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THE PURINERGIC NEUROTRANSMITTER REVISITED: A SINGLE SUBSTANCE OR MULTIPLE PLAYERS?

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Abstract

The past half century has witnessed tremendous advances in our understanding of extracellular purinergic signaling pathways. Purinergic neurotransmission, in particular, has emerged as a key contributor in the efficient control mechanisms in the nervous system. The identity of the purine neurotransmitter, however, remains controversial. Identifying it is difficult because purines are present in all cell types, have a large variety of cell sources, and are released via numerous pathways. Moreover, studies on purinergic neurotransmission have relied heavily on indirect measurements of integrated postjunctional responses that do not provide direct information for neurotransmitter identity. This paper discusses experimental support for adenosine 5'-triphosphate (ATP) as a neurotransmitter and recent evidence for possible contribution of other purines, in addition to or instead of ATP, in chemical neurotransmission in the peripheral, enteric and central nervous systems. Sites of release and action of purines in model systems such as vas deferens, blood vessels, urinary bladder and chromaffin cells are discussed. This is preceded by a brief discussion of studies demonstrating storage of purines in synaptic vesicles. We examine recent evidence for cell type targets (e.g., smooth muscle cells, interstitial cells, neurons and glia) for purine neurotransmitters in different systems. This is followed by brief discussion of mechanisms of terminating the action of purine neurotransmitters, including extracellular nucleotide hydrolysis and possible salvage and reuptake in the cell. The significance of direct neurotransmitter release measurements is highlighted. Possibilities for involvement of multiple purines (e.g., ATP, ADP, NAD⁺, ADP-ribose, adenosine, and diadenosine polyphosphates) in neurotransmission are considered throughout.

Keywords

ATP; NAD; ADP-ribose; Adenosine; Purinergic neurotransmission; Nervous system

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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1. Introduction

Rapid information processing in our nervous system typically relies on the release of chemical substances called neurotransmitters, at cell-to-cell contacts called chemical synapses or neuroeffector junctions (Katz, 1969). In fact, chemical neurotransmission is the primary way signals are transmitted from neuron to several classes of effector cells, including other neurons, smooth muscle cells, interstitial cells and glia. Since the seminal discoveries that introduced acetylcholine (ACh) and norepinephrine (NE) as neurotransmitters (reviewed in Lopez-Munoz & Alamo, 2009), it became increasingly clear that the neurochemical transmission is served by numerous neurotransmitter substances in addition to ACh and NE (Hokfelt et al., 1984; Furness et al., 1989; Lundberg, 1996; Carlsson, 2001). These include biogenic monoamines (e.g., dopamine, histamine and 5-hydroxytryptamine), amino acids (e.g., glutamate, glycine and γ -aminobutyric acid), neuropeptides (e.g., neuropeptide Y, vasoactive intestinal polypeptide and calcitonin gene-related protein), adenine purines [e.g., adenosine 5'-triphosphate (ATP), diadenosine polyphosphates ($A_{p_n}A$ adenosine), β -nicotinamide adenine dinucleotide (NAD^+), and ADP-ribose (ADPR)], and gasotransmitters (e.g., nitric oxide (NO), hydrogen sulfide, and carbon monoxide). It also became clear that one nerve can release multiple neurotransmitters and that different neurotransmitter substances can achieve seemingly similar end effects, causing excitation or inhibition of effector cells. The terms 'plurichemical neurotransmission' (Furness et al., 1989) and 'cotransmission' (e.g., Rossier et al., 1983; Scuvée-Moreau, 1983) were originally utilized to reflect co-presence and co-release of peptides with classical transmitters, and were adopted in discussions of co-release of NE or ACh with ATP in the autonomic nervous system (Burnstock & Sneddon, 1985; Burnstock, 1985; Burnstock, 1990; Lundberg, 1996).

About two decades before the purinergic neurotransmission hypothesis was advanced (Burnstock, 1972), there were indications that adenine compounds may participate in neuronal functions. For example, in the 1950s ATP was found to be present in extract of dorsal roots (Holton & Holton, 1953), the adrenal medulla (Blaschko et al., 1956) and sympathetic nerves (Schumann, 1958), and to possibly serve sensory neurotransmission in the rabbit ear artery (Holton & Holton, 1953; Holton, 1959). The report by Holton, 1959 provided evidence using the firefly luminescence assay that ATP was released during antidromic stimulation of sensory nerves supplying the rabbit ear artery. This was perhaps the first report to demonstrate more directly a possible neural source of ATP as suggested by significantly reduced ATP release after mechanical denervation. In the following years a series of studies suggested the possible role of purines in neurotransmission. These include the initial observations in the 1960s of the existence of non-cholinergic, non-adrenergic and non-adrenergic/non-cholinergic (NANC) inhibitory junction potentials (IJPs) in the guinea-pig taenia coli (Burnstock et al., 1963; Burnstock et al., 1964; Bennett et al., 1966; Burnstock et al., 1966) that later led to introduction of the purinergic neurotransmission hypothesis (Burnstock et al., 1970; Burnstock, 1972). Detailed personal accounts of these early observations can be found in a large number of reviews and commentaries (for examples see Burnstock, 1981; Bennett, 1997; Satchell, 2000; Burnstock, 2006; Burnstock, 2014; Bennett, 2013a; Burnstock, 2013b; Burnstock, 2013c; Bennett, 2013b). The

observations in the guinea-pig taenia coli were followed by studies demonstrating purinergic neurotransmission in rodent vas deferens, which soon became a convenient model tissue for investigating adrenergic and purinergic cotransmission (Westfall et al., 1978; Fedan et al., 1981; Sneddon & Burnstock, 1984b; Sneddon & Westfall, 1984; Todorov et al., 1996; Westfall et al., 1996; Westfall & Westfall, 2001), in blood vessels (Su, 1978; Su, 1983; Sneddon & Burnstock, 1984a; Astrand & Stjarne, 1989; Mutafova-Yambolieva & Westfall, 1998; Bobalova & Mutafova-Yambolieva, 2001a; Burnstock & Ralevic, 2014), in urinary bladder (Burnstock et al., 1972; Burnstock et al., 1978b; Breen et al., 2006; Burnstock, 2011b), and in adrenal chromaffin cells or PC12 pheochromocytoma cells (Wagner, 1985; Njus et al., 1986; Todorov et al., 1996; Yamboliev et al., 2009).

Following the first indications of possible participation of purines in neurotransmission, the last half century witnessed remarkable progress in our understanding of extracellular functions of adenine nucleotides and nucleosides, including the identification of purine receptors on cell membranes, cell- and tissue-specific actions of extracellular purines and underlying signal transduction mechanisms, pathways of extracellular metabolism of purines, and ultimately participation of extracellular purines in complex processes, including cell motility, cell proliferation, cell survival, membrane trafficking and cell-cell communication. Amid this entire advance, possible roles of purines in chemical neurotransmission have attracted particular attention as it was found that in many systems there is a component in neural responses that is inhibited by purine receptor antagonists. The nature of the purine neurotransmitter substance, however, is still uncertain, several decades after the purinergic neurotransmission hypothesis was introduced (Burnstock et al., 1970; Burnstock, 1972; Westfall et al., 1982; Chaudhry et al., 1984; Mutafova-Yambolieva et al., 2007; Hwang et al., 2011; Goyal, 2011; Mutafova-Yambolieva, 2012; Gil et al., 2013; Durnin et al., 2013). This might be due in part to technological obstacles and heavy reliance on indirect evidence from measuring radioactive tracers (i.e., tritiated adenosine or adenine), from assaying adenine nucleotide metabolites (i.e., adenosine and inosine) as a measure of released ATP, from common use of chemiluminescence assays that are restricted to ATP (and hence may overlook the release of other purines), and from widely used assessments of post-junctional electrical or mechanical responses to nerve stimulation or exogenous purinergic receptor ligands. It is now recognized that many of these approaches bear low specificity and are, at best, indirect and imprecise assays of neurotransmitter identity. For example, the use of radioactive tracers (i.e., [³H]-adenosine) may not strictly reflect release of ATP. In fact, it is now clear that multiple adenine nucleotides in addition to ATP can be metabolized to adenosine (Zimmermann et al., 2012; Durnin et al., 2012b) and, importantly, some of these nucleotides are released upon nerve stimulation (e.g., Smyth et al., 2004; Mutafova-Yambolieva et al., 2007; Durnin et al., 2012b; Durnin et al., 2013). Moreover, recent works have demonstrated direct neuronal release of adenosine (Brundege & Dunwiddie, 1998; Manzoni et al., 1994; Begg et al., 2002; Correia-de-Sa et al., 2006; Wong et al., 2006; Wall & Dale, 2007; Wall & Dale, 2008; Lovatt et al., 2012) and therefore, detecting extracellular adenosine cannot universally be assumed to be a result (and a measure) of ATP release (Lovatt et al., 2012). Finally, we now know that multiple substances with adenine and pyrimidine moieties, in addition to ATP, could activate the ionotropic and metabotropic P2 purinergic receptors that mediate postjunctional responses to

purine neurotransmitter(s). As mentioned, some of these substances have recently been demonstrated to be released upon nerve stimulation (Smyth et al., 2004; Breen et al., 2006; Mutafova-Yambolieva et al., 2007; Hwang et al., 2011; Durnin et al., 2012b; Durnin et al., 2013) and in some systems such substances appear to mimic the endogenous neurotransmitters better than ATP (Mutafova-Yambolieva et al., 2007; Hwang et al., 2011; Mutafova-Yambolieva, 2012; Durnin et al., 2013). Taken together, such observations bring about the necessity to reevaluate purinergic neurotransmitter identity. This is an important matter, as knowledge of the chemical nature of the purine neurotransmitter in different systems would ensure a more precise control of nerve-mediated functions. This might also be important for understanding major mechanisms of neuromodulation, since different purine neurotransmitters would generate different metabolites at the neuroeffector junction, some with biological activity. Finally, knowing the identity of the purine neurotransmitter and its metabolites will aid our understanding of the pathogenesis of diseases and will facilitate development of novel targets for therapeutic drugs, and specific biological markers. Notably, purinergic neurotransmission appears to be particularly important in some systems of the human body. For example, in the human distal gastrointestinal (GI) tract, purinergic neurotransmission appears to be more important in mediating inhibitory motor responses (Hwang et al., 2011) than NO, which is significant in animal models (Lyster et al., 1995). The present review is intended, therefore, to briefly review the experimental support for ATP as a neurotransmitter and recent evidence for possible contribution of multiple purines, in addition to or instead of ATP, in chemical neurotransmission in the peripheral and central nervous systems.

2. Storage of purines in synaptic vesicles

An important feature of chemical neurotransmission is that classical neurotransmitter substances are stored in and released from synaptic vesicles (Katz, 1971; Smith, 1972; Kelly et al., 1979; Schneggenburger et al., 2012; Sudhof, 2013). Monitoring uptake and release of [^3H]-labeled adenosine during nerve stimulation has been used in earlier studies in smooth muscles as evidence for vesicular storage of ATP (Su et al., 1971; Westfall et al., 1978). Likewise, uptake of [^3H]-ATP has been demonstrated in adrenal chromaffin granules (Aberer et al., 1978) and in cholinergic synaptic vesicles isolated from *Torpedo* electric organ (Luqmani, 1981). Notably, these studies also describe uptake of [^3H]-ADP, [^3H]-AMP, guanosine and uridine triphosphates, with similar characteristics to ATP, suggesting that nucleotide uptake is not limited to ATP. Quinacrine-binding has also been used to localize ATP and to demonstrate storage of ATP in neurons (Olson et al., 1976; Bock, 1980; Crowe & Burnstock, 1981; Belai & Burnstock, 1994); however, quinacrine appears to also bind to other adenine nucleotides, guanylic acid, nucleic acids, DNA, RNA, prion proteins and acetylcholine receptors (Irvin & Irvin, 1954b; Irvin & Irvin, 1954a; Kurnick & Radcliffe, 1962; Fertuck & Salpeter, 1976; Sumner, 1986; Valenzuela et al., 1992; Yu et al., 2003). Clearly there are specificity problems with the use of radioactive tracers and quinacrine for specific detection of ATP. Firefly luciferin-luciferase chemiluminescence assay (Stanley & Williams, 1969) has provided more direct evidence for storage of ATP in various secretory granules and synaptic vesicles (Hillarp, 1958; Da & Pletscher, 1968; Dowdall et al., 1974; Fried, 1980) and for release of ATP from isolated rat brain

synaptosomes (White, 1977; White, 1978) and small intestine myenteric varicosities (White & Leslie, 1982) in response to membrane depolarization. In fact, it is now believed that ATP is stored in all synaptic vesicles, independently of neurotransmitter type, vesicle size, stage of vesicle formation or 'readiness' for release (Sperlagh & Vizi, 1996; Reigada et al., 2003; Aspinwall & Yeung, 2005; Pankratov et al., 2006), making this molecule a universal marker for vesicular content and secretion (Zimmermann et al., 1993; Reigada et al., 2003; Aspinwall & Yeung, 2005; Aspinwall & Yeung, 2005). Perhaps this universal presence of ATP in secretory vesicles suggests that ATP might also be important for functions different from those it performs as a neurotransmitter. It has been suggested that vesicular ATP might be important for acidification of the vesicle lumen (Sperlagh & Vizi, 1996) or for fueling neurotransmitter uptake mechanisms (Takeda & Ueda, 2012). As discussed, other adenine nucleotides can also be accumulated in synaptic vesicles. For example, diadenosine polyphosphates have been found in secretory granules, synaptic vesicles, and brain synaptic terminals (Rodriguez del Castillo et al., 1988; Pintor et al., 1992), and are released in a Ca^{2+} -dependent manner (Pintor et al., 1992). Presumably their intravesicular concentration is on the order of 5–10 mM, which exceeds their cytoplasmic concentrations by several orders of magnitude (Zimmermann et al., 1993). These substances have been suggested to be neurotransmitters (Miras-Portugal et al., 1998; Delicado et al., 2006). More recent evidence has demonstrated that in addition to ATP, NAD^+ and ADPR are stored in synaptic vesicles of rat pheochromocytoma PC12 cells (Yamboliev et al., 2009) and in isolated rat forebrain synaptosomes (Durnin et al., 2012a). These are the first studies to demonstrate novel intracellular storage sites of NAD^+ and ADPR in synaptic vesicles that had not been identified before.

Accumulation of neurotransmitters in vesicles requires efficient uptake mechanisms. Vesicular transporters mediate accumulation of their respective neurotransmitters through an electrochemical gradient of protons across the membrane generated by vacuolar proton ATPase (see Schuldiner et al., 1995). Many earlier studies have attempted to characterize the nucleotide transporter(s). For example, the uptake of tritiated ATP, ADP, or AMP inside isolated bovine chromaffin granules was inhibited by atractyloside, an inhibitor of mitochondrial nucleotide uptake, suggesting the involvement of a transporter (Aberer et al., 1978). Likewise, the uptake of ATP and ADP in chromaffin granules was sensitive to bafilomycin A1 (Bankston & Guidotti, 1996), which blocks vesicular uptake of neurotransmitters by inhibiting membrane H^+ -ATPase (Moriyama & Futai, 1990). It has been proposed that in sheep brain synaptic vesicles ATP is transported along its concentration gradient and may be directly transported from mitochondria to the synaptic vesicles (Zalk & Shoshan-Barmatz, 2006). However, until recently the identity of the vesicular nucleotide transporter was not clear. Sawada et al., 2008 proposed that the protein encoded by the SLC17A9 gene is a vesicular nucleotide transporter in chromaffin granules. This protein is widely expressed in the adrenal gland, brain, and thyroid gland in both mice and humans and was associated with chromaffin granules as demonstrated by electron microscopy (Sawada et al., 2008). Liposomes that had SLC17A9 reconstituted were able to uptake radiolabeled [^{32}P]-ATP, GTP and ADP. Non-hydrolysable ATP analogues or atractyloside inhibited uptake of ATP into SLC17A9-expressing liposomes, and in PC12 cells, knockdown of SLC17A9 by small interfering RNA suppressed 53% of SLC17A9 and

reduced KCl-stimulated release of ATP by 50% (Sawada et al., 2008). Zymogen granules isolated from pancreatic acini (Haanes & Novak, 2010) and varicosities isolated from murine whole gut (Chaudhury et al., 2012) demonstrated immunoreactivity for vesicular nucleotide transporter. Expression profiling of SLC17A9 in the mouse revealed highest expression in stomach, intestine, liver, skeletal muscle, spleen and blood (Sreedharan et al., 2010). In the brain, SLC17A9 was ubiquitously expressed in all brain regions with highest expression in cerebral cortex, certain areas of the hippocampus, and specific nuclei of the hypothalamus and thalamus, regions where purinergic neurotransmission is thought to be important (Sreedharan et al., 2010). In the hippocampus, immunogold labeling showed co-localization of SLC17A9 with synaptic vesicles in excitatory and inhibitory terminals (Larsson et al., 2012). Knockdown of SLC17A9 inhibited high K^+ -evoked ATP release, suggesting the involvement of this protein in uptake and release of ATP in hippocampal neurons (Larsson et al., 2012). SLC17A9 transports nucleotides not only into neuronal vesicles, but also into secretory vesicles of T-cells (Tokunaga et al., 2010), biliary epithelial cells (Sathe et al., 2011), A549 human adenocarcinoma cells (Takai et al., 2012), pancreatic beta-cells (Geisler et al., 2013), airway epithelial cells (Sesma et al., 2013), human monocyte THP-1 cells (Sakaki et al., 2013), and brain astrocytes (Oya et al., 2013).

Uptake of NAD^+ across plasma membranes has also been demonstrated in different cell types (discussed in *Section 5.2*) and in rat brain synaptosomes (Durnin et al., 2012a). NAD^+ is present in small synaptic vesicles in PC12 cells (Yamboliev et al., 2009) and in isolated brain synaptosomes (Durnin et al., 2012a). Whether SLC17A9 is involved in the transport of NAD^+ remains to be determined. It was demonstrated that the synaptic vesicle protein (SV2) binds nucleotides and shows equal affinity for both ATP and NAD^+ (Yao & Bajjalieh, 2008), whereas a protein that is distantly related to SV2, called SVOP (Janz et al., 1998), demonstrated a higher affinity for NAD^+ than for ATP (Yao & Bajjalieh, 2009). It remains to be determined whether and how nucleotide binding to synaptic vesicle proteins is related to the nucleotide transport in vesicles.

It should be emphasized again that the presence of purines in synaptic vesicles is not conclusive evidence for their roles as neurotransmitters. Some investigators point out that ATP is important for acidifying vesicle lumens which would prevent genuine neurotransmitters from degradation, for creating a proton gradient for vesicular neurotransmitter uptake, for vesicle priming, and for steps in exocytosis itself (Sperlagh & Vizi, 1996; Yao & Bajjalieh, 2008). Thus it is acknowledged that ATP may be present in vesicles for functions other than neurotransmission. It is important to consider mechanisms mediating release of purines along with the extracellular action of released purines in studies on candidate neurotransmitter substances.

3. Evidence for release of purine neurotransmitters

3.1. Principles of characterizing neurotransmitters

With the realization that many pharmacologically active signaling molecules can exist in nerves it has become difficult to identify which substances can be considered genuine neurotransmitters. A set of criteria has been proposed to aid in the identification of novel neurotransmitters and to discriminate between neuromodulators and other signaling

molecules. A substance that is a neurotransmitter is synthesized in the neuron and stored within the synaptic vesicles, is released by Ca^{2+} -dependent vesicle exocytosis in a neural activity-dependent manner, activates specific receptors on the post-synaptic/post-junctional target cell, and when applied exogenously mimics the response to the endogenous neurotransmitter; in addition, mechanisms to terminate the extracellular action of the substance should exist at the neuroeffector junction (Eccles, 1964; Burnstock, 2012). These 'classical' criteria have been broadened with the discoveries that gasotransmitters such as NO, carbon monoxide, and hydrogen sulfide can function as neurotransmitters without strictly conforming to established criteria (Baranano et al., 2001; Boehning & Snyder, 2003). The mode of synthesis, storage, and release of these transmitters differs markedly from those of other neurotransmitters in that they are not stored in synaptic vesicles, thus not released by exocytosis, and instead synthesized when required. Additionally, instead of binding to receptors on postjunctional membranes, gasotransmitters diffuse into target cells, immediately affecting excitability by interacting with their intracellular targets. Thus classical criteria for neurotransmitter status are continually developed as we learn more about intricacies of neurotransmission. Nonetheless, the non-gaseous neurotransmitters such as adenine nucleotides are expected to be stored in and released from synaptic vesicles in response to action potential firings in nerve processes, in Ca^{2+} - and frequency-dependent manners, and to act on receptor proteins in the cell membrane of target cells. In practical terms, studies in complex tissues that are aimed at determining whether a substance is a neurotransmitter attempt to document that: i) the substance is released upon short-pulse electrical field stimulation (EFS) that triggers propagation of action potentials in nerve processes and nerve varicosities; ii) the release of this substance is abolished by inhibitors of fast Na^+ channels such as tetrodotoxin (TTX), which block propagation of action potentials in nerve terminals; iii) the evoked release of the substance depends on extracellular Ca^{2+} and is therefore inhibited by blockers of Ca^{2+} influx through neural voltage-dependent Ca^{2+} channels; iv) the amount of substance released is proportional to the depolarization imposed (i.e., release of the substance is increased with increased frequencies of stimulation); v) exogenously applied substance mimics the effect of the endogenous neurotransmitter that is released upon EFS; vi) the postjunctional responses to the substance are mediated by membrane receptors and intracellular signal transduction pathways that are established for the endogenous neurotransmitter; and vii) inhibitors of nucleotide degradation might increase the effects of nerve stimulation. Ideally, such studies should be reinforced with studies in isolated nerve varicosities and synaptosomes and in purified synaptic vesicles demonstrating the presence of the candidate neurotransmitter substance in these structures, its release upon depolarization of cell membranes that is sensitive to inhibitors of voltage dependent Ca^{2+} channels or to destruction of vesicle proton gradient, and possible uptake of the substance in purified synaptic vesicles. Undeniably, while the list is not exhaustive many of these approaches are experimentally demanding, particularly when the attempt is to identify a purine neurotransmitter in a complex tissue that is composed of multiple types of cells such as smooth muscle (e.g., taenia coli and other GI muscles, blood vessels, vas deferens, urinary bladder, airways, or the uterus) or even the brain. Still, direct measurements of what has been released from nerve processes, in addition to characterization of postjunctional responses, provides more reliable functional evidence that

a substance could be a neurotransmitter than do isolated pieces of information on pharmacological characterization of integrated postjunctional responses.

3.2. Common methodologies for purine neurotransmitter detection

A number of techniques have been utilized to measure purine concentrations in biological samples (Manfredi et al., 2002; Praetorius & Leipziger, 2009; Fields, 2011a; Dale & Frenguelli, 2012a), but few of them have been applied to analyze release of purine neurotransmitters.

As mentioned, early studies monitored the release of [^3H]-nucleosides as a measure of released ATP, after loading tissues with [^3H]-adenosine (e.g., Burnstock, 1972; White, 1988); however, it is now recognized that relying on radioactive tracers pose problems with specificity and source. The luciferin-luciferase chemiluminescence assay (Stanley & Williams, 1969; Wood et al., 1989) is currently perhaps the most widely used method for detecting nerve-stimulation evoked release of ATP. It is based on detecting the bioluminescence of luciferase-mediated oxidation of luciferin that depends on ATP (Wood et al., 1989). The specificity of this method is higher than that of radioactive tracers, but it is restricted to detection of ATP. Thus this method overlooks other purines that might be released during nerve stimulation as well as metabolites produced from released purines, which might have biological activity. Moreover many factors can interfere with the assay including anions, anion transport inhibitors, bacterial contamination, and even P2 receptor antagonists that are commonly used in studies of purinergic neurotransmission (Praetorius & Leipziger 2009). The chemiluminescence assay in conjunction with single-photon imaging improves the spatial and temporal resolution of the assay and has been used to examine the release of ATP in single cell types such as astrocytes (Wang et al., 2000) and dorsal neurons (Fields & Ni, 2010), but has not yet been employed in complex tissues such as smooth muscle.

Microelectrode biosensors have also been developed for sensitive detection of purines. However, these biosensors are specific to ATP or adenosine and are not sensitive to ADP, AMP or any other purine or pyrimidine substances (Llaudet et al., 2005; Dale & Frenguelli, 2012b). ATP probes have been utilized to measure ATP overflow in the brain, skeletal muscle arterioles or gastric mucosa (Dale & Frenguelli, 2012a; Patel et al., 2011; Patel, 2014), but have not yet received wide-spread use for neurotransmitter measurements. Boron-doped diamond electrode (Birbeck & Mathews, 2013) and liquid chromatography-mass spectrometry methodology using benzoyl chloride derivatization (Song et al., 2012) have recently been employed to analyze adenosine in mouse brain tissue and rat brain microdialysis samples, respectively. Spectrophotometric enzyme cycling assays have been used to analyze NAD^+ concentrations in cells or tissues (Bernofsky & Swan, 1973; Berger & Berger, 1986; Umemura & Kimura, 2005) or released from bone marrow-derived human mesenchymal stem cells (Fruscione et al., 2011). However, none of these methodologies have been utilized to analyze neuronal release of purines.

Sensitive detection of multiple purines released during nerve stimulation can be obtained with reversed-phase gradient high-performance liquid chromatography assay with fluorescence detection (HPLC-FLD) coupled with a small-volume superfusion assay (e.g.,

Todorov et al., 1996; Smyth et al., 2004; Breen et al., 2006; Yamboliev et al., 2009; Mutafova-Yambolieva et al., 2007; Durnin et al., 2012). To increase detection sensitivity, chloroacetaldehyde added to samples forms fluorescent 1,*N*⁶-etheno-purine analogs, effectively reducing the detection limits to low femtomolar concentrations of released purines (Levitt et al., 1984; Bobalova et al., 2002). A significant advantage of this methodology is that multiple purines are analyzed simultaneously in a single sample of tissue or cell superfusate. These include ATP, ADP, AMP, adenosine, and the more recently identified extracellular substances that contain a purine moiety NAD⁺, ADPR, and cyclic ADPR. Thus direct comparisons in the mechanisms of release of multiple purines can be accurately achieved. Notably, these methodologies have allowed novel candidate purine neurotransmitter substances, such as NAD⁺ and ADPR, to be introduced (discussed in more detail in *Sections 3.3. and 3.4*).

No existing detection methodologies for purines have the ideal combination of spatial resolution, temporal resolution, high sensitivity, and spectrum to detect precisely released purine neurotransmitters. Nevertheless, direct measurements of what is released from nerves added to an array of functional and molecular approaches to characterize neurotransmitter action would be more informative in identifying the purine neurotransmitter substances in different systems than pharmacological analysis of postjunctional responses.

3.3. In peripheral nervous system

3.3.1. Vas deferens—Vas deferens has become a relatively simple model to study adrenergic and purinergic cotransmission (see Westfall & Westfall, 2001; Burnstock & Verkhatsky, 2010). Sympathetic nerve stimulation in the vas deferens produces a biphasic contraction (Swedin, 1971; Birmingham & Freeman, 1976). The first, fast phase of contraction is mediated by purines, whereas the secondary, slow onset phase is due to NE release (reviewed in Westfall & Westfall, 2001; Burnstock & Verkhatsky, 2010). Studies have shown that the initial, fast contractions in guinea pig vas deferens to sympathetic nerve stimulation are blocked by a P2 receptor antagonist arylazido aminopropionyl ATP (ANAPP3) (Fedan et al., 1981; Sneddon et al., 1982; Sneddon & Westfall, 1984), by purine receptor desensitization with α,β -methylene-ATP (Sneddon & Burnstock, 1984b; Stjarne & Astrand, 1984; Stjarne & Astrand, 1984; Allcorn et al., 1986; Brock & Cunnane, 1988), and by other non-selective P2 receptor antagonists (reviewed in Burnstock, 2013c). In addition, exogenously applied ATP has been shown to mimic electrical stimulation (Fedan et al., 1981; Sneddon et al., 1982; Sneddon & Westfall, 1984; Stjarne & Astrand, 1984), suggesting that ATP could be the co-transmitter with NE. It is believed that ATP that is coreleased with NE from sympathetic nerve terminals acts through P2X receptors on smooth muscle to mediate membrane depolarization (e.g., excitatory junction potentials, EJP) and contraction (Stjarne & Astrand, 1984; Sneddon & Burnstock, 1984b; Allcorn et al., 1986; Mulryan et al., 2000). Although some studies report no purinergic component in the mechanical responses of the human vas deferens to nerve stimulation (Holmquist et al., 1990; Medina et al., 1998; Medina et al., 2010), other studies have suggested a role for the P2X1 receptor in human vas deferens motor activity (Banks et al., 2006), and in the longitudinal smooth muscle layer in particular (Amobi et al., 2012; Donoso et al., 2014).

EFS-evoked release of ATP was suggested using different methodologies for ATP detection. For example, Westfall et al., 1978 first demonstrated TTX-sensitive release of tritium in guinea pig vas deferens tissues pretreated with [^3H]-adenosine and stimulated transmurally; this was interpreted as possible nerve-evoked release of ATP. A number of studies have shown frequency-dependent and TTX-sensitive release of ATP in rodent vas deferens using additional techniques such as the firefly luciferin-luciferase assay (Lew & White, 1987; Kirkpatrick & Burnstock, 1987) or high-performance liquid chromatography (HPLC) techniques coupled with radiochemical detection of [^{14}C]-ATP (Vizi & Burnstock, 1988) or with fluorescence detection of 1, N^6 -ethenoderivatized endogenous purines (Levitt et al., 1984; Todorov et al., 1996). Using the luciferin/luciferase methodology it was suggested that in the guinea-pig vas deferens part of ATP might originate from smooth muscle cells (von Kugelgen & Starke, 1991; Vizi et al., 1992).

Studies have suggested that the release of ATP and NE in the vas deferens might originate from different populations of synaptic vesicles. For example, pharmacological characterization of postjunctional responses have shown differential regulation of the biphasic mechanical responses in rodent vas deferens by prostaglandins (Trachte, 1985; Trachte et al., 1989) or atrial natriuretic peptide (Mutafova-Yambolieva et al., 1993). More direct studies measuring the release of ATP and NE using various detection techniques have demonstrated differential effects on ATP and NE release by prostaglandins (Ellis & Burnstock, 1990), angiotensin (Ellis & Burnstock, 1989), β -adrenoceptor activation (Goncalves et al., 1996), and endothelin-3 (Mutafova-Yambolieva & Westfall, 1995). One-minute stimulation of the guinea-pig vas deferens causes phasic release of ATP and tonic release of NE (Todorov et al., 1994; Todorov et al., 1996), which is different from the parallel release of ATP and catecholamines in isolated bovine chromaffin cells (Todorov et al., 1996). Therefore, the temporal dissociation of ATP and NE release described in the guinea-pig vas deferens might not be universal for the sympathetic nervous system. For example, analysis of excitatory junction currents (EJCs) to measure the release of ATP and differential pulse amperometry to measure the release of NE suggested that ATP and NE were likely released in parallel in the mouse vas deferens (Stjarne et al., 1994; Msghina et al., 1998), whereas in the guinea-pig it was proposed that the release of ATP and NE may originate from different classes of small vesicles with different Ca^{2+} sensitivities at low and high frequencies of stimulation (Stjarne, 2001). Furthermore, analysis of electrical and mechanical responses to electrical stimulation has demonstrated that in mouse vas deferens N-, P- and Q-type voltage-dependent calcium channels are involved similarly in the release of ATP and NE (Waterman, 1997), whereas ATP and NE overflow studies in the guinea-pig vas deferens suggest differential modulation of ATP and NE release by N-type and P-type Ca^{2+} channels (Westfall et al., 1996). It has been suggested that the sympathetic nerves of the guinea pig vas deferens store and release ATP together with soluble/releasable nucleotidase(s) responsible for the inactivation of this neurotransmitter (Todorov et al., 1997; Mihaylova-Todorova et al., 2001; Mihaylova-Todorova et al., 2002). Inactivation of purine neurotransmitters by enzymes that are released during nerve stimulation might be tissue- and/or species-specific (see *Section 5.1*).

3.3.2. Blood vessels—Neural release of purines in blood vessels has been a focus in purine neurotransmission research for many years. It is largely believed that sympathetic vasoconstrictor neurons typically utilize the cotransmitters NE, ATP and neuropeptide Y and that the relative roles of these cotransmitters vary considerably between blood vessels or vascular regions and depend on the level of sympathetic nervous system activity (Burnstock, 1990; Lundberg, 1996). In many arteries EFS causes biphasic membrane depolarization consisting of fast EJPs or excitatory junction currents (EJCs) that are inhibited by P2 receptor antagonists and hence are mediated by a released purine, and a slow depolarization that is inhibited by α -adrenoceptor antagonists and hence is mediated by released NE. Therefore, the fast EJPs have been used as a measure of ATP release. For example, Sneddon & Burnstock, 1984a demonstrated that fast depolarizations in rat tail artery were resistant to α -adrenoceptor antagonists but were abolished by α,β -methylene-ATP, which was the basis to propose that ATP is a co-transmitter with NE in tail artery. Characterization of EFS-elicited EJPs in blood vessels and inhibition with P2 receptor antagonists has become a popular approach in investigating purinergic (presumably mediated by ATP) neurotransmission in numerous blood vessels including the rat tail artery (Astrand et al., 1988; Astrand & Stjarne, 1989; Stjarne & Stjarne, 1989; Jobling & McLachlan, 1992; McLaren et al., 1995; Brock & Cunnane, 1999), cutaneous arteries of isolated guinea-pig ear (Morris et al., 1998), mesenteric arteries (Muir & Wardle, 1988; Thapaliya et al., 1999; Keef et al., 1991; Haddock & Hill, 2011), and rabbit saphenous artery (Nally & Muir, 1992; see Burnstock & Ralevic, 2014). Low-frequency EFS-evoked Ca^{2+} transients in smooth muscle cells in rat mesenteric small arteries are assumed to represent Ca^{2+} entering smooth muscle cells through P2X1 receptors activated by ATP released from sympathetic nerves (Lamont & Wier, 2002; Lamont et al., 2006). Some studies also analyzed the P2 receptor-mediated vascular contractions to investigate purinergic neurotransmission (e.g., Muramatsu, 1986; Brizzolara & Burnstock, 1990), although the EJPs in blood vessels may not always be directly associated with contraction (Surprenant, 1980; Sneddon & Burnstock, 1984a; Hirst et al., 1992). Notably, release of ATP or other purines was not measured in these studies. In general, lack of release measurements is regarded as lack of direct evidence for a transmitter role of candidate neurotransmitter substances, including ATP (McLachlan & Keast, 1993), and this has prompted investigators to conclude in a variety of systems that the responses were mediated by ‘ATP or a related compound’ (Stjarne & Astrand, 1984; von Kugelgen & Starke, 1985; Serio et al., 2003; De Man et al., 2003; Heppner et al., 2005; Zizzo et al., 2007; Lomax & Vanner, 2010).

The significance of measuring release of purines in studies on neurotransmission is illustrated in the case of neural control of arteries and veins of the mesenteric circulation. As in most arteries, sympathetic nerve stimulation initiates rapidly rising EJPs in the guinea-pig mesenteric artery (Fujioka & Suzuki, 1985; Kreulen, 1986; Hottenstein & Kreulen, 1987) (Fig. 1A), that are presumably mediated by released ATP (Ishikawa, 1985; Onaka et al., 1997) acting on P2X receptors (Evans & Surprenant, 1992). In contrast to arteries, fast EJPs are not initiated during sympathetic nerve stimulation in veins (Makita, 1983; Kreulen, 1986) (Fig. 1A), perhaps with the exception of dog mesenteric vein (Suzuki, 1984; Yamboliev & Mutafova-Yambolieva, 2005) and rabbit saphenous vein (Cheung, 1985). Rather, repetitive sympathetic nerve stimulation in the guinea-pig mesenteric vein typically produces a

depolarization which is slow in onset, long lasting (Suzuki, 1981) and resistant to purinoreceptor desensitization with α,β -methylene ATP (Hirst & Jobling, 1989). The slow depolarization observed in vein is associated with contraction and both events are reduced by the α_1 -adrenoceptor antagonist prazosin (Suzuki, 1981; Hottenstein & Kreulen, 1987; Van Helden, 1988), making NE a likely candidate for these responses.

It has been suggested that differences in the electrical responses to nerve stimulation in the guinea-pig artery and vein (Fig. 1A) might reflect a wide separation between the site of transmitter release and the receptors which are present on the effector cell membrane in the vein (Suzuki, 1981). However, the density of neuromuscular junctions and the width of the synaptic cleft in guinea-pig mesenteric arteries and veins appear to be very similar (Luff & McLachlan, 1989; Klemm et al., 1993) making this explanation untenable. Mesenteric veins also differ from mesenteric arteries in that veins contract in response to lower frequencies of nerve stimulation than do mesenteric arteries (Karim & Hainsworth, 1976; Hottenstein & Kreulen, 1987), but this was unlikely due to differences in the resting membrane potentials of the smooth muscles or in muscle mechanics of the two vessels (Hottenstein & Kreulen, 1987). Neurons in the inferior mesenteric ganglion of the guinea-pig that innervate arteries appear to be different from those that innervate veins (Browning et al., 1999; Kreulen, 2003). Therefore, the composition of the neurotransmitter cocktail may differ in the two blood vessels. The lack of EJPs in the vein implied that ATP is not involved in the junctional events in veins and therefore is not released during nerve stimulation in veins. However, a P2 receptor-mediated component in the contractile responses to EFS can be revealed in mesenteric vein isolated from reserpine-treated guinea-pigs (Smyth et al., 2000), suggesting that ATP and/or other purines are likely released upon action potential firings in both arteries and veins. Important answers to these questions were provided by the first direct measurements and quantitative comparison of nerve-evoked purine release in the two blood vessels (Bobalova & Mutafova-Yambolieva, 2001a) almost 20 years after the first observations that the mesenteric artery and vein differ in their electrical responses to nerve stimulation. Thus, using HPLC methodologies it was demonstrated that guinea-pig mesenteric veins, as well as arteries, release ATP (Fig. 1B) along with NE (Bobalova & Mutafova-Yambolieva, 2001a). Moreover, release of both ATP and NE in the veins exceeded release of ATP and NE in the arteries, suggesting that threshold concentrations of neurotransmitter may be achieved at lower frequencies of stimulation in the veins. These findings offer an explanation of why veins constrict proportionally more at lower frequency than do arteries (Hottenstein & Kreulen, 1987). Clearly, the lack of EJPs in veins is not due to absence of ATP release in response to nerve stimulation and may, instead, be due to differences in the complement of the postjunctional P2 receptors (e.g., fewer P2X1 receptors in the vein) that mediate the smooth muscle cell response to ATP (Mutafova-Yambolieva et al., 2000; Galligan et al., 2001; Kreulen, 2003).

Only a limited number of studies in blood vessels have measured neurogenic release of purines (primarily ATP) although it is recognized that this would be the most direct way to demonstrate ATP sympathetic cotransmission (see Burnstock & Ralevic, 2014). These include studies on release of tritium after incubation of vessel segments with [3 H]-adenosine in rabbit aorta, ear artery and portal vein (Su, 1975; Katsuragi & Su, 1980), dog basilar artery (Muramatsu et al., 1981), and rat tail artery (Muir & Wardle, 1989). Rump & von

Kugelgen, 1994 used firefly luciferin-luciferase assay to demonstrate TTX-sensitive release of ATP in human saphenous vein, but could not demonstrate a role of neurally released ATP in vasoconstriction elicited by electrical field stimulation. TTX-sensitive ATP overflow was also demonstrated using an amperometric microelectrode biosensor for ATP in the first-order resistance arteriole in the rat gastrocnemius red muscle (Kluess et al., 2010). The study concluded that in this blood vessel ATP originates from a number of sources including nerve terminals, endothelium, and smooth muscle. Finally, as discussed above, highly-sensitive HPLC methodologies for purine detection proved to be instrumental in characterizing purinergic neurotransmission and cotransmission in a number of blood vessels, including the guinea-pig mesenteric artery and vein (Bobalova & Mutafova-Yambolieva, 2001a), rat tail artery (Westfall et al., 1987; Mutafova-Yambolieva & Westfall, 1998), rabbit aorta (Sedaa et al., 1990), rabbit pulmonary artery (Mohri et al., 1993), and canine mesenteric arteries and veins (Bobalova & Mutafova-Yambolieva, 2001b; Bobalova et al., 2002; Smyth et al., 2004; Smyth et al., 2009). The EFS-evoked release of ATP was reduced by TTX or guanethidine in the rabbit aorta (Sedaa et al., 1990), rat tail artery (Mutafova-Yambolieva & Westfall, 1998), and guinea-pig and canine mesenteric arteries and veins (Bobalova & Mutafova-Yambolieva, 2001a; Bobalova & Mutafova-Yambolieva, 2001b). This detection technique has also been used to study purine release in the bladder (Smyth et al., 2004; Breen et al., 2006; Durnin & Mutafova-Yambolieva, 2011), gastro-intestinal muscles (McConalogue et al., 1996; Mutafova-Yambolieva et al., 2007; Hwang et al., 2011; Durnin et al., 2012b; Durnin et al., 2013), PC12 cells (Yamboliev et al., 2009), and rat brain synaptosomes (Durnin et al., 2012a).

Similar to guinea-pig vas deferens, studies in mesenteric blood vessels have suggested that the release of ATP and NE might originate from different populations of synaptic vesicles. For example, in the canine mesentery, α_2 -adrenoceptor agonists significantly reduced the release of NE but had no effect on ATP release at low frequencies of EFS, suggesting release from different sites (Bobalova & Mutafova-Yambolieva, 2001b). In addition, Smyth et al., 2009 showed in canine mesenteric blood vessels that release of NE was primarily regulated by N-type Ca^{2+} channels whereas release of ATP was regulated mainly by P/Q-type Ca^{2+} channels. Therefore NE and ATP may not be stored in and released from the same population of synaptic vesicles in mesenteric vasculature. It should be pointed out that in the canine mesenteric blood vessels EFS evoked release of NAD^+ along with ATP and NE, in a frequency-dependent and neurotoxin-sensitive manner (Smyth et al., 2004; Smyth et al., 2009). Interestingly, the release of NAD^+ , like NE, was reduced by the N-type Ca^{2+} channel inhibitor ω -conotoxin GVIA, whereas the release of ATP was not affected by ω -conotoxin GVIA in either artery or vein (Smyth et al., 2009). Therefore, different mechanisms appear to regulate the release of ATP and NAD^+ (and NE) in canine mesentery blood vessels. Unlike mesenteric vessels, studies in rat tail artery suggest that NE and ATP are released in parallel and might be co-stored in the same synaptic vesicles (Msghina et al., 1998; Brock & Cunnane, 1999; Msghina et al., 1999). For example, Brock & Cunnane, 1999 found that depolarization responses to both ATP and NE were mediated by N-type Ca^{2+} channels in rat tail artery. Msghina et al., 1999 concluded that in the absence of drugs ATP and NE are likely released in parallel, although the effects of α_2 -adrenoceptor antagonists seem to suggest dissociated release. The studies by Brock & Cunnane, 1999 and Msghina et al.,

1999 examined EJs evoked by nerve stimulation as a measure of ATP 'release,' whereas NE release was analyzed by measuring NE oxidation currents and fractional overflow of [3]NE. Mutafova-Yambolieva & Westfall, 1998 demonstrated with HPLC methodologies that endothelin peptides modulated in parallel the nerve-evoked release of ATP and NE in rat tail artery. Therefore temporal dissociation of NE and ATP release as observed in vas deferens and mesenteric blood vessels might not be universal to all sympathetic nerves.

It is important to note that release of ATP has been shown in all major cell types of the vessel wall including vascular endothelium (Sedaa et al., 1990; Hassessian et al., 1993; Shinozuka et al., 1994) and smooth muscle (Vizi & Burnstock, 1988; Sedaa et al., 1990; Vizi et al., 1992), as well as circulating erythrocytes and platelets (Sprague et al., 1996; Sprague & Ellsworth, 2012) (see also Lohman et al., 2012). The endothelium appears to be the major source of purines in the vasculature. Sedaa et al., 1990 demonstrated using HPLC-FLD that 90% of EFS-evoked release of purines originates from endothelium, 7% from smooth muscle and 3% from neuronal sources. Therefore, it could be technically demanding to detect purine release from perivascular nerve terminals. Instead, blood vessels represent an excellent model for extraneuronal release of ATP by various mechanisms (see Mutafova-Yambolieva, 2012 and Burnstock & Ralevic, 2014).

3.3.3. PC12 cells—PC12 cells are ontogenetically related to sympathetic neurons and can serve as a single cell line model for sympathetic neurotransmission and cotransmission (Greene & Tischler, 1976; Wagner, 1985). This cell line was established from rat adrenal pheochromocytoma and responds reversibly to nerve growth factor (NGF). NGF treatment results in increased content of a number of nucleotides including NAD⁺, AMP, ADP, ATP, GTP, GDP, GMP, UTP, UDP, UDP-glucose, and UDP-galactose (Morelli et al., 1986). A significant number of studies has focused on effect of extracellular nucleotides in modulating NGF-induced neurite outgrowth (Aono et al., 1990) as well as catecholamine release in these cells (Sela et al., 1991; Moskvina et al., 2003; Lechner et al., 2004).

However, a limited number of studies report direct measurements of released ATP or other purines upon membrane depolarization in PC12 cells. Instead, content and secretion of catecholamines (epinephrine and NE) and adenine nucleotides (AMP, ADP, ATP, and adenosine) have been analyzed in adrenal chromaffin cells from different animals. For example, early studies have suggested that the 'heavy' granules in chromaffin cells are rich in nucleotides (primarily AMP) that are involved in the acute secretory process (Douglas et al., 1965); in this study efflux of nucleotides (i.e., ATP, ADP, and AMP) was measured by spectrophotometric and fluorimetric techniques in cat adrenal gland perfusates upon stimulation of the entire gland with acetylcholine, nicotine, carbamoyl choline or high K⁺. Similar observations were made by stimulating the adrenal medulla through the splanchnic nerve and it was concluded that AMP and related substances (ADP and ATP) are released from 'heavy', nucleotide-rich, chromaffin granules (Douglas & Poisner, 1966); in this study adenosine was determined spectrophotometrically with adenosine deaminase, AMP was determined by enzymatic assays, and ATP was determined by the luciferase method. Early studies have also shown uptake of [³H]-labeled nucleotides in chromaffin granules (Aberer et al., 1978); Weber & Winkler, 1981). Studies in cultured chromaffin cells have shown that [³H]ATP and [³²P]ATP are distributed differently within the cells (vesicles vs. cytosol) and

stimulation of cell cultures with nicotine or scorpion venom released catecholamines and [^3H] or [^{32}P] in different ratios (Corcoran et al., 1986). Therefore, the choice of radiolabelled tracer may affect interpretation of results. In bovine adrenal chromaffin cells Todorov et al., 1996 demonstrated with HPLC techniques that KCl- or nicotinic acetylcholine receptor activation-evoked release of purines (calculated as sum of ATP, ADP, and AMP) and catecholamines (calculated as sum of epinephrine and NE) occurred with the same time course and constant ratio, suggesting that both purines and catecholamines originated from a homogenous population of exocytotic vesicles. In this study the overflow of individual purines tested was ADP>AMP>ATP, but no significant overflow of adenosine was observed. Therefore, conclusions about intracellular sources of nucleotides in chromaffin cells appear to depend on the detection methodologies.

It is believed that PC12 cells have high concentrations of ATP (up to 125 mM) in dense-core vesicles (Njus et al., 1986). Others have proposed that P2X1 receptor-mediated currents in PC12 cells correspond to vesicular ATP release (Hollins & Ikeda, 1997), although release of ATP was not measured directly. Membrane depolarization-dependent release of ATP from PC12 cells has been demonstrated using the luciferin-luciferase assay (Fabbro et al., 2004; Mahapatra et al., 2006). Sawada et al., 2008 also demonstrated with luciferin-luciferase membrane depolarization-dependent release of ATP from PC12 cells that was dependent on intact SLC17A9. In fact, PC12 cells were instrumental in validating SLC17A9 as a vesicular nucleotide transporter (Sawada et al., 2008). Using activation of P2Y12 receptor as a biosensor for nucleotide release, it was suggested that spontaneous nucleotide release from PC12 cells occurs independently of vesicle exocytosis, whereas depolarization-evoked nucleotide release relies predominantly on exocytosis mechanisms (Hussl et al., 2007); however, the nature of released nucleotides was not determined in this study. Gardner et al., 2005 demonstrated using HPLC-FLD that ω -conotoxin GVIA had no effect on the KCl-evoked release of ATP in PC12 cells. In addition, they showed that endothelin-1 differentially modulated the release of ATP and dopamine, suggesting different sites or mechanisms of release of these substances in PC12 cells. Using HPLC techniques with electrochemical and fluorescence detection Yamboliev et al., 2009 evaluated simultaneously secretion of dopamine, ATP, ADP, AMP, adenosine, NAD^+ , and ADPR from NGF-differentiated rat PC12 cells. They demonstrated that ATP is co-stored with dopamine in both small synaptic vesicles and large dense core vesicles in PC12 cells, but unlike dopamine the release of ATP was not dependent on intact SNARE protein synaptosomal-associated protein 25 (SNAP-25). It is important to note that NAD^+ was also found present in both small synaptic vesicles and large dense core vesicles and, like dopamine, membrane depolarization evoked release of NAD^+ via SNAP-25-mediated exocytosis (Fig. 2). Therefore, this study strengthens the argument that NAD^+ might be a novel neurotransmitter in sympathetic neurons as suggested by studies in vascular and visceral smooth muscles (Smyth et al., 2004; Mutafova-Yambolieva et al., 2007; Hwang et al., 2011). This study also demonstrated that neurotransmitters may have different preferential sites of release, including sites associated with SNAP-25 (dopamine and NAD^+) and sites non-associated with SNAP-25 (ATP).

3.3.4. Urinary bladder—Purine neurotransmitters also appear to be released from parasympathetic motor nerves (together with ACh) and sensory nerves in the bladder. In guinea pig urinary bladder exogenous ATP caused a contraction that was similar to the non-cholinergic contraction evoked by nerve stimulation. ATP was more potent than ADP, GTP, and inosine triphosphate, and AMP and adenosine caused relaxation (Burnstock et al., 1972). Therefore ATP was proposed to be the excitatory cotransmitter of ACh causing non-cholinergic contractions in the bladder (Burnstock et al., 1972; Dean & Downie, 1978; see Burnstock, 2013d) through activation of P2X (mainly P2X1) receptors (Vial & Evans, 2000). It was also suggested that a purine that is released from 6-hydroxydopamine-sensitive nerves contributes to the excitatory motor transmission in the guinea-pig urinary bladder (Westfall et al., 1983). In mice, neurotransmission to urinary bladder detrusor muscle consists of almost equal muscarinic and purinergic components, but in healthy adult humans neurotransmission to detrusor appears to be mediated primarily by muscarinic transmission (Sibley, 1984). Likewise, Tagliani et al., 1997 reported that atropine-resistant contractions in human bladder detrusor muscle were unaffected by inhibition of purinergic receptors with suramin or pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), and suggested that perhaps atropine caused incomplete block of cholinergic transmission or that the atropine-resistant contractions are mediated by another non-cholinergic, non-purinergic substance. In fact, the reports about contribution of ATP to motor neurotransmission in the bladder are somewhat conflicting. For example, it was suggested that the purinergic component of human bladder contraction increases with age (Yoshida et al., 2004), but a recent study in human bladder found no evidence of that (Fry et al., 2011). It was also suggested that the purinergic component of human bladder contractions increases in bladder disease (e.g. interstitial cystitis, outflow obstruction and neurogenic bladder). For example, bladder preparations from interstitial cystitis patients showed increased sensitivity of contractions to purine receptor desensitization with α,β -methylene ATP (Palea et al., 1993). Likewise, bladder detrusor preparations from obstructed bladders demonstrated significant atropine-resistant contractions that were abolished by TTX or by α,β -methylene ATP (Bayliss et al., 1999). P2X1 receptors are believed to be the predominant receptor mediating purinergic contractions in human bladder, and their expression appears to be increased in obstructed bladders compared with control bladders (O'Reilly et al., 2001; O'Reilly et al., 2002). The greater atropine-resistant contractions in detrusor from unstable bladders might also be due to reduced extracellular hydrolysis of released ATP (Harvey et al., 2002). However, possible increased release of ATP in such bladders has not been ruled out. For example, Yoshida et al., 2001 reported that postjunctional contractile responses to exogenous ATP were not changed in older human bladder detrusor muscles, but atropine-resistant contractions to EFS increased with age and were inhibited by α,β -methylene ATP. A recent study compared contractions elicited in isolated human detrusor smooth muscle preparations by EFS, agonist application (carbachol and α,β -methylene ATP), and high K^+ solutions or direct muscle electrical stimulation and concluded that in the human bladder there is no evidence for a decline of intrinsic muscle contractility or excitability as a function of age, gender, or presence of pathology, but in the pathology groups (i.e., bladder outlet obstruction and idiopathic detrusor overactivity) there was evidence for a decline of functional innervation with age (Fry et al., 2011). This study also demonstrated that

atropine-resistant and α,β -methylene ATP-sensitive contractions were greatest in samples of bladders from patients with idiopathic detrusor overactivity (Fry et al., 2011).

In general, the experimental approaches that have supported the idea that ATP is the non-cholinergic excitatory neurotransmitter in the bladder are similar to those discussed for other smooth muscles, and include almost exclusively analyses of smooth muscle responses to nerve stimulation or exogenous ATP. In addition to mechanical studies, electrophysiological approaches have also been used to evaluate purine-mediated responses in the bladder. For example, local Ca^{2+} transients that were resistant to atropine but were inhibited by P2X receptor desensitization with α,β -methylene ATP or by suramin are proposed to be elementary signals of ATP release from nerve varicosities (Heppner et al., 2005). Spontaneous EJPs that are mediated by P2X1 receptors are used to characterize neurogenic release of ATP in the mouse urinary bladder (Young et al., 2008). The purinergic pathway appears to play a greater role at lower stimulation frequencies (Werner et al., 2007).

A limited number of studies have attempted to detect directly neurogenic release of ATP and other purines. Using the luciferase bioassay, Chaudhry et al., 1984 demonstrated EFS-evoked release of ATP in rabbit detrusor muscle strips, but this release was entirely resistant to TTX. Therefore, it was concluded that ATP is not a non-adrenergic non-cholinergic neurotransmitter in the rabbit bladder. However, in the guinea-pig urinary bladder Burnstock et al., 1978a demonstrated using the firefly luciferin-luciferase reaction nerve stimulation-evoked release of ATP that was inhibited by TTX. Based on quinacrine binding as well as chemical sympathectomy with 6-hydroxydopamine or guanethidine, it was suggested that ATP was likely released from a population of nerves that is different from adrenergic or cholinergic nerves, called purinergic nerves (Burnstock et al., 1978a). The presence of excitatory purinergic nerves in the bladder has not been unequivocally demonstrated. Based on indirect evidence from pharmacological studies (see Burnstock, 2013d) it was also suggested that ATP may originate from parasympathetic nerves in the bladder, but this, too, has not been definitively established. Using HPLC detection of etheno-derivatized purines, Tong et al., 1997b reported that in rat urinary bladder muscle strips EFS evokes release of ATP, ADP, AMP, and adenosine. The sum of the four substances, called 'total purines,' was found to depend on the frequency of EFS and to be inhibited by TTX. It should be noted, however, that AMP and adenosine can be formed from other endogenous nucleotides that are released in addition to ATP (see Zimmermann et al., 2012; Durnin et al., 2012b) and therefore presence of AMP and adenosine does not reflect exclusively release of ATP. Using similar detection methodologies (i.e., HPLC coupled with fluorescence detection) overflow of purines including ATP, ADP, AMP, adenosine, NAD^+ , cyclic ADPR, and ADPR was reported in bladder detrusor smooth muscles of dog, rabbit, guinea-pig, rat, and mouse at rest and evoked by nerve stimulation (Smyth et al., 2004). This was also the first study to demonstrate neural release of NAD^+ in bladder and blood vessels. In the mouse bladder EFS evoked TTX-sensitive release of both ATP and NAD^+ (Durnin & Mutafova-Yambolieva, 2011). Neuronal release of purines (i.e., ATP, ADP, AMP, adenosine, NAD^+ , ADPR, and negligible amounts of cyclic ADPR) was also demonstrated in human bladder detrusor muscle preparations (Breen et al., 2006). EFS evoked a particularly prominent release of NAD^+ , which exceeded greatly the release of ATP (Fig. 3A), and was inhibited by TTX, ω -conotoxin GVIA, and botulinum neurotoxin A, but not by guanethidine. Therefore, NAD^+

likely originated from non-sympathetic nerves. Capsaicin increased the overflow of NAD^+ , suggesting that sensory nerves may at least in part participate in release of NAD^+ , but at present the nerves that release NAD^+ in the bladder are not defined. Interestingly, NAD^+ reduced the frequency and amplitude of contractions in human detrusor smooth muscle strips (Fig. 3B), suggesting that NAD^+ is rather an inhibitory factor in the human bladder. This might be a particularly important observation considering the recently proposed purinergic inhibitory regulation of murine detrusor muscles that is mediated by P2Y1 receptors and SK channels on platelet-derived growth factor receptor α -positive (PDGFR α^+) interstitial cells (Lee et al., 2014). These cells appear to be associated with varicose nerve processes in detrusor muscles, suggesting that the cells are likely innervated and receive neurotransmitter signals (Koh et al., 2012a; Monaghan et al., 2012). Earlier studies also demonstrated that EFS or exogenous ATP induces a biphasic response consisting of a P2X-mediated contraction followed by a sustained P2Y-mediated relaxation in urinary bladders from mice (Boland et al., 1993), rats (Bolego et al., 1995; Aronsson et al., 2010), marmoset (McMurray et al., 1998), and pigs (Tong et al., 1997a; Noda et al., 2002; Hernandez et al., 2009). Nerve stimulation-evoked bladder relaxation was not associated with NO, peptides, K^+ channel openers, or activation of β -adrenoceptors in the trigone and proximal urethra of pig bladder (Noda et al., 2002) and in the pig bladder neck, P2Y1 receptors were proposed to partially mediate the relaxations to endogenously released purines (Hernandez et al., 2009). P2Y1 receptors were proposed to be involved in inhibition of detrusor tone and the micturition reflex in urethane-anaesthetized rats (King et al., 2004). Likewise, apamin-sensitive small-conductance Ca^{2+} -activated K^+ channels, SK channels, appear to be involved in ongoing or tonic inhibition of bladder muscles (Herrera et al., 2003; Thorneloe et al., 2008). NAD^+ (and its primary extracellular metabolite ADPR) was found to activate P2Y1 receptors in GI smooth muscles and to cause activation of SK channels and hyperpolarization (Mutafova-Yambolieva et al., 2007; Hwang et al., 2011; Durnin et al., 2012b). NAD^+ also activates P2Y1 receptors and SK channels in PDGFR α^+ cells from murine colon (Kurahashi et al., 2011). Therefore, NAD^+ might contribute to inhibitory neural regulation of bladder excitability by activation of P2Y1 receptors and SK channels in PDGFR α^+ cells. It should be pointed out that AMP and adenosine also cause relaxation of rat urinary bladder (Brown et al., 1979). Therefore, additional pathway that could be involved in bladder inhibition by NAD^+ or ADPR might be through AMP and adenosine that are produced from released NAD^+ . The functional relevance of such mechanisms could be that neurogenic NAD^+ and ADPR may participate in the purinergic mechanisms of stabilization of excitability during bladder filling (Lee et al., 2014).

As in blood vessels, ATP was found to be released from multiple cell types in the bladder, the urothelium being the most prominent source. Thus, using luciferase bioluminescence assay Ferguson et al., 1997 demonstrated that EFS of rabbit bladder strips or mechanical stretch released ATP. Release occurred from urothelial cells and was not vesicular, as it was increased in the absence of Ca^{2+} and was not abolished by TTX. Vlaskovska et al., 2001 also demonstrated using the luciferase bioluminescence assay that ATP was released from urothelial cells during distention in mouse bladder and the amount of ATP released was proportional to the extent of distention. Stretch-evoked TTX-insensitive ATP release was also detected in pig and human bladders (Kumar et al., 2004). Thus it was proposed that

ATP is released from urothelium as a sensory mediator for the degree of distension of the bladder. It was also proposed that distension of bladder leads to release of ATP from lining of epithelium which then acts on P2X2/3 receptors on subepithelial sensory nerves to convey information to the central nervous system (CNS). Therefore ATP released from urothelium would be able to initiate bladder voiding (Burnstock, 1999). P2X3 receptors are present in urothelium and specifically on afferent nerve fibers. P2X3 receptor knockout mice had marked urinary bladder hyporeflexia with reduced voiding frequency and increased voiding volume, suggesting that these receptors are involved in mechanosensory transduction underlying activation of afferent fibers that control voiding reflexes during bladder filling (Cockayne et al., 2000). Age-related changes in afferent pathways were demonstrated in male mouse bladder (Daly et al., 2014): bladders from aged mice showed increased voiding frequency and enhanced low-threshold afferent nerve activity concurrent with increased purinergic receptor sensitivity and raised P2X3 receptor expression in the urothelium and increased levels ATP, but decreased levels of acetylcholine in the bladder lumen. Increased stretch-activated ATP release from bladder urothelium was demonstrated in patients with interstitial cystitis (Sun et al., 2001), painful bladder syndrome (Kumar et al., 2007), and idiopathic detrusor overactivity (Kumar et al., 2010). In general, the major source of ATP in bladders following mechanical stretch (which is probably a more physiological stimulus) is nonneuronal (Kumar et al., 2007). Urothelial ATP release following mechanical stretch shows greater sensitivity to TTX in bladders from patients with neurogenic detrusor overactivity (Kumar et al., 2010), suggesting that this condition might be associated with increased release of ATP from neuronal sources. In a recent study, intravesical instillation of onabotulinumtoxin A in distended mouse bladder inhibited ATP release from the urothelium, but the site of action of this neurotoxin was not determined (Collins et al., 2013). Interestingly, the concentrations of ATP in the bladder lumen are lower in patients with refractory overactive bladder with bacteriuria than in patients without bacteriuria, but the underlying mechanisms are currently unknown (Walsh et al., 2013). It should be noted that the majority of studies on purine release upon distension use the luciferase bioluminescence assay that is restricted to ATP. Therefore, at present it is unclear whether or not other adenine nucleotides are released from bladder urothelium and contribute to processes triggered by mechanical stretch during bladder filling. Bladder urothelium is also a source of non-neuronal acetylcholine (Yoshida et al., 2006; Collins et al., 2013; see Birder & Andersson, 2013).

3.4. In enteric nervous system

Since the first observations of NANC hyperpolarization and relaxation in the guinea-pig taenia coli (Burnstock et al., 1964; Burnstock et al., 1966) and the proposal that ATP or a related purine is a NANC neurotransmitter in GI smooth muscle and other systems (Burnstock et al., 1970; Burnstock, 1972), a large number of studies have been focused on characterization of purinergic electrophysiological, mechanical, and secretory responses in the GI tract (see Burnstock, 2013c). In the enteric nervous system (ENS) purines can act as inhibitory neurotransmitters from motor neurons to the circular smooth muscle (via P2Y receptors), excitatory neurotransmitters between enteric interneurons and from interneurons to motor neurons (via P2X and P2Y receptors), and sensory mediators from epithelial cells to intrinsic sensory nerve terminals (see Galligan et al., 2000, Bertrand, 2003; Galligan &

North, 2004; Bornstein, 2008; Roberts et al., 2012; Burnstock, 2013c). In addition, adenosine through P1 receptors modulates release of neurotransmitters, responsiveness of smooth muscle cells to extracellular stimuli, and intestinal motility (Christofi & Wood, 1993; Christofi et al., 2001; Duarte-Araujo et al., 2004; Correia-de-Sa et al., 2006; Bornstein, 2008; Zizzo et al., 2011). Therefore purines participate in essentially all aspects of intestinal function, from the sensory transduction of stimuli from the gut lumen to the subsequent initiation and propagation of enteric reflexes.

3.2.1. Inhibitory motor neurotransmission—On the subject of motility control it is now recognized that purines released during stimulation of myenteric motor nerve terminals mediate relaxation of circular smooth muscle, which is necessary for functions such as sphincter relaxation, gastric accommodation, and the peristaltic reflex (see Bennett, 1997). Spontaneous purine release from motor neurons may mediate constant tonic inhibition of the circular muscle at rest (Dickson et al., 2010b), which keeps the muscle in a hyperpolarized state for normal transit and stool formation (Dickson et al., 2010a). Purines have also been suggested to regulate synaptic activity and strength by acting as retrograde transmitters that facilitate acetylcholine release in the myenteric plexus (Hons et al., 2012), or to participate in neuron-to-glia signaling (Gulbransen & Sharkey, 2009; Gulbransen et al., 2010).

Purinergic neuromuscular transmission participates in the peristaltic reflex by contributing to relaxation of the receiving segment of the bowel, and purines appear to be the primary inhibitory neurotransmitter(s) in the distal parts of the GI tract, including the large intestine, particularly in humans (Hwang et al., 2011). Stimulation of colonic muscles results in inhibitory junction potentials (IJs) that consist of a fast purinergic component and a slower nitrergic component (Pluja et al., 1999; Xue et al., 1999; Serio et al., 2003; Hirst et al., 2004; Gallego et al., 2006; Mutafova-Yambolieva et al., 2007; Gallego et al., 2008a; Zhang et al., 2010; Hwang et al., 2011). IJs are the electrophysiological basis for smooth muscle relaxation. Purine-mediated relaxation in GI smooth muscles from various species has been documented (De Man et al., 2003; Gallego et al., 2006; Mutafova-Yambolieva et al., 2007; King & Townsend-Nicholson, 2008). The purinergic relaxation likely contributes to propulsive motility because P2Y1 receptor inhibition reduces pellet velocity in guinea-pigs (Strong et al., 2010) and pellet transit is significantly delayed in colon segments from *P2ry1*^{-/-} mice (Hwang et al., 2012). Purinergic IJs are significantly attenuated, while the slow nitrergic IJs remain intact in experimental models of colitis (Strong et al., 2010; Roberts et al., 2013), suggesting that aberrant purinergic neurotransmission might contribute to motility disorders in disease states. The fast IJs are sensitive to apamin (Taylor & Bywater, 1986; Bridgewater et al., 1995; Rae & Muir, 1996; Spencer et al., 1998b; Gallego et al., 2006; Mutafova-Yambolieva et al., 2007) and are therefore coupled to the opening of SK channels. The fast IJs are also mediated by the P2Y1 receptor, because they are inhibited by selective P2Y1 receptor antagonists (Gallego et al., 2006; Mutafova-Yambolieva et al., 2007; Wang et al., 2007; Gallego et al., 2008b; Hwang et al., 2011) and absent in colonic preparations from *P2ry1*^{-/-} mice (Hwang et al., 2012; Gallego et al., 2012). Therefore, the signal transduction mechanisms underlying the purinergic IJs involve activation of P2Y1 receptors and subsequent stimulation of phospholipase C and Ca²⁺ release from intracellular stores which then activates SK channels (Bayguinov et al., 2000;

Kong et al., 2000; Bowler et al., 2003; Zizzo et al., 2006; Wang et al., 2007). Non-nitroergic apamin-resistant IJPs were also described in the mouse colon (Spencer et al., 1998a) and apamin-resistant P2Y/P2Y1 receptor-mediated IJPs were described in human colon (Gallego et al., 2006; Gallego et al., 2008a; Hwang et al., 2011) and in mouse internal anal sphincter (McDonnell et al., 2008) and colon (Zhang et al., 2010). The nature of this additional ion conductance that is associated with P2Y receptor activation in GI muscles remains to be determined. Collectively, the discovery that the endogenous non-nitroergic inhibitory neurotransmission in the GI tract is mediated by P2Y1 receptors coupled with K⁺ channels on effector cells represents a significant advance of knowledge and provides strong support to the purinergic neurotransmission concept in the GI system. However, as in other systems, characterization of postjunctional responses does not shed light on the identity of the purine neurotransmitter. The purine that is responsible for purinergic neurotransmission in the gut is still controversial. Although ATP is a long standing candidate for transmission (e.g., Burnstock, 2011a) recent studies have demonstrated that in the gut other purines meet criteria for a neurotransmitter better than ATP (discussed below).

3.2.2. Purinergic inhibitory neurotransmitters—As in other systems, the notion that ATP is a neurotransmitter in the motor nerves has been developed primarily from pharmacological characterization of integrated responses of effector cells (i.e., IJPs and smooth muscle tone) to neuronal stimulation or analysis of responses to exogenous purines (most frequently ATP) and synthetic analogues. In a similar fashion, the notion that ATP is a neurotransmitter in interneurons has been based primarily on pharmacological characterization of synaptic potentials, application of ATP, expression and localization of P2 receptors in ENS, and electrophysiological characterization of P2X receptors (see Galligan, 2002a; Galligan, 2002b; Ren & Bertrand, 2008). Occasionally release of purines in response to nerve stimulation in smooth muscles from GI tract has been measured. For example, an early study demonstrated uptake of tritiated ATP, ADP, AMP, and adenosine that exceeded the uptake of tritiated adenine and inosine in guinea-pig taenia coli (Su et al., 1971). [³H] was released upon transmural stimulation of tissues preincubated with [³H]-adenosine, the [³H] overflow was reduced by guanethidine, and approximately 60% of [³H]-adenosine was converted to and retained as [³H]-ATP. It was concluded that ATP can be released from both non-adrenergic and adrenergic inhibitory nerves in the taenia coli. Later Burnstock et al., 1978b reported, using the luciferin-luciferase assay, that ATP release from guinea-pig taenia coli during nerve stimulation was TTX-sensitive. In contrast, White et al., 1981, also using luciferin-luciferase assay, reported TTX-resistant release of ATP in guinea pig taenia coli. White & Leslie, 1982 demonstrated using the firefly luciferin-luciferase technique high K⁺ depolarization- and veratridine-induced release of ATP from guinea-pig small intestine preparations enriched with myenteric varicosities. The high K⁺-evoked release appeared to be Ca²⁺-dependent, as release was reduced in the presence of EGTA to chelate Ca²⁺. However, as pointed out by the authors, EGTA also reduces the sensitivity of the luciferin-luciferase assay, which makes it unclear whether reduced ATP was due to reduced extracellular Ca²⁺ or to methodological aberrations. The veratridine-evoked release of ATP was sensitive to TTX, indicating that it was mediated by Na⁺ channel openings. The authors concluded that ATP might be released from myenteric varicosities as a typical neurotransmitter, but they also suggested that ATP might also be released during vesicular

release of the primary neurotransmitters acetylcholine and NE, since ATP is found stored with acetylcholine and NE within their respective synaptic vesicles (Dowdall et al., 1974; Geffen & Livett, 1971). Release of other purines was not determined in these studies. Neuronal release of adenosine has also been suggested in ENS. For example, using adenosine specific microelectrode biosensors, EFS-evoked TTX-sensitive release of adenosine in myenteric plexus-longitudinal muscle preparations of the guinea-pig ileum was detected (Begg et al., 2002). Likewise, Ca^{2+} - and action potential-dependent release of adenosine was reported from myenteric plexus-longitudinal muscle preparations isolated from rat ileum using HPLC detection techniques (Correia-de-Sa et al., 2006). Using HPLC methodologies coupled with fluorescence detection McConalogue et al., 1996 showed frequency-dependent and TTX-sensitive release of purines from guinea-pig taenia coli. The release of purines (ATP, ADP, AMP, and adenosine) in this study was also reduced by atropine. However, recent studies in mouse (Mutafova-Yambolieva et al., 2007) and human and monkey colons (Hwang et al., 2011), using HPLC methodologies with fluorescence detection, demonstrated that the EFS-evoked release of ATP was not frequency-dependent and was not inhibited by TTX or ω -conotoxin GVIA. Therefore, ATP was released during EFS, but it did not meet essential presynaptic criteria for a neurotransmitter in these smooth muscles, suggesting that the majority of ATP might be released from sites different from nerve varicosities. It is important to note that EFS of murine, monkey, and human colons also caused overflow of NAD^+ and ADPR in addition to ATP, ADP, AMP, and adenosine. In contrast to ATP, the release of NAD^+ was frequency-dependent and was inhibited by TTX and ω -conotoxin GVIA. Notably, in both studies all purines, including ATP and NAD^+ , were analyzed simultaneously in the same tissue superfusate samples. Therefore, in the mouse, monkey and human colon NAD^+ appeared to meet important presynaptic criteria for a neurotransmitter better than ATP (Mutafova-Yambolieva et al., 2007; Hwang et al., 2011). Moreover, studies in primate colons have demonstrated that in preparations containing only nerve processes and varicosities (i.e., neurotransmitter release sites) the release of NAD^+ , but not of ATP, met presynaptic criteria for a neurotransmitter (Hwang et al., 2011). CD38, the primary protein that degrades extracellular NAD^+ (De Flora et al., 2004), was found present in the mouse colon (Mutafova-Yambolieva et al., 2007). Exogenous NAD^+ was also shown to be rapidly metabolized in mouse and primate colons (Durnin et al., 2012b). Therefore, mechanisms for NAD^+ degradation and removal appear to be present in the colon. ADPR, a primary extracellular metabolite of NAD^+ (Lee, 2001), was also found released in response to EFS of mouse, monkey, and human colons (Mutafova-Yambolieva et al., 2007; Hwang et al., 2011). Therefore together, NAD^+ and ADPR are strong candidates for mediators of neurotransmission. It remains to be determined whether ADPR is only a metabolite of released NAD^+ or ADPR can also be released as an independent purine neurotransmitter.

Electrical field stimulation is a common approach for stimulating neural activity, but it often fails to differentiate the precise sources of released molecules. For example, EFS could trigger release of substances from neuronal cell bodies or axons or from non-neuronal sources such as glia. Durnin et al., 2013 used an alternative method to stimulate motor neurons in the ENS by selectively activating ligand-gated ion channel receptors localized on cell bodies of myenteric motor neurons. Activation of these receptors stimulated the release

of ATP and NAD⁺ via different mechanisms and thus likely from different parts of the neuron. The release of NAD⁺ required nerve action potentials and Ca²⁺ influx via N-type voltage-dependent Ca²⁺ channels, verifying release from nerve terminals. However the majority of ATP appears to be released directly from the nerve cell bodies or glia, as its release was not altered by TTX or ω -conotoxin GVIA and hence did not require action potential propagation to the nerve terminal (Durnin et al., 2013). Figure 4 illustrates the differential release of NAD⁺ and ATP upon stimulation of serotonergic 5-HT₃ receptors in non-human primate colon (Durnin et al., 2013). Monitoring purine release caused by direct stimulation of enteric motor nerve cell bodies represents a significant refinement of the experimental approach demonstrating that ATP is unlikely to be the motor neurotransmitter in the colon. This study also demonstrates the need of further research to better understand this inhibitory pathway in the gut (Smith, 2013). Release of ATP from nerve cell bodies was also suggested in another recent study in mouse colon. In a mouse model of experimental colitis, chronic activation of neuronal P2X7 receptors led to activation of pannexin-1 channels, expressed on neuronal cell bodies and processes, and to release of ATP through pannexins, which then activated enteric glia (likely via its extracellular conversion to ADP). In this study, inhibiting ATP degradation with sodium polyoxotungstate (POM1) reduced glial activation and therefore the authors concluded that the Ca²⁺ responses in glia were mediated by ADP that was produced from released ATP. The Ca²⁺ responses elicited in glial cells were not sensitive to TTX, suggesting action potential-independent release of ATP from neuronal cell bodies and/or processes (Gulbransen et al., 2012).

NAD⁺ and ADPR were not only demonstrated to meet presynaptic criteria for candidate neurotransmitters, but were also found to mimic postjunctionally the endogenous purine neurotransmitter better than ATP. Thus locally applied NAD⁺, ADPR, ATP, and ADP caused membrane hyperpolarization in circular muscle of murine (Mutafova-Yambolieva et al., 2007; Hwang et al., 2012) or primate (Hwang et al., 2011; Durnin et al., 2012b) colons. However, only the responses to NAD⁺ and ADPR were inhibited by selective and specific P2Y1 receptor antagonists (Mutafova-Yambolieva et al., 2007; Hwang et al., 2011) and were absent in colons from *P2ry1*^{-/-} mice (Hwang et al., 2012), as were the fast IJPs in response to endogenously released neurotransmitter. In contrast, responses to ATP (Mutafova-Yambolieva et al., 2007; Hwang et al., 2011) or ADP (Durnin et al., 2012b) were not affected by P2Y1 receptor antagonists in colon muscles. Likewise, colon muscles from *P2ry1*^{-/-} mice responded with hyperpolarization to locally applied ATP or ADP, but not to EFS (Hwang et al., 2012). These are surprising observations considering the fact that ADP and ATP are well-known potent agonists of the P2Y1 receptor (see von Kugelgen, 2006). ATP appeared to be even a more potent activator of Ca²⁺ transients in HEK293 cells transfected with guinea-pig P2Y1 receptor cDNA than NAD⁺ (Mutafova-Yambolieva et al., 2007), but it is unknown whether this is true for the mouse, monkey or human P2Y1 receptor. Most likely exogenous ATP and ADP activate one or more additional receptors that mediate membrane hyperpolarization in colonic muscles. NAD⁺, applied with the perfusion solution in murine colon, caused membrane hyperpolarization that was inhibited by the selective P2Y1 receptor antagonist (1*R**,2*S**)-4-[2-Iodo-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate (MRS2500) (Gallego et al., 2012). In a similar fashion, previous studies demonstrated that

local application of NAD⁺ from a pressure pipette (to mimic conditions of released neurotransmitter) caused hyperpolarization that was mediated by P2Y1 receptors (Mutafova-Yambolieva et al., 2007). A large part of the hyperpolarization response to NAD⁺ applied in perfusion solution was eliminated in colons from P2Y1 receptor knockout mice, but a small response (~3 mV hyperpolarization) remained unchecked (Gallego et al., 2012). It was suggested that the residual response to NAD⁺ was likely mediated by additional receptors. Gil et al., 2013 reported that the hyperpolarization responses to NAD⁺, applied with the perfusion solution, persisted in cecum from P2Y1 receptor knockout mice. The authors suggested that receptors different from P2Y1 receptors mediate the hyperpolarization response to exogenous NAD⁺. Responses to ATP, ADP, or other authentic purines or NAD⁺ metabolites in murine cecum were not demonstrated in this study. Purine release measurements or identification of NAD⁺ metabolites and characterization of their activity in the cecum might help to understand the discrepancy between EFS evoked purine-mediated responses in murine cecum (Gil et al., 2013) and colon (Mutafova-Yambolieva et al., 2007; Gallego et al., 2012; Hwang et al., 2012; Durnin et al., 2012b). It is currently unknown whether enteric inhibitory purinergic neurotransmission in different parts of the GI system could be served by different neurotransmitter substances as discussed in Goyal et al., 2013.

3.5. In central nervous system

3.3.1. In neurons—Early hints of a potential role of ATP in the CNS transmission came from studies in the late 1970s using the firefly luciferin-luciferase assay. These studies demonstrated that ATP was released from isolated rat whole brain synaptosomes when stimulated with high K⁺ solutions or with the Na⁺ channel activator, veratridine (White, 1977; White, 1978). The veratridine-evoked release of ATP was sensitive to TTX. Release was also thought to be partially Ca²⁺-dependent as it was reduced by the Ca²⁺ chelator EGTA (White, 1977). Early studies also measured uptake and release of [¹⁴C]- and [³H]-labeled nucleotides in cortical and hypothalamic synaptosomes to suggest that purines are released from neuronal structures in the CNS (Kuroda & McIlwain, 1974; Fredholm & Vernet, 1979). These were followed by studies showing Ca²⁺-dependent release of ATP during high K⁺-induced membrane depolarization in guinea-pig and rat cortical synaptosomes (White et al., 1980; Salgado et al., 1997), rat and mouse hippocampal synaptosomes (Terrian et al., 1989; Wieraszko et al., 1989), and dorsal spinal cord synaptosomes (Sawynok et al., 1993). Experiments in the early 1980s demonstrated that exogenously applied ATP could elicit inward currents in dorsal horn neurons (Jahr & Jessell, 1983) and in isolated sensory neurons (Krishtal et al., 1983), but it appeared that only subpopulations of neurons responded to ATP. For example, 27% of dorsal horn neurons responded to exogenous ATP whereas 96% of neurons responded to glutamate application (Jahr & Jessell, 1983). However, no evidence for purinergic neurotransmission at central synapses existed at the time that these studies were performed therefore they could not support a potential neurotransmitter role of released ATP in the CNS. It wasn't until 1992 that the first endogenous P2 receptor-mediated currents were recorded in rat medial habenula neurons (Edwards et al., 1992). This study demonstrated that, in the presence of inhibitors of glutamate, γ -aminobutyric acid, and acetylcholine receptors, electrical stimulation produced TTX-sensitive fast postsynaptic excitatory currents. The currents were sensitive to suramin and to P2X receptor desensitization with α,β -methylene-ATP and were

mimicked by local fast application of α,β -methylene-ATP (Edwards et al., 1992). Since the currents were mediated by P2X receptors it was concluded that ATP was a fast neurotransmitter in the rat medial habenula (Edwards et al., 1992). Since then P2X-receptor-mediated synaptic currents have been identified in a number of brain regions including the locus coeruleus (Nieber et al., 1997), hippocampus (Pankratov et al., 1998; Mori et al., 2001), spinal neurons (Bardoni et al., 1997; Jo & Schlichter, 1999), cultured coeliac ganglion neurons (Silinsky et al., 1992; Evans et al., 1992), hypothalamic neurons (Jo & Role, 2002), and the somatosensory cortex (Pankratov et al., 2002; Pankratov et al., 2003), although some researchers question the significance of fast purinergic signaling in the brain due to the small, infrequent currents activated by purines that require strong stimuli to evoke (see Khakh & North, 2012). In all of these studies mimicry of the nerve-evoked response by exogenous ATP and inhibition of the response by P2 receptor antagonists caused researchers to conclude almost exclusively that ATP was the purine responsible for excitatory currents in the brain (see Burnstock, 2013a). However, none of these studies measured directly release of ATP or other purines.

In rat hypothalamic slices, electrical stimulation evoked the release of ATP and [^3H]-NE during low and high frequency stimulation (Sperlagh et al., 1998). The release of both ATP and [^3H]-NE was frequency-dependent and Ca^{2+} -sensitive and the majority of release was inhibited by TTX, which suggested that ATP and NE might be co-released in the hypothalamus. However, in other brain regions the release of ATP did not appear to be vesicular. For example, in guinea-pig cortical synaptosomes White et al., 1980 found that the release of ATP was unaffected by cleavage of SNAP-25 with botulinum neurotoxin A, whereas the release of acetylcholine in the same preparations was reduced by 60%. In rat brain slices, Hamann & Attwell, 1996 demonstrated that the release of ATP during stimulation of the hippocampus, cerebellum, or habenula was neither Ca^{2+} -dependent nor TTX-sensitive. Salgado et al., 1997 used the scorpion toxin tityustoxin to depolarize rat brain cortical synaptosomes by stimulating Na^+ channels and found that the release of ATP was only partially sensitive to TTX. In each of these studies the release of ATP was measured using the firefly luciferin-luciferase assay. Using single-photon imaging microscopy in conjunction with the firefly luciferin-luciferase assay, which improves spatial and temporal resolution of ATP measurements (Wang et al., 2000; Fields, 2011b), a recent study demonstrated that stimulation of axons of dorsal root ganglion neurons evoked TTX-sensitive and Ca^{2+} -dependent ATP release (Fields & Ni, 2010). Interestingly, however, the release of ATP was not affected by pretreatment of neurons with bafilomycin A1 to inhibit the proton gradient of synaptic vesicles or by botulinum neurotoxin, indicating that release was not vesicular. Instead, activation of voltage-gated anion channels during stimulation-evoked axon swelling released ATP, demonstrating again a non-vesicular mechanism for ATP release during action potential firings in dorsal root ganglion neurons (Fields & Ni, 2010). It is clear from this study that determining only TTX-sensitivity or Ca^{2+} -dependence of release may not be sufficient to confirm vesicular release of putative neurotransmitter substances from neurons. Using HPLC-FLD methodologies it was recently demonstrated that NAD^+ is released during high- K^+ membrane depolarization in isolated rat forebrain synaptosomes (Durnin et al., 2012a). KCl-evoked release of NAD^+ and ADPR was proportional to the membrane depolarization (i.e., KCl concentration-dependent) and

required intact vesicle exocytosis machinery. Thus release was attenuated by botulinum neurotoxin A, by inhibition of N-type voltage dependent Ca^{2+} channels with ω -conotoxin GVIA, and by disrupting the filling of synaptic vesicles with neurotransmitters with bafilomycin A1 (Durnin et al., 2012a). In cultured cortical neurons, localized application of exogenous NAD^+ (and ADPR) caused rapid Ca^{2+} transients, suggesting that endogenously released NAD^+ could participate in neuron-to-neuron communication in the brain. Application of ATP also caused rapid Ca^{2+} transients, but close examination of cell responses suggested that NAD^+ and ATP likely stimulate different populations of cortical neurons (Durnin et al., 2012a). Increased adenosine overflow in response to high- K^+ stimulation was also detected in brain synaptosome superfusates (Durnin et al., 2012a) and part of it might be generated by released NAD^+ , ADPR, and ATP. Adenosine has been shown to provide inhibitory neuromodulation in the brain and its presence at the extracellular space has commonly been attributed to the presence of 5'-nucleotidase that degrades AMP (formed from ATP) to adenosine (Dowdall, 1978; Richardson et al., 1987; Schoen et al., 1999; Sebastiao et al., 1999). Recent studies have proposed, however, that adenosine is released directly from neurons during electrical stimulation in the hippocampus (Lovatt et al., 2012) and cerebellum (Wall & Dale, 2007). In the hippocampus, 5'-nucleotidase did not play a role in adenosine-mediated synaptic depression, suggesting that adenosine effects are not due to metabolism of nucleotides; adenosine release likely occurred via nucleoside transporters ENT1 and ENT2 expressed on these neurons (Lovatt et al., 2012). In the cerebellum, release of adenosine detected with an adenosine biosensor was frequency-, TTX-, and Ca^{2+} -dependent, whereas release of ATP in the same preparation, detected with an ATP biosensor, could not be measured (Wall & Dale, 2007). Evidently, multiple endogenous purine substances in addition to ATP are released extracellularly and could participate in synaptic transmission in the brain.

3.3.2. In glia—While fast neuron-to-neuron communication mediated by purines in the CNS seems apparent at least in some brain regions, purine nucleotides and nucleosides may play wider roles as neuromodulators of central synaptic function. ATP has emerged as one of the primary extracellular signaling molecules activating Ca^{2+} responses in CNS glia (Guthrie et al., 1999; Bowser & Khakh, 2007) and it has been shown in numerous studies that glial cells themselves are prominent sources of ATP in the brain. Using the firefly luciferin-luciferase assay studies have demonstrated release of ATP during activation of glutamate receptors on cultured astrocytes (Queiroz et al., 1997) or after mechanically stimulating calcium waves in cultured astrocytes (Guthrie et al., 1999; Coco et al., 2003). Using a luciferin-luciferase assay and a biosensor technique Liu et al., 2008 demonstrated that ischemic stress induces ATP release from astrocytes.

Different mechanisms have been reported to regulate the release of ATP from glia. For example, ATP can be released from glia through connexin hemichannels (Cotrina et al., 2000; Stout et al., 2002; Torres et al., 2012), through P2X7 receptor pores (Suadicani et al., 2006), through maxi-anion channels (Liu et al., 2008), via lysosomal exocytosis (Oya et al., 2013), and through volume-regulated anion channels (Darby et al., 2003; Sabirov & Okada, 2005; Lazarowski, 2012). Accumulating evidence suggests that ATP can also be released from glial cells by vesicular exocytosis, as a 'gliotransmitter' (Bal-Price et al., 2002; Coco et

al., 2003; Pascual et al., 2005; Imura et al., 2013; Lalo et al., 2014). For example, using the luciferin-luciferase assay, release of ATP from cultured rat cortical astrocytes was shown to be botulinum neurotoxin-sensitive (Bal-Price et al., 2002). Imura et al., 2013 demonstrated, also with the luciferase assay, vesicular release of ATP from cultured microglia that was dependent on Ca^{2+} , vesicular H^{+} -ATPase, and SNARE proteins, but independent of connexin or pannexin hemichannels. Pascual et al., 2005 used an inducible transgenic mouse line that expresses a dominant-negative SNARE domain selectively in astrocytes to show that release of ATP from hippocampal astrocytes was dependent on intact SNARE proteins and is therefore vesicular. Similarly, Lalo et al., 2014 demonstrated using transgenic mice SNARE protein-dependent release of ATP from acutely isolated mouse cortical astrocytes. In this study, a 'sniffer cell' approach was used to determine ATP release whereby isolated astrocytes were placed over HEK293 cells expressing GFP-tagged P2X2 receptors and currents evoked in HEK293-P2X2 receptor cells after astrocyte stimulation were recorded. Since the responses were mediated by the P2X2 receptor it was concluded that ATP was the purine released. Glial ATP appears to be an important regulator of synaptic strength and plasticity, modulating the activity of both neurons and neighboring glia. ATP released from hippocampal astrocytes is a predominant modulator of intracellular calcium waves in neighboring astrocytes (Bowser & Khakh, 2007). Release of ATP from mouse cortex astrocytes was shown to directly activate P2X-receptor-mediated currents in neighboring neurons (Lalo et al., 2014). In addition, glial ATP can influence synaptic plasticity via its metabolic products. For example, Pascual et al., 2005 demonstrated that adenosine produced from glia-released ATP tonically suppressed synaptic transmission in the hippocampus. NAD^{+} might also have regulatory functions in the brain. For example, Verderio et al., 2001 demonstrated that NAD^{+} is also released from cultured hippocampal astrocytes and early studies proposed a neuromodulatory role of NAD^{+} in the rat and guinea-pig hippocampus (Richards et al., 1983; Snell et al., 1985; Galarreta et al., 1993). Thus, NAD^{+} was found to depress synaptic transmission in hippocampal neurons (Richards et al., 1983; Snell et al., 1985; Galarreta et al., 1993) and to reduce the release of glutamate from synaptic terminals (Galarreta et al., 1993). The mechanisms involved in glial release of NAD^{+} include release through connexin hemichannels (Verderio et al., 2001; Ying et al., 2003) and possibly through P2X₇ receptor pores (Lu et al., 2007). Whether vesicular release of NAD^{+} occurs from glial cells has not been established.

4. Postsynaptic and postjunctional targets of purine neurotransmitters

4.1 Receptor targets

The discovery and cloning of purinergic receptors was a significant advancement in understanding purine signaling mechanisms and reinforced the purinergic neurotransmission hypothesis. Burnstock, 1976 proposed the existence of purine receptors that were distinct from receptors for classical neurotransmitters and that there were likely to be different receptors for nucleotides and nucleosides due to different time course of responses to ATP and ADP vs. adenosine in the intestine (Burnstock, 1972; Burnstock, 1976) and the bladder (Burnstock, 1972; Burnstock, 1978). Later cloning of receptors enabled major breakthroughs confirming endogenous expression of receptors specific to purine nucleotides/nucleosides (Libert et al., 1989; Lustig et al., 1993; Webb et al., 1993; Brake et al., 1994; Valera et al.,

1994). Purine receptors are classified as P1 adenosine receptors and P2 nucleotide receptors. P1 adenosine receptors are G-protein-coupled receptors comprised of A1, A2A, A2B, and A3 receptor subtypes (Fredholm et al., 2001; Abbracchio et al., 2009). Typically A1 and A3 couple to the $G_{i/o}$ family of G proteins which inhibit cyclic AMP production, whereas A2A and A2B subtypes stimulate cyclic AMP production via G_s . P2 receptors are characterized as ionotropic P2X or metabotropic P2Y receptor families. Seven P2X (P2X1-7) and eight P2Y (P2Y1,2,4,6,11-14) receptors are expressed in the mammalian nervous system (Abbracchio et al., 2009). P2X receptors are classical ligand-gated ion channel receptors permeable to Na^+ , K^+ , and Ca^{2+} ions (North, 2002). G-protein coupled P2Y receptors can be subdivided into P2Y1, 2, 4, 6, and 11 receptors that primarily couple to G_q/G_{11} to activate phospholipase C/inositol triphosphate intracellular Ca^{2+} release pathway. The P2Y11 receptors also couple to G_s resulting in activation of adenylyl cyclase. P2Y12, 13, and 14 receptors couple to $G_{i/o}$ and inhibit adenylyl cyclase (Abbracchio et al., 2006). Moreover, specific receptors for diadenosine polyphosphates, called P4 receptors or dinucleotide receptors, that are not sensitive to ATP, UTP, adenosine, or their respective analogues, have been proposed (Pintor & Miras-Portugal, 1995; Miras-Portugal et al., 1998; Pintor et al., 2000; Gualix et al., 2013).

ATP responses are mediated by P2 receptor activation. However it becomes increasingly clear that in addition to ATP, other nucleotides also activate these receptors. Importantly, some of these nucleotides appear to meet criteria for a neurotransmitter. Thus P2X receptors can be activated by ATP, Ap_nA , NAD^+ , and ADPR. In the guinea-pig vas deferens, Ap_4A and Ap_5A were proposed to elicit contraction via P2X1 receptors (Westfall et al., 1997). In the cerebellum, Ap_5A was reported to activate P2X3 receptors (Leon et al., 2006) and Ap_4A has been shown to activate recombinant P2X2 receptors (Pintor et al., 1996). In fact, diadenosine polyphosphates have been shown to activate recombinant P2Y1, P2Y2, P2Y4, P2Y12, and P2Y13 receptors (Lazarowski et al., 1995; Schachter et al., 1996; Communi et al., 1996; Zhang et al., 2002) and recombinant homomeric P2X1, P2X2, P3X3, and P2X4 receptors (Pintor et al., 1996; Wildman et al., 1999; Pintor et al., 2000). P2X7 receptors were activated by NAD^+ via the ADP-ribosylation of arginine in murine T-lymphocytes (Seman et al., 2003; Hong et al., 2009), and in human monocytes NAD^+ increased intracellular Ca^{2+} via activation of P2X1, P2X7 and, to a lesser extent, P2X4 receptors (Grahner et al., 2009). In rat cultured cortical neurons the rapid dynamics of Ca^{2+} responses to localized application of NAD^+ and ADPR suggested possible activation of P2X receptor-mediated currents by these purines (Durnin et al., 2012a). P2Y receptors are also activated by multiple nucleotides including adenine, pyridine, and pyrimidine nucleotides (Harden et al., 2010), and each subtype exhibits different agonist binding profiles. P2Y1 receptors are preferentially activated by ADP (Palmer et al., 1998; Waldo & Harden, 2004). NAD^+ and ADPR are also ligands for P2Y1 receptors (Mutafova-Yambolieva et al., 2007; Klein et al., 2009; Hwang et al., 2011; Gustafsson et al., 2011; Hwang et al., 2012; Durnin et al., 2012b) and so is the dinucleotide, Ap_3A (Pintor et al., 1996). P2Y2 receptors are activated equipotently by UTP and ATP (Nicholas et al., 1996; Lustig et al., 1993). Human P2Y4 and P2Y6 receptors are preferentially activated by UTP and UDP, respectively (Nicholas et al., 1996), however species differences have been reported. For instance, the rat P2Y4 receptor is equipotent for UTP and ATP (von Kugelgen, 2006). ATP (Communi et al., 1997) and

NAD⁺ (Moreschi et al., 2006; Klein et al., 2009; Fruscione et al., 2011) are agonists at P2Y₁₁ receptors. Finally, P2Y₁₂ and P2Y₁₃ receptors are activated by ADP (Communi et al., 2001; Hollopeter et al., 2001) and P2Y₁₄ by UDP/UDP-sugars (Chambers et al., 2000; Fricks et al., 2008; Carter et al., 2009). Clearly, examination of the receptors involved in purinergic response is not sufficient for determining the identity of the purine mediating the response. Likewise, inhibition of responses with purinergic receptor antagonists is not adequate to assign the function to ATP only.

In some smooth muscles P2X₁ receptors predominantly mediate contractile responses to neurogenic purines and are, therefore, the specific receptor target for released purinergic neurotransmitter. Thus, nerve-evoked purinergic contractions are absent in mice lacking P2X₁ receptors in the vas deferens (Mulryan et al., 2000), urinary bladder (Vial & Evans, 2000), and blood vessels (Vial & Evans, 2002). As mentioned earlier, some studies have demonstrated a purinergic relaxation to nerve stimulation that follows the initial contraction in the urinary bladder (Boland et al., 1993; Bolego et al., 1995; Tong et al., 1997a; Hernandez et al., 2009), and P2Y₁ receptors are reported to mediate the relaxation response (Hernandez et al., 2009; Lee et al., 2014). Purines released from enteric motor neurons mediate relaxation also via activation of P2Y₁ receptors (Gallego et al., 2006; Mutafova-Yambolieva et al., 2007; Gallego et al., 2008a; Hwang et al., 2011; Hwang et al., 2012; Gallego et al., 2012; Durnin et al., 2012b). Pharmacological characterization of the receptor involved in enteric inhibitory responses has recently been confirmed using *P2ry1^{-/-}* mice (Hwang et al., 2012; Gallego et al., 2012). *P2ry1^{-/-}* mice also lack purinergic transmission in the gastric antrum (Gil et al., 2013), and Baker et al., 2013 reported that Ca²⁺ transients to NAD⁺ in the murine gastric fundus were absent in *P2ry1^{-/-}* mice. Therefore P2Y₁ receptors may mediate inhibitory responses to purines throughout the GI tract. Neurally released purines also mediate a portion of synaptic excitation between enteric interneurons and from descending interneurons to inhibitory motor neurons (Galligan, 2002a), predominantly via P2X₂ and P2X₃ receptors (Galligan, 2002a; Ren et al., 2003; Bian et al., 2003; Galligan & North, 2004). Recently a role of P2Y₁ metabotropic receptors in interneuronal excitation has been reported in the myenteric plexus of guinea-pig ileum (Thornton et al., 2013).

In the CNS, fast synaptic responses to purines are mediated largely by P2X receptors. However it is not entirely clear which receptor subtypes are involved maybe due to a lack of studies utilizing knockout mice. Souslova et al., 2000 reported that purine-mediated currents in dorsal neurons are absent in mice lacking P2X₃ receptors but expression of this receptor is limited to certain brain regions (Vulchanova et al., 1997). In cortical, hippocampal, cerebellum, and spinal cord neurons, P2X₂ and P2X₄ receptors are the most widely distributed subtypes (Kanjhan et al., 1999; Norenberg & Illes, 2000; Bo et al., 2003) but functional roles in fast purinergic neurotransmission have not been confirmed. In rat cultured cortical neurons, P2X receptor desensitization with α,β -methylene-ATP reduced but did not abolish the responses to NAD⁺ and ADPR, which could suggest partial involvement of α,β -methylene-ATP-sensitive receptors (i.e., P2X₁ and P2X₃ receptor subtypes, North, 2002) in the responses (Durnin et al., 2012a). Interestingly, subpopulations of neurons appeared to preferentially respond to either NAD⁺ or ATP, suggesting different receptors could be involved in the responses to NAD⁺ versus ATP in the brain (Durnin et al., 2012a).

Presynaptic P2X7 receptors have been shown to be targeted by purines in the brain. For example, activation of P2X7 receptors on medullary and spinal cord neurons facilitated glutamate release (Deuchars et al., 2001; Deng & Fyffe, 2004; see Sperlagh et al., 2006). Functional P2X1/P2X5, P2X7, P2Y1, P2Y2 as well as all subtypes of adenosine P1 receptors are expressed on CNS astrocytes and other glia cell types (Brodie et al., 1998; Biber et al., 1999; Fam et al., 2000; Fumagalli et al., 2003; Trincavelli et al., 2004; Wittendorp et al., 2004; Lalo et al., 2008; Nagasawa et al., 2009; Verkhratsky et al., 2009; Abbracchio et al., 2009; Lecca et al., 2012). Thus there is diverse expression of purinergic receptors on neurons and glial cells in the CNS that could regulate a wide range of responses to extracellular purines.

In general, functional characterization of P2 receptor-mediated responses is based on the use of receptor agonists and antagonists. However, selectivity and specificity of many pharmacological agents is sometimes questionable. For example, as discussed throughout, α,β -methylene-ATP is commonly used to either activate or desensitize P2X1 (and possibly P2X3) receptors. Recent studies, however, have suggested that α,β -methylene-ATP could also activate the P2Y1 receptor (Auli et al., 2008; Aslam et al., 2013), which would cause very different responses from responses mediated by ligand-gated P2X receptors. Therefore, studies relying exclusively on pharmacological characterization of purine responses should be interpreted with caution.

4.2. Cell targets

In contrast to direct synaptic communication between neurons, there is a lack of clear synaptic specializations between nerves and smooth muscles. Therefore the term ‘volume transmission’ (also referred to as non-synaptic or non-junctional transmission) has been used to describe neurotransmission at smooth muscle neuroeffector junctions (Vizi, 1984; Burnstock, 2008). While this may be valid for some smooth muscles, at other neuromuscular junctions close juxtaposition of nerve terminals and specialized postjunctional cell targets allows communication over a short distance similar to that of a synapse. Thus transmission can occur at organized compartments where released transmitters interact with restricted pools of receptors clustered on the postjunctional membrane (Hirst et al., 1992). Smooth muscle cells, interstitial cells, neural cells and glial cells are possible postjunctional cell targets of neurogenic purines. Three possible types of purinergic neuroeffector junctions are discussed below (depicted in Fig. 5).

4.2.1. Smooth muscle cell targets—Traditionally, smooth muscle cells are presumed to be the postjunctional target cell type at nerve-smooth muscle junctions. For example, expression of P2X1 receptors on the smooth muscle cells of the vas deferens (Vulchanova et al., 1996; Liang et al., 2001), urinary bladder (Vial & Evans, 2000), and blood vessels (Vial & Evans, 2002) suggests that neurogenic purines are likely to elicit effects by directly targeting the smooth muscle (Fig. 5A). P2X1 receptors in regions adjacent to sympathetic nerve varicosities in the vasculature have been described (Hansen et al., 1999; Vial & Evans, 2005). Some studies have suggested the presence of vascular interstitial cells between neurons and the smooth muscle (Povstyan et al., 2003; Harhun et al., 2005) but it has not been established whether these cells have functional significance in neurotransmission.

Unlike in the vasculature, in the vas deferens P2X1 receptors were distributed uniformly around the circumference of smooth muscles irrespective of the presence or absence of adjacent sympathetic axon varicosities (Liang et al., 2001), suggesting that purines might elicit effects via volume transmission in this smooth muscle. Smooth muscle cells do not appear to be the direct target for neurogenic purines mediating relaxation in the ENS and possibly in the urinary bladder. For example, exogenous purines activated non-selective cation currents in smooth muscle cells isolated from primate colons (Hwang et al., 2011) and transient inward currents in smooth muscle cells from mouse urinary bladder (Lee et al., 2014), which cause membrane depolarization, not hyperpolarization as one would expect from inhibitory neurotransmitters. Additionally, there is very little expression of P2Y1 receptors or of apamin-sensitive SK channels in smooth muscle cells from colon (Peri et al., 2013) and bladder (Lee et al., 2013; Lee et al., 2014), suggesting that purines likely target other cell types (i.e., interstitial cells) to elicit muscle relaxation.

4.2.2. Interstitial cell targets—Without directly targeting smooth muscle, motor transmitters in the ENS can elicit muscle responses by activating receptors localized on interstitial cells that are in close apposition to varicosities of enteric motor neurons (Sanders et al., 2010). Thus interstitial cells mediate a portion of motor input from the ENS to the smooth muscle. There are at least two types of interstitial cells in the *tunica muscularis* of the GI tract: interstitial cells of Cajal (ICC) and PDGFR α ⁺ cells (Komuro et al., 1999; Pieri et al., 2008; Sanders et al., 2010; Kurahashi et al., 2011). The anatomical localization of these cells close to neurotransmitter release sites is suggestive of physiological functions in enteric neurotransmission and together, smooth muscle cells, ICC, and PDGFR α ⁺ cells constitute the enteric SIP syncytium (Koh et al., 2012b). ICC are localized in close proximity to enteric neurons in the GI tract and connect to the smooth muscle via gap junctions (Komuro et al., 1999; Sanders et al., 2010). These cells have been shown to mediate responses to nitrergic (Burns et al., 1996) and cholinergic (Ward et al., 2000) motor transmission. Purinergic motor neurotransmission, however, does not appear to be mediated by ICC (Alberti et al., 2007) and in fact enhanced non-nitrergic apamin-sensitive responses (Burns et al., 1996) and increased expression of purinergic P2Y1 receptors (Sergeant et al., 2002) have been described in the *W/W^v* mutant mouse lacking intramuscular ICC, perhaps as a compensatory mechanism to overcome reduced nitrergic transmission in *W/W^v* mutants (Burns et al., 1996; Ward et al., 2004; Ward & Sanders, 2006; Alberti et al., 2007). ICC may mediate responses to extracellular UTP and UDP as these cells express highly *P2ry2*, *P2ry4*, and *P2ry6* (Peri et al., 2013) however this needs to be functionally verified. ICC have also been identified in the urinary bladder and morphologically resemble gut ICC (McCloskey & Gurney, 2002; McCloskey, 2010), however the functional significance of bladder ICC in relation to purinergic signaling has not been established.

Enteric PDGFR α ⁺ cells, previously referred to as fibroblast-like cells, make close contacts with enteric nerve varicosities (Zhou & Komuro, 1992) and connect to smooth muscle with gap junctions (Horiguchi & Komuro, 2000; Fujita et al., 2003). Characterization of these cells was facilitated significantly by the use of transgenic mice with bright green fluorescent protein expressed specifically in cells expressing PDGFR α (Kurahashi et al., 2011; Peri et al., 2013; Lee et al., 2014). PDGFR α ⁺ cells of the GI tract express apamin-sensitive SK3

channels (Iino & Nojyo, 2009; Iino et al., 2009; Kurahashi et al., 2011; Kurahashi et al., 2012; Peri et al., 2013) and P2Y1 receptors (Kurahashi et al., 2011; Peri et al., 2013). In fact, the genes for P2Y1 receptors and SK3 channels were enriched in PDGFR α ⁺ cells in relation to SMC and ICC (Peri et al., 2013). Thus the molecular apparatus involved in purinergic inhibitory responses in the gut is expressed by PDGFR α ⁺ cells. Moreover, exogenous application of purines, including ATP and NAD⁺, activates apamin-sensitive, large outward currents in isolated PDGFR α ⁺ cells that are blocked by P2Y1 receptor antagonists (Kurahashi et al., 2011). This strongly suggests a role of PDGFR α ⁺ cells in transducing purinergic inhibitory inputs to the smooth muscle in the gut (Fig. 5B). Unfortunately *Pdgfra* mutant mice are embryonic lethal (Sun et al., 2000) hampering studies of the role of PDGFR α ⁺ cells in purine motor transmission. Future experiments using cell-specific knockouts of P2Y1 receptors in PDGFR α ⁺ cells may help confirm whether these cells are primary mediators of enteric inhibitory purinergic neurotransmission. Recent studies have identified PDGFR α ⁺ cells in the urinary bladders of mice (Koh et al., 2012a), guinea-pigs and humans (Monaghan et al., 2012). Similar to the colon, these cells are closely associated with nerve processes in detrusor muscles and highly express SK3 channels (Lee et al., 2013) and P2Y1 receptors (Lee et al., 2014). Exogenous ATP activated large outward currents in urinary bladder PDGFR α ⁺ cells that were inhibited by blockers of SK channels and P2Y1 receptors, and responses to ATP were largely absent in PDGFR α ⁺ cells from *P2ry1*^{-/-} mice ((Lee et al., 2014). Thus PDGFR α ⁺ cells may be important mediators of purinergic inhibitory responses in the bladder. NAD⁺ is a potent inhibitory mediator in human detrusor muscles (Breen et al., 2006) but it is currently not known whether the effects of NAD⁺ in the bladder are mediated by P2Y1 receptors and PDGFR α ⁺ cells. Clustering of P2Y1 receptors on specialized PDGFR α ⁺ cells close to neurotransmitter release sites would allow purinergic transmission to occur within a limited volume in the interstitium. Thus purinergic neurotransmission in both the ENS and bladder would rather conform with synaptic transmission.

4.2.3. Neural cell targets—The general perception is that purinergic fast synaptic transmission in the CNS is mediated by neurally released purines targeting receptors localized on adjacent neural cells. Thus this mode of transmission is synaptic in nature. Figure 5C depicts the neuron-neuron model of neuroeffector junction. In the cerebellum and hippocampus Rubio & Soto, 2001 demonstrated that P2X2 and P2X4 receptors were not localized in synapses directly opposite neurotransmitter release sites and were instead present at the edges of the postsynaptic density. Therefore it is possible that neurally released purines target P2X2 and P2X4 receptors via volume transmission in these brain regions or, alternatively, these receptors are not involved in fast synaptic communication; P2X2 and P2X4 receptors might be targeted by purines released from non-neural sources such as astrocytes. In the ENS, fast interneuronal communication likely involves a direct action of released purines on adjacent nerve cells.

4.2.4. Glia cell targets—As discussed earlier (*Section 4.1*), multiple subtypes of purine receptors are expressed by glial cells in the CNS and potentially mediate responses to neurogenic purines (Brodie et al., 1998; Biber et al., 1999; Fam et al., 2000; Fumagalli et al., 2003; Trincavelli et al., 2004; Wittendorp et al., 2004; Lalo et al., 2008; Nagasawa et al.,

2009; Verkhatsky et al., 2009). Thus glial cells are an additional target for purines in the CNS (depicted in Fig. 5C). Similarly, enteric glial cells are additional targets for neurogenic purines in the ENS (Gulbransen & Sharkey, 2009; Gulbransen et al., 2010; Gulbransen et al., 2012). For example, the release of ATP through pannexin-1 channels on the cell bodies and processes of enteric neurons was shown to activate enteric glia during experimental colitis (Gulbransen et al., 2012). ATP, ADP, and UTP elicited robust Ca^{2+} responses in enteric glia which are likely mediated by P2Y1 or P2Y4 receptors (Gulbransen & Sharkey, 2009; Gulbransen et al., 2012). Enteric glia are also activated by purines released from sympathetic fibers innervating the myenteric plexus of mouse colon (Gulbransen et al., 2010). Thus multiple cell targets exist for neurogenic purines in the ENS (Fig. 6).

5. Termination of neurotransmitter action

Efficient control of synaptic transmission requires a rapid mechanism for terminating the actions of released neurotransmitters. Small molecule neurotransmitters like amino acids and monoamines (e.g., NE, dopamine, and 5-hydroxytryptamine) are taken back up into the nerve cell by specific transporters. Part of the monoamine neurotransmitters are also broken down in the extracellular space by membrane-bound enzymes. Acetylcholine is first metabolized in the extracellular space by acetylcholine esterase and then choline is taken up by the cell. Likewise, ATP is hydrolysed in the extracellular space by multiple enzymes and then adenosine is taken up by the cell or inactivated by adenosine deaminase and purine nucleoside phosphorylase reactions. Cell-surface located enzymes (ecto-nucleotidases) typically hydrolyze nucleoside tri-, di-, and monophosphates and dinucleoside polyphosphates, and produce nucleoside diphosphates, nucleoside monophosphates, nucleosides, phosphate, and inorganic pyrophosphate (PP_i) (see Zimmermann, 1996; Zimmermann, 2006; Yegutkin, 2008; Zimmermann et al., 2012). Nucleosides can in turn be recycled by cellular reuptake via specific high-affinity transporters (King et al., 2006). Notably, breakdown products (e.g., ADP, ADPR, and adenosine) might have biological activity and add to the complexity of purinergic neurotransmission. Along with uptake and metabolism, simple diffusion of neurotransmitters away from the synaptic cleft and subsequent dilution in surrounding extracellular fluids could also play a role in transmitter inactivation (Gerschenfeld & Stefani, 1968; Bevan & Su, 1973; Iversen, 1971); however, the roles of such mechanisms have not been elucidated for purine neurotransmitters.

5.1. Breakdown

Enzymes located on the cell surface with catalytic sites facing the extracellular space catalyze the hydrolysis of released purine neurotransmitters and thus limit their spatio-temporal activity (see Zimmermann, 1996; Zimmermann, 2000; Yegutkin, 2008; Zimmermann et al., 2012). Extracellular nucleotide hydrolysis is surprisingly complex. The enzymes involved include ecto-nucleoside triphosphate diphosphohydrolases (NTDPases), ecto-nucleotide pyrophosphatase/phosphodiesterases (NPPs), alkaline phosphatases, 5'-nucleotidase, and monoamine oxidase. Members of these families have been characterized in molecular and functional terms, but the considerable diversity of individual family members makes it difficult to assign identified enzymes to particular purinergic signaling pathways (Zimmermann, 2000; Zimmermann et al., 2012). Moreover, members of

nucleotide-degrading enzymes are ubiquitously distributed in the nervous system (Zimmermann, 1996; Zimmermann, 2006; Robson et al., 2006), demonstrate differing prevalence in brain locations (Kukulski et al., 2004; Langer et al., 2008), and are expressed in diverse cell types that are associated with neurotransmission (e.g., Marti et al., 1996; Zimmermann & Braun, 1999; Braun et al., 2004; Smyth et al., 2006a; Lavoie et al., 2011; Peri et al., 2013).

ATP is degraded sequentially to ADP, AMP and adenosine. The primary enzymes involved in ATP catabolism are NTPDases, alkaline phosphatase, NPPs, and 5'-nucleotidase (Zimmermann et al., 2012). These enzymes differ in pH optimum, ion dependence, membrane anchoring, catalytic properties, substrate specificity, and product formation. Most of these enzymes are cell membrane-bound extracellularly directed proteins (Zimmermann et al., 2012), although some enzyme families also have soluble members in the cytosol (Zimmermann, 1996; Skladanowski, 2013) that could be released in response to different stimuli (e.g., Champagne et al., 1995; Hicks-Berger et al., 2000). Increased degradation of ATP during electrical field stimulation has been demonstrated in the guinea-pig vas deferens (Todorov et al., 1997; Mihaylova-Todorova et al., 2001; Westfall et al., 2002), mouse and rat vas deferens (Westfall et al., 2000), guinea-pig urinary bladder (Westfall et al., 2000), and canine mesenteric arteries (Bobalova & Mutafova-Yambolieva, 2003). Todorov et al., 1997 proposed that the degradation of the neurotransmitter ATP is performed by a releasable ATPase activity. Functional characterization of this activity in the guinea-pig vas deferens showed similarities to NTPDases and 5'-nucleotidases (Mihaylova-Todorova et al., 2002; Westfall et al., 2002) that are possibly released from 'a putative ATP storage vesicle' (Mihaylova-Todorova et al., 2001). The protein(s) that carry out these activities has not yet been identified. Enhanced degradation of ATP was not observed during nerve stimulation in guinea pig taenia coli (Westfall et al., 2000), canine mesenteric veins (Bobalova & Mutafova-Yambolieva, 2003) and murine and monkey colons (Durnin et al., 2012b), suggesting that releasable ATPase activity may not be a universal mechanism for neurotransmitter removal. Interestingly, electrical field stimulation-evoked release of adenosine deaminase has been shown in longitudinal muscle-myenteric plexus preparations of the rat ileum (Correia-de-Sa et al., 2006).

Extracellular NAD^+ is degraded by multiple enzymes to multiple products. NAD^+ is first degraded to ADPR and cyclic ADPR by NAD glycohydrolase and ADP-ribosyl cyclase, respectively, and cyclic ADPR is hydrolyzed to ADPR by ADP-ribosyl hydrolase. The three enzyme activities are associated with CD38, a type II integral membrane glycoprotein with multiple functions (Zocchi et al., 1993; Sauve et al., 1998; Lee, 2001; De Flora et al., 2004). Its catalytic site faces the extracellular space (De et al., 1997; Munshi et al., 2000; Lee, 2001), providing a mechanism for the degradation of released NAD^+ . CD157 shares several characteristics with CD38 and is proposed to be involved in the metabolism of NAD^+ especially in the immune system (Ortolan et al., 2002). After its formation from NAD^+ , ADPR is further degraded to AMP and adenosine by NPPs and 5'-nucleotidase, respectively (Zimmermann, 2000). In addition, NPPs can produce nicotinamide mononucleotide (NMN) and AMP from NAD^+ , which are further degraded to adenosine (Zimmermann, 2000; Zimmermann et al., 2012). Therefore, in the neuroeffector junction adenosine can be formed from either ATP or NAD^+ , or both (Durnin et al., 2012b), or be directly released from

neurons (Wall & Dale, 2007; Lovatt et al., 2012). Likewise, adenosine is produced from diadenosine polyphosphates in the brain (Asensio et al., 2007).

There has been only a small number of studies on the functions of CD38 in the nervous system. CD38 is expressed on plasma membranes and cell organelles of neurons and astrocytes in rat brain (Yamada et al., 1997). CD38 appears to be important for the basal survival of brain microglia (Ma et al., 2012). Presence of CD38 in both intracellular compartment and membranes of brain synaptosomes has been recently demonstrated, and functional evidence for degradation of extracellular NAD^+ in brain synaptosomes was provided (Durnin et al., 2012a).

In blood vessels, a functional role of CD38 in the metabolism of neurally released NAD^+ was suggested by 1) the presence of NAD^+ metabolites, ADPR, cyclic ADPR, AMP, and adenosine in overflow samples collected during nerve stimulation (Smyth et al., 2004; Smyth et al., 2006b); 2) the degradation of exogenous NAD^+ after contacting smooth muscles; and 3) the presence of CD38 exclusively at nerve terminals in canine vasculature, demonstrated by co-localization with tyrosine hydroxylase and the vesicle proteins synaptotagmin and SV2 (Smyth et al., 2006a). However in other smooth muscles, CD38 is apparently not the only protein involved in the degradation of extracellular NAD^+ . For example, in the murine colon NAD^+ metabolism to ADPR and adenosine was equal in wild type mice and in mice lacking *Cd38* (Durnin et al., 2012b); in the same study the absence of CD38 in the *Cd38*^{-/-} mice was verified by the lack of ADP-ribosyl cyclase activity and by PCR techniques. The nature of the enzyme(s) that carry out NAD -glycohydrolase activity in the colon remains to be elucidated, but as discussed above, NPPs and 5'-nucleotidases also participate in extracellular NAD^+ metabolism (Zimmermann et al., 2012). Both NPPs and 5'-nucleotidases are ubiquitously distributed in the nervous system.

5'-Nucleotidases in particular were found at the surface membranes of axons and dendrites (Bernstein et al., 1978) and are associated with glial membranes in the central nervous system (Schoen & Graybiel, 1992). Localization of ecto- Ca-ATPase and 5'-ectonucleotidase activities was shown in caveolae of smooth muscle cells of the guinea-pig vas deferens and their role in neurotransmission has been suggested (Kittel & Bacsy, 1994). Interestingly, it has recently been proposed that human CD73 (which carries out 5'-nucleotidase activity) is capable of processing NAD^+ and its metabolite NMN (Garavaglia et al., 2012; Grozio et al., 2013), suggesting novel pathways in NAD^+ metabolism. The possible role of these enzymatic pathways in metabolism of neurally released NAD^+ has not been determined.

Extracellular Ap_nA are degraded in an asymmetrical manner by surface-located NPP1, NPP2 and NPP3 to generate AMP and Ap_{n-1} from Ap_nA (Vollmayer et al., 2003; Asensio et al., 2007). The mononucleotides generated from Ap_nA are further degraded to adenosine by 5'-nucleotidases.

As discussed previously, adenosine is formed by alkaline phosphatases that hydrolyze nucleoside 5'-tri-, di-, and monophosphates (Millan, 2006) and by ecto5'-nucleotidases that dephosphorylates nucleoside monophosphates and thus produces adenosine from extracellular AMP (see Hunsucker et al., 2005; Yegutkin, 2008). Removal of adenosine

from the extracellular compartment can occur by metabolism to its inactive derivative, inosine, by adenosine deaminase; further metabolism of inosine by purine nucleoside phosphorylase leads to hypoxanthine, which can be either transformed into uric acid by xanthine oxidase or salvaged into mononucleotides (Franco et al., 1997; Yegutkin, 2008). Adenosine deaminase does not appear to play a major role in adenosine removal in some smooth muscles, such as guinea-pig vas deferens (Todorov et al., 1997) and murine and monkey colons (Durnin et al., 2012b). In addition, adenosine uptake transporters do not appear to be involved in adenosine removal in murine and monkey colons (Durnin et al., 2012b), suggesting that another mechanism might be involved in adenosine removal in the enteric nervous system.

5.2. Neural Uptake

The common perception is that extracellular ATP is removed by enzymatic degradation to ADP, AMP, and adenosine, which in turn enters back into the nerve cells via high activity nucleoside transporters and replenishes cytosolic ATP levels. Similar mechanisms likely apply to other nucleotides. Nucleoside transporters are divided into two broad categories: concentrative Na^+ -dependent transporters use the transmembrane driving force of the Na^+ gradient to allow cells to concentrate nucleosides, and equilibrative transporters move nucleosides by facilitated diffusion (Thorn & Jarvis, 1996). Equilibrative transporters are ubiquitously expressed and are capable of transporting both purine and pyrimidine nucleosides (Baldwin et al., 2004), whereas expression of concentrative transporters appears to be restricted to liver, kidney, and the GI tract (Gray et al., 2004). Nucleoside transporters play critical roles in nucleoside salvage pathways, where they mediate the first step of nucleotide biosynthesis (Zimmermann et al., 1979).

Trans-membrane transport of NAD^+ or NADH has been demonstrated in NIH3T3 fibroblasts, cultured murine astrocytes, human neuroblastoma SH-SY5Y, HeLa, HaCaT, K562, and RAW 264.7 cell types (Bruzzzone et al., 2001b; Ying et al., 2003; Billington et al., 2008). Some of the proposed mechanisms include connexin 43 (Bruzzzone et al., 2001a), CD38 or unidentified dinucleotide transport carrier (Zocchi et al., 1999), and P2X7 receptor pore (Lu et al., 2007). Incubation of rat brain synaptosomes with a fluorescent NAD analogue caused appearance of this substance in the intra-synaptosomal compartment (Durnin et al., 2012a). Gap 27, a connexin 43 inhibitor, had no significant effect on the synaptosomal uptake of etheno- NAD^+ (Durnin et al., 2012a). The nature of the NAD^+ transport system in synaptosomes is currently unknown. Transport of NAD^+ back into the neuron would terminate its extracellular action, but it would also help replenish intracellular NAD^+ levels.

Another possible way to replenish cellular NAD^+ is through uptake of its precursor(s). Cellular NAD^+ is synthesized either *de novo* from tryptophan or through any of the 'salvage' pathways, starting from nicotinic acid, nicotinamide or nicotinamide riboside (Magni et al., 1999; Houtkooper & Auwerx, 2012; Bieganski & Brenner, 2004). Extracellular NAD^+ precursors like nicotinic acid, nicotinamide, nicotinamide riboside, and nicotinic acid riboside are shown to be taken up by yeast (Belenky et al., 2009) and by human cells (i.e., HeLaS3, 293 and HepG2 cells) (Nikiforov et al., 2011). If such

mechanisms are present in nerve cells, then intracellular NAD⁺ levels can be replenished by uptake of its extracellular metabolites, which are also its precursors. However, such mechanisms have not yet been reported for cells that are known to participate in neuroeffector transmission.

6. Therapeutic potential of drugs targeting purinergic neurotransmission

The definitive goal of basic research is to advance our understanding of fundamental mechanisms of control of body functions and to see translation of this work to treatment of human diseases. There is increasing interest in developing purine-related drugs for treatment of a wide range of pathological conditions, because purinergic signaling is involved in virtually all functions in the body. These include rapid synaptic transmission, neuromuscular transmission, exocrine and endocrine secretion, cell proliferation, differentiation, migration, and death, reproduction, and embryonic development. The roles of purines might be altered in pathological states in many systems of the body, as has been suggested for pathologies of the urinary bladder (discussed in *Section 3.3.4*). For example, a higher cotransmitter role for ATP in sympathetic nerves supplying blood vessels has been reported in spontaneously hypertensive rats (Brock et al., 1995) and altered expression of purinergic receptors has been suggested in disease states such as inflammatory bowel disease (Yiangou et al., 2001; Rybaczyk et al., 2009) and congestive heart failure (Hou et al., 1999). It is believed that both P1 and P2 receptors can be targeted in pathological conditions such as cardiovascular diseases, thrombosis, pain, eye diseases (i.e., glaucoma and dry eye), inflammation, respiratory distress, gastrointestinal motility disorders, urogenital diseases, infertility, skin disorders, and malignancies (e.g., Burnstock 2013c, Burnstock 2013d).

Numerous review articles have indicated the potential of purinergic mechanisms as therapeutic targets (e.g., Burnstock 2013c, Burnstock 2013d). Strategies that are currently used by medicinal chemists in search of purinergic drugs include development of 1) selective agonist and antagonist ligands for the P2X and P2Y receptors that respond to extracellular nucleotides and for the adenosine receptors (Jacobson 2013), 2) inhibitors of extracellular catabolism of purines, and 3) modulators of nucleotide and nucleoside transport (Burnstock, 2013d). Such drugs would be beneficial in treating organ system-specific aberrations in purinergic neurotransmission by increasing or decreasing local concentrations of purinergic neurotransmitters at target cells, by inhibiting or accelerating neurotransmitter clearance at neuroeffector junctions, by affecting mechanisms of storage and release of neurotransmitters or by modulating the postjunctional responses to released neurotransmitters. Some purinergic drugs are already in clinical trials or have been used in clinical practice. For example, drugs such as dipyridamole and methotrexate act by enhancing the activation of adenosine receptors (Chen et al., 2013) and P2Y₁₂ receptor antagonists (e.g., clopidogrel and related) as antithrombotic agents are validated clinically (Oh et al., 2012). Admittedly, however, purinergic drug discovery faces significant challenges in terms of selectivity and specificity and drugs that selectively target neural control mechanisms mediated by purinergic neurotransmitters are lacking. A great challenge in developing purinergic drugs is that purinergic signaling is so widespread so that development of potent and stable tissue-specific agents proved to be particularly difficult (Burnstock 2013c, Burnstock 2013d, Chen et al., 2013). Nevertheless, there are new

concepts (e.g., novel drug delivery, allosteric modulation, prodrug approaches, molecular docking, biased agonism, and use of multiple-target drugs for purinergic signaling) and continued medicinal chemistry efforts to develop novel purine receptor ligands with refined structure-activity relationships and to improve *in vivo* stability and bioavailability, and tissue selectivity of purinergic drugs (Jacobson, 2012, Chen et al., 2013). Advances in purinergic drug discovery, hopefully over the next decade, will facilitate our understanding of mechanisms of purinergic neurotransmission and will improve the neural control of body functions when needed.

7. Concluding remarks

Purinergic neurotransmission is involved in an array of system-specific responses. The integrated neural control by purines is determined by multiple factors, including the release of purine neurotransmitter, responsiveness of target cells that is mediated by different types of receptors with diverse transduction pathways, and inactivating mechanisms. Together, these mechanisms govern the type, duration, and magnitude of effector cell responses. The data that have been discussed in this review highlight the importance of purine substances in neurotransmission and justify re-examination of current concepts of neurotransmitter identity.

In particular, extensive research has shown that ATP can be released in a regulated manner from many cell types by many mechanisms, and might be a neurotransmitter in some systems but not in others. Older and recent data demonstrate the ability of multiple purine substances to meet presynaptic and postsynaptic criteria for a neurotransmitter. Pharmacological characterization of purine responses is not sufficient anymore to assigning a neurotransmitter role to a particular purine substance. It becomes increasingly evident that multiple purine substances are responsible for mediating neurotransmission (Fig. 6), perhaps in organ- and species-specific manners. Likewise, multiple cell types can be the immediate targets of purine neurotransmitters. Understanding the mechanisms of purinergic neurotransmission is critical for understanding the fundamental mechanisms of neural regulation and may also have important implications therapeutically. Resolving the identity of the purine neurotransmitter will help to develop treatments that target steps in biosynthesis, release, degradation or action that are specific for the particular purinergic neurotransmitter. Clearly, understanding the identity of the purine neurotransmitters in different systems and identifying the exact mechanisms by which the network of purine neurotransmitters regulates local and systemic functions will be an area of intense interest in the future.

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Abbreviations

ACh	acetylcholine
ADP	adenosine 5'-diphosphate

ADPR	adenosine 5'-diphosphate ribose
AMP	adenosine 5'-monophosphate
Ap_nA	diadenosine polyphosphates
ATP	adenosine 5'-triphosphate
CNS	central nervous system
EFS	electrical field stimulation
EJC	excitatory junction current
EJP	excitatory junction potential
ENS	enteric nervous system
FLD	fluorescence detection
GI	gastrointestinal
GJ	gap junction
HPLC	high-performance liquid chromatography
ICC	interstitial cells of Cajal
IJP	inhibitory junction potential
NAD	nicotinamide adenine dinucleotide
NANC	non-adrenergic/non-cholinergic
NE	norepinephrine
NGF	nerve growth factor
NMN	nicotinamide mononucleotide
NO	nitric oxide
NPP	ecto-nucleotide pyrophosphatase/phosphodiesterase
NTDPases	ecto-nucleoside triphosphate diphosphohydrolases
PDGFR	platelet-derived growth factor receptor
SK	small-conductance Ca ²⁺ -activated potassium channel
SMC	smooth muscle cell
SNAP-25	synaptosomal-associated protein 25
SNARE	soluble N-ethylmaleimide sensitive factor attachment protein receptor
TTX	tetrodotoxin

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Figure 1. Lack of fast excitatory junction potentials in guinea-pig mesenteric veins is not due to an absence of ATP release in response to nerve stimulation

A. Comparison of intracellular responses in [guinea-pig] mesenteric artery and vein to stimulation of lumbar colonic nerves. A1 and B1 are computer average of 25 consecutive responses evoked at 0.3 Hz. A2 and B2 are single traces of responses in same cells at a slower sampling rate. Stimulus artefacts in B2 are a result of variable capture of their peaks at slow sampling rates. Resting membrane potential in artery -69 mV, in vein -77 mV (reproduced from Kreulen, 1986, with permission from the American Physiological Society). **B.** Original chromatograms [obtained with HPLC-FLD analysis] illustrating the electrical field stimulation (EFS)-evoked overflow of adenine nucleotides and adenosine from superfused guinea-pig inferior mesenteric artery (a) and vein (b). Note that these chromatograms are made from samples collected from an artery (8.9 mg) and vein (2.2 mg) from the same animal. [When normalized to tissue weight EFS-evoked release of ATP in

vein exceeded release of ATP in artery]. LU, luminescence units. Reproduced from Bobalova & Mutafova-Yambolieva, 2001a with permission from John Wiley and Sons Ltd.

Figure 2. Membrane depolarization-evoked release of NAD⁺, but not ATP, from NGF-differentiated rat PC12 cells requires intact SNAP-25-mediated exocytosis

A. Original chromatograms from samples collected during perfusion of the PC12 cells with either 5 mM KCl (pre-stimulation sample) or in the presence of 60 mM KCl for different time periods. KCl 60 mM evoked overflow of ATP, ADP, AMP, adenosine (ADO) and a mixture of β -NAD, cADPR, and ADPR. [β -NAD is the predominant compound in the β -NAD+cADPR+ADPR mixture as determined by HPLC fraction analysis: see original text in Yamboliev et al., 2009 for reference]. Inset: chromatographic peaks generated by etheno-derivatized purine standards (1 pmol each). Note that β -NAD has significantly lower fluorescence coefficient in generating eADPR than ATP, ADP, ADPR, AMP and ADO in generating corresponding e-purines. **B.** Excerpts of original chromatograms of superfusates from botulinum neurotoxin A (BoNT/A)-pretreated cells with an emphasis on ATP and the mixture of β -NAD, ADPR, and cADPR. **C–H.** Quantified data from 4–5 experiments expressed as the mean $\pm$ SEM. Data are averaged for each individual purine as well as for the sum of all purines (total purines). * P <0.05 vs. controls at 0' (KCl 5 mM). One-way

ANOVA followed by Bonferroni's multiple comparison tests. [#]P<0.05 vs. 0' in controls. Unpaired Student's t-test. Note that the evoked release of most purines, except for ATP, was significantly reduced by BoNT/A. In BoNT/A-treated cells secretion of β -NAD⁺ADPR +cADPR at 5–20' was below the initial levels. Reproduced from Yamboliev et al., 2009, with permission from John Wiley and Sons Ltd.

Figure 3. In human urinary bladder EFS evokes a prominent release of NAD^+ that exceeds the release of ATP. Exogenous β -NAD inhibits the human detrusor muscle contractions

A. Electrical field stimulation (EFS; 0.1 ms, 15 V, 60 s) evokes frequency-dependent overflow of adenine nucleotides in human isolated bladder detrusor smooth muscle. Original chromatograms are shown from HPLC-FLD analysis of purines in tissue superfusate samples collected before (prestimulation, PS) and during EFS (ST) at 4 and 16 Hz. Aliquots were derivatized with 2-chloroacetaldehyde (80°C, pH 4, 40 min) to 1, N^6 -etheno (e)-nucleotides (i.e., eATP, eADP, eAMP) and 1, N^6 -etheno-(e)ADO. At all frequencies of stimulation, 1, N^6 -etheno-(e)ADPR with elution time of 11.2 min is also observed. eADPR stands for β -NAD, because β -NAD represents 90–95% of the eADPR peak as discussed in

the main text of Breen et al., 2006. The EFS (16 Hz)-evoked release of purines is significantly reduced in the presence of TTX (0.3 $\mu\text{mol/l}$ for 30 min). Scales apply to all chromatograms. Note that $\beta\text{-NAD}$ has a significantly lower fluorescence coefficient in generating eADPR than ATP generating eATP as demonstrated in Figure 2 inset. **B.** Original traces showing that exogenous $\beta\text{-NAD}$ (1 $\mu\text{mol/l}$) inhibits both the frequency and amplitude of muscle contractions in a human detrusor muscle segment exhibiting spontaneous contractile activity. Reproduced from Breen et al., 2006, with permission from The American Physiological Society.

Figure 4. Direct stimulation of enteric motor nerve cell bodies evoked differential release of NAD⁺ and ATP in monkey colon; NAD⁺ was released from enteric nerve terminals in an action potential- and Ca²⁺-dependent manner whereas ATP was released from nerve cell bodies independent of action potentials and Ca²⁺ entry

A. Purines released by stimulation of serotonergic 5-HT₃ receptors in monkey colon whole muscle preparations. (A) Chromatograms of tissue superfusates collected before (control) and during activation of 5-HT₃ receptors with SR57227 (500 μmol L⁻¹ for 30 s) in the absence and presence of ondansetron (10 μmol L⁻¹, 30 min), tetrodotoxin (TTX, 0.5 μmol L⁻¹, 30-min superfusion), or ω-conotoxin GVIA (ω-CtxG, 50 nmol L⁻¹, 30-min superfusion) in monkey colon. Small amounts of ATP, ADP, β-NAD⁺, AMP, and ADO were present in superfusate samples in the absence of agonist. Stimulation of 5-HT₃ receptors with SR57227 evoked additional release of purines that was inhibited by the 5-HT₃ receptor antagonist ondansetron. SR57227-evoked release of β-NAD⁺, but not ATP, was reduced by the neural blockers TTX and ω-CtxG. Scale applies to all chromatograms. LU, luminescence units. **B.** Averaged data are means ± SEM and show release of ATP,

ADP, AMP, ADO, β -NAD⁺, and total purines (calculated as ATP+ADP+AMP+ADO+ β -NAD⁺) during activation of 5-HT₃ receptors with SR57227 (SR) and in the presence of ondansetron (Ond, 10 μ mol L⁻¹, 30 min), TTX (0.5 μ mol L⁻¹, 30 min), and ω -CtxG (50 nmol L⁻¹, 30 min). Overflow (femtomoles per milligram of tissue) is the overflow during 5-HT₃ receptor activation less spontaneous overflow. Each peak was calibrated to individual etheno-derivatized purine standards. Asterisks denote significant differences from SR57227-evoked release (i.e. control release) (**P* < 0.05, ***P* < 0.01, ****P* < .001); number of experiments in parenthesis. Note that the release of β -NAD⁺, but not ATP, was significantly inhibited by TTX and ω -CtxG. Reproduced from Durnin et al., 2013, with permission from John Wiley and Sons Ltd.

Figure 5. Schematic models of purinergic neurotransmission at three potential types of neuroeffector junctions

A. Neuron-to-smooth muscle effector junction. Purines (i.e., ATP) released from sympathetic nerve terminals (e.g., in vas deferens and blood vessels) and possibly from parasympathetic nerve terminals in the urinary bladder, act through P2X, mainly P2X1, receptors on smooth muscle cells (SMC) to mediate membrane depolarization (excitatory junction potentials, EJP) and contraction. In blood vessels P2X1 receptors are clustered in regions adjacent sympathetic nerve varicosities (Hansen et al., 1999; Vial & Evans, 2005), suggesting that released purines bind receptors in a limited volume in the interstitium, in a synapse-like space. In the vas deferens, however, P2X1 receptors do not appear to form clusters on SMC (Liang et al., 2001), and therefore, volume transmission might apply to this smooth muscle. There is evidence that NAD^+ is released during stimulation of sympathetic nerves in blood vessels (Smyth et al., 2004) and from unidentified nerves in the urinary bladder (Breen et al., 2006). It is not clear if ATP and NAD^+ are stored in and released from the same population of vesicles in whole tissues. Co-storage of both purines was demonstrated in vesicles from NGF-differentiated PC12 cells (Yamboliev et al., 2009). Differential modulation of ATP and NAD^+ release in blood vessels by neuronal Ca^{2+} channel inhibitors has been shown as well (Smyth et al., 2009). The postjunctional receptor targets of NAD^+ in blood vessels and bladder have not yet been determined. **B. Neuron-to-astrocyte effector junction.** Purines can elicit muscle responses by first activating receptors localized on astrocytes that are in close apposition to nerve varicosities and connect to SMC via gap junctions (GJ). There are at least two types of astrocytes in the enteric nervous system: astrocytes of Cajal and platelet-derived growth factor receptor alpha-positive (PDGFR α^+) cells. Depicted here is purinergic inhibitory motor transmission

in the enteric nervous system. NAD^+ released from enteric inhibitory motor nerve terminals activates P2Y1 receptors localized on closely apposed $\text{PDGFR}\alpha^+$ cells. P2Y1 receptors mediate activation of phospholipase C and release of Ca^{2+} from intracellular stores (sarcoplasmic reticulum, SR) which activates small-conductance K^+ channels (SK3). Inhibitory junction potentials (IJP) conduct to SMCs via GJ between $\text{PDGFR}\alpha^+$ cells and SMC. IJPs reduce excitability and cause muscle relaxation. ADPR also activates enteric P2Y1 receptors causing hyperpolarization and muscle relaxation. ADPR can be produced from NAD^+ (Durnin et al., 2012b), but it is not known if ADPR is also released from enteric nerve varicosities. $\text{PDGFR}\alpha^+$ cells may also mediate purinergic relaxation in the urinary bladder via activation of P2Y1 receptors and SK channels (Lee et al., 2014). It is currently unknown whether the inhibitory effects of NAD^+ in the bladder (Breen et al., 2006) are mediated by P2Y1 receptors and $\text{PDGFR}\alpha^+$ cells. Clustering of P2Y1 receptors on $\text{PDGFR}\alpha^+$ cells close to neurotransmitter release sites (Kurahashi et al., 2011) allows purinergic transmission to occur in a synapse-like space. **C. Neuron-to-neuron effector junction (synapse).** Neuron-to-neuron communication involves a direct action of released transmitters on receptors localized in the postsynaptic nerve membrane. In the central nervous system, ATP, NAD^+ , and diadenosine polyphosphates (Ap_nA) released from nerves could mediate fast synaptic transmission (fast excitatory postsynaptic potentials, fEPSP) by targeting P2X receptors localized on adjacent neural cells. Similarly, in the enteric nervous system purines mediate communication between interneurons via P2X receptors, predominantly P2X2 and P2X3 subtypes, on the adjacent nerve cells. Neuronal release of adenosine (not depicted) (Brundege & Dunwiddie, 1998; Manzoni et al., 1994; Lovatt et al., 2012) may mediate a widespread purinergic presynaptic inhibition. As depicted, purines could also target glial cells. Furthermore, glial cells release purines, including ATP and NAD^+ , which could target P2 receptors on nearby nerve cells. Studies have also suggested transport of NAD^+ into glial cells (Verderio et al., 2001; Ying et al., 2003).

Figure 6. Multiple purine neurotransmitters act on various types of effector cells

Schematic model demonstrating that multiple purines can be released from neurons, which can act at different postjunctional target cell types, including smooth muscle cells, interstitial cells (e.g., interstitial cells of Cajal and platelet-derived growth factor receptor α -positive cells), other neural cells, and glial cells. The identity of neurotransmitter substance and target cells could vary between systems and species. ADP, adenosine 5'-diphosphate; ADPR, ADP-ribose; ATP, adenosine 5'-triphosphate; NAD⁺, nicotinamide adenine dinucleotide; Np_nN, dinucleoside polyphosphate.