only the latter receptor type is probably present. We conclude by proposing a model that can explain how purinergic and/or opioid feedback modulation during bursts can mediate differences in the control of neurohypophysial AVP vs. OT release.

Keywords

Vasopressin; Oxytocin; Hypothalamus; Calcium channels; Peptide release; Neurotransmitters; ATP; Adenosine; Opioids

1. The hypothalamic-neurohypophysial system

Depolarization–secretion coupling is the primary mechanism [1–3] for transforming electrical activity into chemical signals [4]. It is particularly important to study this mechanism at nerve terminals, which are specifically differentiated for release, rather than just at neuronal cell bodies [5]. Our primary goal is to understand how specific patterns of electrical activity generated at somata regulate Ca^{2+} -entry and subsequent transmitter release at nerve terminals.

1.1. Bursting patterns of activity

At the neuromuscular junction level, there is no simple linear relationship between the amount of acetylcholine (ACh) released and the number of action potentials (AP) stimulating the motor ending [4]. Other systems, in particular peptide-releasing neurons, maximally release transmitter only in response to specific “bursting” patterns of activity [6–12]. In the bullfrog sympathetic ganglia bursts of APs are necessary to specifically elicit peptide (such as LHRH) release. While single APs do not release LHRH, they do release ACh [13]. Similarly, in chromaffin cells, trains of APs (bursts) are needed to elicit the “fight or flight” response, *i.e.* large amounts of norepinephrine release, while a single AP only releases ACh [14].

Much is known about the physiological bursting patterns of the HNS (reviews: [15,16]). OT neurons are characterized by a high frequency (up to 200 Hz) discharge during suckling which leads to the pulsatile release of OT into the blood and to subsequent milk-ejection [17]. 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 [7,8,18]. A “typical” AVP burst [8,19,20] has a low frequency (<5 Hz) for the first 2–3 s, then high to low frequency (60–10 Hz) for the next 20–60 s. Interestingly, interburst silent periods of greater than 21 s have been shown to be critical for maximal peptide release [7,8].

1.2. Hypothalamic-neurohypophysial system

The two neuropeptides, arginine vasopressin (AVP) and oxytocin (OT) are mainly synthesized in magnocellular neurons (MCN) located in the hypothalamus. AVP is a vasoconstrictor and an antidiuretic and, thus, is involved in fluid homeostasis. OT has recognized functions in parturition and lactation [21], and has an emerging role as a natriuretic agent [12]. Both hormones may also be central neurotransmitters and have been implicated in sexual behavior [22], stress, learning, memory processes [23,24], the development and maintenance of tolerance to ethanol [25], and also in several pathophysiological functions [24].

Neuroendocrine MCNs are located principally in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus [26–28]. Almost all the neurons within this system project to the neurohypophysis [29]. The NH nerve terminals have a mean diameter of about 2 μm , although much larger (*ca.* 5–10 μm) terminals can be observed [19].

1.3. Electrophysiology of terminals *in situ*

Direct electrophysiological measurements of individual nerve terminals have been very difficult. Even though loose patch-clamp recordings from intact, whole rat HNS have become possible [30,31], only in the large sized squid giant, rat NH, and crab sinus gland terminals has it been possible to record endogenous electrical activity *via* intracellular electrodes [5,9,11,33,34]. Optical recording techniques have yielded much data on murine NH terminals, but not at the individual terminal level [35,36]. Neither intracellular nor optical recordings have allowed specific characterization and localization of the ionic channels responsible for individual nerve terminal electrical activity in the intact HNS. Thus, these questions remain: how and where does Ca^{2+} enter each type of terminal in response to the specific patterns of electrical stimulation and by what process(es) does Ca^{2+} entry lead to the regulated exocytotic release of the different peptide transmitters *in situ*?

1.4. Isolated neurohypophysial terminals

In order to address such questions, we have developed a preparation of isolated neurohypophysial terminals [19,37–40]. Electron microscopy [29] and immunocytochemistry [19,20] have been used to characterize this preparation. The integrity and purity of the isolated NH terminal preparation is important, and in the intact NH, blood vessels, pituicytes, and other cells are distinguishable from the terminals [20]. It is certain that this preparation contains only nerve terminals with minimal contamination from the other components and numerous studies have confirmed that the physiological functioning of isolated terminals is the same as in the intact NH [29,41–44]. In addition, we can confirm by immunoblotting [42,45], or specific ELISAs [32] after patch-clamp recordings and Ca^{2+} measurements, whether the individual terminal is vasopressinergic or oxytocinergic. Recently, transgenic rats tagged by a visible fluorescent protein have been developed and successfully employed to study the physiology of AVP (enhanced green fluorescent protein), OT (enhanced cyan fluorescent protein) and OT (monomeric red fluorescent protein) in neurons and terminals [24,47–51]

1.5. Intraterminal $[\text{Ca}^{2+}]_i$

It is possible to show, using fluorescent probes, that depolarization of populations of isolated nerve terminals is associated with an increased Ca^{2+} concentration in their cytoplasm [52]. It has also become feasible to measure $[\text{Ca}^{2+}]_i$ levels in individual terminals to determine the role of Ca^{2+} in various physiological functions in rats [46,53–65], as well as in mice [60,66,67] neurohypophyses. These techniques are complementary and confirm findings made using electrophysiological characterization of Ca^{2+} effects in these terminals.

2. Ca^{2+} currents in terminals

There are two main voltage-dependent outward currents in NH terminals. One is a fast, inactivating current that can be blocked with 4-aminopyridine [68], and the other is a non-inactivating current which can be blocked with tetrandrine [69]. The pharmacology and kinetics of the initial transient outward current identify it as an A-current. The second, slower developing outward current, which is dependent upon increase in $[\text{Ca}^{2+}]_i$, shows no steady-state inactivation [42,70]. Interestingly, this Ca^{2+} -activated K^+ (BK) current can be blocked by extracellular ATP [71]. There are fast, voltage-dependent inward currents that are blocked by tetrodotoxin (TTX), as well as slow, inward currents that are not blocked by TTX but disappear in low Ca^{2+} buffer suggesting that they are, respectively, voltage-dependent Na^+ and Ca^{2+} currents [71,74,75].

2.1. Different Ca²⁺-channel subtypes

There are two kinetic components to the slower inward Ca²⁺ current, which are differentially inactivated depending upon the holding potential [71]. The two Ca²⁺ current kinetic components appear to be composed of distinct subtypes of voltage-gated Ca²⁺ channels (VGCC) in NH terminals. VGCCs, classified as L-, N-, P-, Q-, R-, and T-type based on their functional and pharmacological properties, are critical for many cellular functions including muscular contraction, neurotransmitter release, and excitability. To date, nine neuronal Ca²⁺ channel genes have been identified and termed α_{1A} through α_{1I} [76].

2.2. VGCC subtypes in AVP vs. OT terminals

Our previous findings show that there is heterogeneity of Ca²⁺ channel types in the NH terminals [71–75]. Perforated patch-clamp measurements of Ba²⁺ currents in NH terminals (see Fig. 1) classify them into two groups: one in which the GVIA- and nicardipine-resistant component is sensitive to ω -AgaIVA/SNX-230 (Fig. 1A) and one in which it is insensitive (Fig. 1B). The former channel is not found, or not functional, in about half of the terminals. Immuno-histochemistry of isolated NH terminals labeled with AVP or OT vs. P/Q subtype VGCC (Fig. 2) indicates that the P/Q channel is found only in AVP terminals. Thus, Q-type Ca²⁺ channels are preferentially located on AVP-containing NH terminals [56].

The biophysical properties of the GVIA- and nicardipine-resistant component (Fig. 1B) are that of a high-voltage activated, transient, Ca²⁺ current which shows moderate steady-state inactivation. These properties are most consistent with the “R”-type Ca²⁺-channel. At maximally effective concentrations (20 nM), SNX 482, a polypeptide toxin able to specifically block α_{1E} (R) sub-type Ca²⁺ currents expressed in HEK cells [76,78], abolished the resistant component of OT release and the resistant component of the neurohypophysial Ca²⁺ current from these terminals (see Fig. 1B). These results lead to the hypothesis that the R-type channels are preferentially localized on OT peptide-containing nerve terminals and can thus preferentially regulate the release of OT [57].

Immunohistochemistry of isolated NH terminals labeled with AVP or OT vs. the R-type channel (Figs. 2 and 3A) indicates, however, that the R-type VGCC is found in both OT- and AVP-containing terminals. The latter channel, however, might not be functional or could be a splice variant that is not blocked by SNX-482 [76,77]. This is confirmed since low doses of SNX 482, can block this resistant component of the neurohypophysial Ca²⁺ current (see Fig. 1B), but not the one in cerebellar granule cells [79].

Thus, there appear to be three main functional subtypes of Ca²⁺ channels in each kind of peptidergic terminal: L, N, and Q in vasopressinergic terminals [56] vs. L, N, and R in oxytocinergic terminals [57].

2.3. VGCC and burst patterns

For the past couple of decades we have been studying the voltage-dependent ionic currents involved in HNS release at the terminals, and thus far, their biophysical properties [56,57] alone cannot explain the differential efficacy of these bursting patterns on AVP vs. OT release [72,75]. That is, the L, N, and Q VGCC in vasopressinergic terminals do not have substantial differences in their voltage-dependence or kinetic properties vs. L, N, and R VGCC in oxytocinergic terminals (see Fig. 3B), certainly none that would explain the differences in burst pattern efficacies in terms of release [72]. An important point is that although the different burst patterns are generated in the HNS somata [6,15,17], it is at the HNS terminals that they are transduced or “interpreted” into the release of the two neuropeptides [75]. Thus the questions become: why is AVP release greatest when using long, low frequency bursts, but maximal OT release requires short, high frequency burst

stimulations [7,8]? Furthermore, any mechanism underlying these different OT vs. AVP release efficacies should have widespread importance, since bursting patterns of action potentials are necessary for the release of not only peptides but also many other neurotransmitters at central and peripheral nervous system synapses.

3. Purinergic effects

Since the biophysical properties of the distinctive terminal VGCC cannot explain the OT vs. AVP release “efficacy” of these different bursting patterns, the focus shifted to looking for other possible physiological mechanisms at the nerve terminal level that could help in explaining these differences in release. ATP is one such candidate since it is co-released from nerve terminals [90] and it has been shown to modulate the release of only AVP from HNS terminals [46].

3.1. ATP

The importance of ATP as a neurotransmitter is demonstrated by its involvement in multiple processes throughout the nervous system including modulation of long-term potentiation, pain transduction, bladder control, modulation of vascular tone, and control of the gastrointestinal system [80]. ATP receptor-mediated synaptic currents in the brain were first described almost two decades ago [81] and, later on, described in various specific regions, such as the hippocampus [82]. ATP can enhance both long-term potentiation and long-term depression, but may differentially affect plasticity depending upon which type of receptor it activates and whether that receptor is located pre- or post-synaptically [82–84]. Thus, although ATP receptor localization at CNS synapses is critical, for the most part, little is known about this.

Extracellular ATP communicates physiological signals through the activation of P2X ligand-gated cation channels (ionotropic) and P2Y G-protein-coupled (metabotropic) receptors. Of the seven P2X receptor subunits (P2X1–P2X7), evidence supports a major role for the P2X2 and P2X3 subunits, which can form homomultimeric P2X2 or P2X3, or heteromultimeric P2X2/P2X3 receptors, in mediating the primary sensory effects of ATP [85–87]. However, the relative contribution of these receptor subtypes to the afferent functions of ATP *in vivo* is poorly understood [88].

Although ATP can modulate hippocampal synaptic plasticity [82,89,90], the roles of specific P2X receptor proteins in long-term potentiation and/or long-term depression are unknown. This is because these studies relied on the use of pharmacological agents, such as P2X receptor agonists and antagonists or agents that reduce extracellular ATP levels [91]. The use of P2X2 and P2X3 receptor knockouts [88,92] has allowed the elucidation of pain mechanisms in sensory neurons. These receptor knockouts are precisely what we have recently taken advantage of to study purinergic effects (see Section 3.3). Importantly, it has been demonstrated that ATP, in the HNS, acts in a positive feedback mechanism on only AVP release [93] by activation of probably P2X2 and P2X3 receptors [73] and by inhibiting calcium-activated K⁺ channels [72].

3.2. Adenosine

Adenosine, which is a product of ATP hydrolysis, is present in the neurohypophysis due to the presence of ecto-nucleotidases (see Fig. 3B) localized to the outer surface of AVP-containing NH terminal plasma membranes [94]. This purine has been shown to interact with a family of adenosine (A1, A2a, A2b, and A3) receptors that exert effects *via* G-protein-coupled intracellular pathways [95]. Each of the known adenosine receptors [96] contains seven transmembrane domains. The A1 receptor is strongly and widely expressed in the brain, particularly in the cortex, cerebellum, thalamus and hippocampus [97].

Adenosine-mediated effects that occur *via* the A1 receptor include depression of neurotransmission, sleep induction, antinociception, ethanol-induced motor incoordination, autonomic control of cardiac function, bronchoconstriction, negative chronotropy, inotropy and dromotropy, anti- β -adrenoceptor action and renal sodium retention [96].

Adenosine receptors are major targets of caffeine, the most commonly consumed drug in the world. There is growing evidence that they could also be promising therapeutic targets in a wide range of conditions, including cerebral and cardiac ischemic diseases, sleep disorders, immune and inflammatory disorders and cancer [98]. The use of pharmacological inhibitors has suggested a key role for purinergic receptors in synaptic plasticity and possible roles in Parkinson's and Huntington's diseases; however, the known antagonists are not specific enough for individual members of the purinergic receptor family [91]. New genetic and epigenetic tools have emerged that facilitate the elucidation of the function of these receptors with greater specificity than is generally possible with traditional antagonist drugs (see below).

3.3. Purinergic effects in terminals

Neither the slowly inactivating L-type nor the transient Q- and R-type Ca^{2+} channels have been shown to be affected by purines. However, it is clear that the transient N-type Ca^{2+} current can be inhibited *via* an adenosine A1 receptor leading to the decreased release of both AVP and OT from isolated neurohypophysial terminals [59]. Thus, a specific adenosine receptor, A1 (Fig. 3B), appears to be functional in HNS terminals [59,93].

Furthermore, in HNS terminals, ATP dose-dependently evokes an inactivating, inward, $\text{Ca}^{2+}/\text{Na}^{+}$ current [99]. In contrast, in HNS somata ATP evokes a sustained inward current that displays a different dose-dependence. The differences in EC_{50} , inactivation, and desensitization of the ATP responses in terminals *vs.* somata of the HNS indicate that different P2X receptors mediate the responses in the two compartments of these CNS neurons. The HNS somata receptor(s) appears to be a P2X2, P2X4, or P2X6 [100] while the HNS terminal receptor(s) appears to be a mixture of P2X2, P2X3, P2X4, and P2X7 receptors localized on AVP terminals [32,99] and probably only P2X7 is on OT terminals ([101]; see Fig. 3B). Of interest, in nerve terminals, ATP is also able to induce actin disaggregation by a Ca^{2+} dependent mechanism [102].

We have recently confirmed this receptor distribution by using specific P2X receptor knockout (rKO) mice to determine the P2X receptor subtype responsible for endogenous ATP induced potentiation of electrically stimulated neuropeptide release [103]. Treatment of WT mouse NH with suramin/PPADS significantly reduced electrically stimulated AVP release. A similar inhibition by suramin was observed in electrically stimulated NH from P2X3 and P2X7 rKO mice but not from P2X2/P2X3 rKO mice, indicating that the endogenous ATP facilitation of electrically stimulated AVP release is mediated primarily by the activation of the P2X2 receptor.

We thus speculate that this difference in purinergic receptors reflects the specific functions of co-released ATP in these two compartments of HNS neurons and in bursting efficacy [104]. We propose a model that can explain how the autocrine effects on VGCCs can directly mediate differences in the control of neurohypophysial OT and/or AVP release (see Section 5).

4. Opioid effects

Opioids are thought to primarily affect the brain by influencing the amount of neurotransmitter present at synapses through interactions with specific μ (μ), κ (κ) or

delta (δ) receptors [105]. Whether opiates functionally reduce or increase the effective amount of transmitter release directly (presynaptically) and/or indirectly (postsynaptically) is still an active area of research, however.

The effects of opioids on ionic currents have been investigated using the perforated 'whole-cell' patch-clamp technique on isolated terminals of the HNS ([61], see [106] for review). κ -[107] and μ -opioid receptors (see, *e.g.*, Fig. 3A) have been identified in the somata and nerve endings of the HNS using pharmacological [65], autoradiographic [105], and immunohistochemical [106] techniques.

Locally released agonists (*e.g.*, Dynorphin-A [108] and Met-enkephalin [109], are thought to activate opioid receptors in OT- and AVP-containing NH terminals to regulate the influx of Ca^{2+} during depolarizing stimuli. The net Ca^{2+} influx is determined by the sum of all types of voltage-gated Ca^{2+} channels mediating the stimulated release of each neuropeptide [106]. The sensitivity of OT to the μ -receptor agonist DAMGO comes as a result of the general inhibition of all channels (R-, N-, and L-type), but in particular the R-type [110] controlling its release. In an analogous way, the reduced sensitivity of AVP containing terminals to low concentrations of a μ -agonist, could also be explained by a limited block of L-, and P/Q-type channels, coupled to an unusual potentiation of the N-type calcium current [111]. Therefore, the relative sensitivity to μ -opioid inhibition of OT release, particularly at lower agonist concentrations, is due to a net inhibitory action on all voltage-gated channels mediating Ca^{2+} influx into an OT terminal. In contrast, an AVP terminal will respond with little or no change in release to lower concentrations of a μ -opioid agonist, as some of the voltage-gated channels controlling Ca^{2+} influx are inhibited and others are potentiated. Kappa-opioid effects are seen on all channels in both types of terminals, however. Furthermore, in contrast to μ -opioids, κ -opioid effects are *via* a membrane-delimited pathway [112].

5. Conclusions

Feedback effects by *endogenous* opioids/purines on their receptors could thus explain the differential facilitation by bursting patterns of action potentials on neuropeptide release from HNS terminals. We have proposed (see Fig. 4) that endogenously co-released opioids, 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 opioid/purinergeric feedback mechanisms could explain the efficacy of such patterns in facilitating AVP *vs.* OT release.

We have proposed and have evidence for the following series of events: during the initial period (20–50 Hz frequency [17]) of a physiological AVP-like burst (Fig. 4A,) ATP that is co-released with the neuropeptides inhibits BK [72] and opens P2X2 and P2X3 receptor channels [99]; these cation ($\text{Ca}^{2+}/\text{Na}^{+}$) channels would further depolarize the membrane potential and, in parallel, increase intraterminal $[\text{Ca}^{2+}]$; [46]. Because these receptors are exclusively on AVP terminals [101], this would thus potentiate subsequent AVP release, in particular [46]. Then, ecto-nucleotidases found only on the AVP terminals [94], hydrolyze the extracellular ATP to adenosine, which acts on A1 receptors on both types of terminals, activating an unidentified G-protein complex that specifically inhibits the N-type calcium channel [59]. Since this channel is found on both types of HNS terminals [56,57], this would inhibit any subsequent physiologically stimulated release of both neuropeptides.

Accumulation of endogenously released Dynorphin A [107] would also directly inhibit, *via* κ -opioid receptors (KOR), VGCC and the subsequent release of AVP in the later portion of such a burst of action potentials. Since both adenosine and Dynorphin A act *via* voltage-

regulated membrane-delimited pathways [112], neither would be active during the higher frequencies at the beginning of bursts.

In contrast, during a physiological OT-like burst (50–200 Hz frequency [113]) co-released ATP probably does not have a positive feedback effect on OT release since those terminals only express the less-sensitive P2X7 receptor (Fig. 3B). Furthermore, since there are no ecto-nucleotidases on OT terminals [94], no effects by adenosine on release of OT are expected. Accumulation of endogenously released Met-enkephalin [109] would indirectly inhibit, *via* the μ -opioid receptor (MOR), the VGCC and the subsequent release of OT during such a burst of action potentials [106]. In both cases, interburst silent periods are necessary for the clearance of these accumulated opioids and purines, so that the next burst can facilitate neuropeptide release again.

In general, targeting specific channels, such as those with Ca^{2+} permeability (*e.g.*, VGCC and P2XR) that directly regulate synaptic release, appears to be a very efficient way to achieve control of different transmitters.

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Abbreviations

HNS	hypothalamic-neurohypophyseal system
ATP	adenosine tri-phosphate

AVP	arginine-vasopressin
OT	oxytocin
VGCC	voltage-gated Ca^{+2} channels
MCN	magnocellular neurons
AP	action potentials
SON	supraoptic nuclei
PVN	paraventricular nuclei
BK	calcium-activated K^{+}
TTX	tetrodotoxin
CNS	central nervous system
GVIA	ω -conotoxin GVIA
ω-AgaIVA	ω -agatoxin IVA
DAMGO	D-Ala ² , MePhe ⁴ , Glyol ^[5] enkephalin
rKO	receptor knockout, μOR , κOR

Fig. 1.

Ca^{2+} -currents in isolated NH terminals. Perforated patch-clamp measurements of Ba^{2+} currents in rat isolated neurohypophysial nerve terminals. The terminals appear to fall into two groups: one in which the nicardipine-(red) and GVIA-(green) resistant component is sensitive to ω -AgaIVA/SNX-230 (gray) (A) and one in which it is sensitive to SNX482 (gray) (B). The biophysical properties of the resistant component in group B are that of a high-voltage (-4.2 mV) activated, transient ($\tau_i = 25.6$ ms), Ca^{2+} current which shows moderate steady-state inactivation ($V_{1/2} = -58.8$ mV). Both of these terminals were tested for peptide content [32] and were identified as AVP^{+ve} (A) and OT^{+ve} (B). Modified from [107] and [106].

Fig. 2.

P/Q type and R-type VGCC co-localized with AVP and OT terminals, respectively. (A) R (Cav2.3) and (B) P/Q (Cav2.1) VGCC-dependent immunoreactivity in rat isolated NH terminals. Isolated NH terminals were fixed and exposed simultaneously to primary antibodies against neurophysin I (OT; green; P38, a generous gift from H. Gainer), neurophysin II (AVP; blue: V15 from SCB) and either an anti-Cav2.1 or anti-Cav2.3 (red: Alomone ACC-001 and ACC-006, respectively). IgGs conjugated to Alexa-Fluor probes were used as secondaries. Images were obtained and deconvoluted using Axiovert 4.0 (V. 4.6.3.0) software. Bright field images (DIC) of each individual terminal are shown on left, top corners. Scale bars = 2 μ m.

Fig. 3.

(A) Co-localization of μ -opioid receptor, R-type VGCC, and OT in the same NH terminal. An isolated terminal was fixed and labeled with primary antibodies directed against μ -opioid receptor (green), R-type Ca^{2+} -channel (orange) and OT (red). Secondary antibodies, labeled with different wavelength Alexas, were raised in donkeys against the different species-specific primary antibodies (goat, rabbit, and mouse). The controls for this primary Abs were IgG2b (100 $\mu\text{g}/\text{ml}$) for OT monoclonal, IgG normal goat (500 $\mu\text{g}/\text{ml}$) for μ -OR, IgG normal rabbit (400 $\mu\text{g}/\text{ml}$) for R-type channel. This representative NH terminal was 3D imaged using a digital imaging microscope together with deconvolution. Terminal diameter = 5 μm . (B) Model of the established exogenous and the proposed endogenous purinergic effects on neurohypophysial terminals. Different physiological burst patterns regulate OT (high frequency) vs. AVP (low frequency) release. The biophysical properties of the VGCC (N, L, R on OT and N, L, Q on AVP terminals), alone, however, cannot explain the differential effects of such bursts. Thus, we propose that endogenous co-released ATP activates P2X2, P2X3, P2X4, and P2X7 receptors localized on AVP terminals, while activating only P2X7 receptors on OT terminals. The flux of Ca^{2+} through these receptors increases $[\text{Ca}^{2+}]_i$ and, thus, neuropeptide release. The ATP is then broken down to adenosine by ecto-nucleotidases, which are present only on AVP terminals. Adenosine, which acts on A1 receptors, present on both terminal types, directly inhibits N-type Ca^{2+} -channels and subsequent neuropeptide release.

Fig. 4.

Summary Burst models. Feedback effects by *endogenous* opioids and/or purines on their receptors can explain the differential facilitation by bursting patterns of action potentials on neuropeptide release from HNS terminals. (a) AVP Burst Model Table describes the modulators' (*Modul.*) effects, at different [concentrations], at the *beginning* (20–50 Hz frequency [17]), during the *change* (10–20 Hz), at the *End* (1–10 Hz) of a long (30 s) burst, and during *interval* (0 Hz) between bursts. Maximum effect (↑ or ↓) on *release* and by what type of *effect* are given. (i) During the initial period of physiological AVP-like burst co-released ATP has a positive feedback effect *via* P2X2 and/or P2X3 receptors only on AVP release. (ii) Hydrolysis of ATP to adenosine by ecto-nucleotidases, found exclusively on AVP terminals, inhibits, *via* the A1 receptor acting directly on N-type VGCC, the release of both neuropeptides in the later portion of a burst of action potentials. (iii) Accumulation of endogenously released Dynorphin A also directly inhibits, *via* the κ-opioid receptor (KOR), VGCC and the subsequent release of AVP in the later portion of such a burst of action potentials. (iv) Since both adenosine and Dynorphin A act *via* a voltage-regulated membrane-delimited pathway [112], both would be inactive or “knocked off” (“k.o.”) during the higher frequencies at the beginning of bursts. (v) Interburst silent periods are necessary for the clearance of both the accumulated purines and opioids. Relative [amounts] of AVP released and physiological effects are below line. (b) OT Burst Model Table describes the modulators' (*Modul.*) effects, at different concentrations, at the *beginning* (50–100 Hz frequency [113]), during the *change* at the end of the short (5 s) high frequency burst, and during *interval* (0 Hz) between bursts. Maximum effect (↑ or ↓) on *release* and by what type

of *effect* is given. (i) In contrast to AVP bursts, during a physiological OT-like burst, co-released ATP probably does not have a positive feedback effect on OT release since those terminals only express the less-sensitive P2X7 receptor (Fig. 3B). (ii) Furthermore, since there are no ecto-nucleotidases on OT terminals [94], no effects by adenosine are expected. Any paracrine adenosine effects would be “k.o.” (iii) Accumulation of endogenously released Met-enkephalin would indirectly inhibit, *via* μ -opioid receptors (MOR), VGCC and the subsequent release of OT during such a burst of action potentials [106]. (iv) Interburst silent periods are necessary for the clearance of both the accumulated purines and opioids. Relative [amounts] of OT released and physiological effect are below line.