Acupuncture-Induced Analgesia: A Neurobiological Basis in Purinergic Signaling

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Abstract
Chronic pain is a debilitating and rather common health problem. The present shortage in analgesic drugs with a favorable spectrum but without remarkable side effects furthered the search for alternative therapeutic manipulations. Increasing evidence from both basic and clinical research on acupuncture, a main alternative therapy of traditional Chinese medicine, suggests that chronic pain is sensitive to acupuncture procedures. Clarification of the underlying mechanisms is a challenge of great theoretical and practical significance. The seminal hypothesis of Geoffrey Burnstock and the astounding findings of Maiken Nedergaard on the involvement of purinergic signaling in the beneficial effects of acupuncture fertilized the field and led to an intensification of research on acupurines. In this review, we will summarize the state-of-the-art situation and try to forecast how the field is likely to develop in the future.

Keywords
acupuncture analgesia, purinergic signaling, ATP, adenosine, P2X receptors, P2Y receptors, adenosine receptors

Introduction
Acupuncture (AP; from Latin acu “with a needle”; Latin punctura, from pungere “to prick”), the word invented by Wilhelm ten Rhyne in his Latin treatise De Acupuncture is originally characterized by the insertion of fine, solid metallic needles into or through the skin at specific sites, so called acupuncture points (also named acupoints) (Berman and others 2010; Johnson 2006). It has probably been used in traditional Chinese medicine (TCM) as a therapeutic manipulation for the past 3000 to 4000 years, although the first document called The Yellow Emperor’s Classic of Internal Medicine (known as the Huangdi Neijing) that unequivocally described AP as an organized system of diagnosis and treatment is dating from about 100 BCE (Cheng 2010; White and Ernst 2004). During the Ming Dynasty (1368-1644), The Great Compendium of Acupuncture and Moxibustion (Zhenjiu Dacheng) was published, which is a synthesis of classic AP and forms the basis for its use in modern times.

To date, AP has gained popularity since the landmark NIH Consensus Conference in 1997; it has been employed in more than 180 countries and regions around the world based on a report issued by the World Federation of Acupuncture-Moxibustion Societies (WFAS) in 2013. Currently, the term acupuncture should refer to a family of procedures involving physical or chemical stimulation at acupoints using a variety of techniques (NIH Consensus Conference 1998; see also below).

In traditional AP, needles may be manipulated manually (Fig. 1A,B) or stimulated electrically, the latter is called electroacupuncture (EAP). Manipulation of the needles causes deqi, a sensation of soreness, numbness, distension, aching, or heaviness, which is thought to be essential for the therapeutic outcome (Loyeung and Cobbin 2013). Moxibustion means the burning of a cone-shaped preparation of moxa (made of dried mugwort) on or near the skin, often but not always near to an acupoint (Fig. 1A,B; Fu and others 2016). Cupping therapy is an ancient Chinese form of alternative medicine in which a local suction is created on the skin (Fig. 1A,B; Cao and others 2012). In this publication, the term acupuncture will be used in its broad sense to include traditional body needling, moxibustion, electroacupuncture, laser acupuncture, and so on (Fig. 1A; World Health Organization 2002). Onetime sessions of AP last for about 30 minutes,
are usually repeated twice a week at the beginning, and continued once a week later, for a minimum of 12 sessions. AP treatment is favored by the relatively low incidence of major adverse effects, which can be ascribed less to the procedure itself than to the moderate skills of the acupuncturist (Berman and others 2010; Ernst and others 2011).

In 1979, the World Health Organization conducted a symposium in Beijing, China, to identify the conditions that might benefit from acupuncture treatment. The international participants drew up a list of 43 suitable diseases. In 1996, the number was updated to 64 in another meeting in Milan, Italy. In 2002, the World Health Organization published a book titled Acupuncture: Review and Analysis of Reports on Controlled Clinical Trials, in which 29 conditions were identified for which acupuncture has been proved, through controlled trials, to be an effective treatment. Among the 29 conditions, 12 were associated with pain. In the PubMed database, we found that in a total of 24,430 acupuncture publications, 2,236 (9.15%) focus on acupuncture analgesia (AA) and 6,553 (26.82%) on acupuncture and pain. Furthermore, increasing evidence from both basic and clinical research on AP has indicated that pain is particularly sensitive to acupuncture (Berman and others 2010; Lu and others 2016; Staud and Price 2006; Vickers and others 2012; Vickers and Linde 2014; Zhang and others 2014). In this article, we will summarize the available data on acupurines, that is, the role of purines and purinergic signaling in acupuncture, from research published in the recent years on the mechanism of AA.

Clinical Effectiveness and Neurobiological Basis of Acupuncture Analgesia

The clinical effectiveness of AP in various pain conditions is a much debated issue with powerful advocates and similarly powerful opponents. The opponents’ arguments are basically the following (Colquhoun and Novella 2013; Madsen and others 2009): (1) Large multicenter clinical trials conducted in Germany and the United States consistently revealed that true (verum) and sham AP (stimulation at nonspecific acupoints) do not differ in their effectiveness in decreasing pain levels across multiple chronic pain disorders; (2) the existing differences between the AP (verum or sham) and non-AP groups in improvement were only minor and therefore
could be due to a placebo effect; (3) it is hard to understand why AP relieves some types of pain while leaving other types of probably similar etiology unaffected; (4) interestingly, the strongest evidence for a positive outcome of AP treatment is in case of postoperative nausea and vomiting, a condition not related to pain.

However, the arguments of the advocates of AP appear to be also meaningful. (1) Numerous authors published meta-analyses of randomized, sham-controlled clinical investigations or evaluated Cochrane reviews, which are internationally recognized as the highest standard of evidence. They concluded that AP is effective in the short-term management of low back pain, neck pain, and osteoarthritis involving the knee, but ineffective in the management of dental pain, coloscopy pain, and intraoperative analgesia (Johnson 2006; Lee and Ernst 2011; Wang and others 2008). It was stated that the majority of early reviews arrived at negative conclusions (Johnson 2006), while the majority of recent reviews were positive, probably indicating a biased or incomplete evaluation of the early studies (Ernst and others 2011). (2) Neuroimaging techniques such as positron emission computed tomography (PET), single photon emission computed tomography (SPECT), and functional magnetic resonance imaging (fMRI), all suggested that AP results in significant activation of multiple brain areas of healthy human volunteers, including the hypothalamus, primary somatosensory cortex and rostral anterior cingulate cortex, important for pain sensation in higher brain centers (Lewith and others 2005; Staud and Price 2006; Wang and others 2008).

Figure 2. The neuronal purinergic system. 1. ATP is released from healthy cells into the extracellular space by vesicular release (as a transmitter or co-transmitter substance) or via ion channels/hemichannels/transporters. 2. Spontaneous efflux of purines occurs from injured or dying cells resulting in pathological concentrations of ATP in the extracellular space. 3. Ecto-enzymes rapidly hydrolyze or interconvert the extracellular nucleotides thereby either terminating their action or producing an active metabolite of altered receptor selectivity. 4. Postsynaptic ionotropic P2X and metabotropic P2Y receptors mediate fast and slow synaptic responses, respectively. 5. Extracellular purines activate pre- and postsynaptic P2X and P2Y receptors; adenosine stimulates its own P1 receptor type. These receptors (further divided into subtypes) modulate the effects of classic neurotransmitters (e.g., glutamate acting at its ionotropic receptors); whereby, purines represent a complex neuromodulatory system involved in fine-tuning of neurotransmission. In addition, purines of course also function as signaling molecules between cells of non-neuronal origin. Modified from Köles and others (2016).
sensory nerve terminals to suppress noiceception (Cabot and others 1997; Zhang and others 2014). In studies, with uninjured rats, low frequency EAP released β-endorphin and enkephalins, while high-frequency EAP released dynorphins to suppress noiceception as assessed by the tail flick test (Han 2003). Eventually, it has been shown that EAP inhibits the sensory dimension of pain by targeting many sites of the brain such as periaqueductal gray, locus coeruleus, habenula, nucleus accumbens, and caudate nucleus (Zhao 2008). It has been hypothesized that the periaqueductal gray is neuronally connected with the raphe nuclei which send descending serotonergic fibres to the spinal cord dorsal horn activating enkephalinergic, analgesia-mediating interneurons (Takeshige and others 1992). (4) AP activates a number of non-opioid pain-relevant neuronal systems in the CNS which operate via serotonin, noradrenaline, cholecystokinin, glutamate, neuropeptide Y, and so on (Irnich and Beyer 2002; Zhao 2008).

The Purinergic System

Since the discovery of purinergic neurotransmission in the mammalian organism (Burnstock 1972), and the observation that adenine and uridine nucleotides as well as their enzymatic degradation products (e.g., adenosine) have a widespread signaling function coordinating the activities of almost all neuronal and non-neuronal cells (see Ralevic and Burnstock 1998), research activities of many scientists have concentrated on purinergic/pyrimidinergic mechanisms. It has been noticed relatively early that purines play an important role in the sensation of noxious stimuli in the periphery and the transmission/modulation of such impulses by neuronal pathways in the CNS (Burnstock 2006, 2009a, 2016).

In this context, ATP was identified as an autacoid released into the extracellular space from the interior of damaged cells via holes in their plasma membrane caused by noxious stimuli, or from healthy cells in consequence of shear stress, stretch, osmotic swelling or metabolic limitation (Fig. 2; Lazarowski 2012). Subsequently, ATP acts at its receptors or is degraded by the ectonucleotidase families E-NPTDases (ectonucleoside triphosphate diphosphohydrolases), E-NPPs (ectonucleotide pyrophosphatase and/or phosphodiesterases), alkaline phosphatases and ecto-5′-nucleotidase (Yegutkin 2008). The individual enzymes differ in their substrate specificity and product formation. E-NPTDases and E-NPPs hydrolyze ATP and ADP to AMP, which is further hydrolyzed to adenosine by ecto-5′-nucleotidase (Abbracchio and Burnstock 1994; Abbracchio and others 2009). Alkaline phosphatases equally hydrolyze nucleoside tri-, di-, and monophosphates. Adenosine is generated either extracellularly from AMP by ecto-5′-nucleotidase or intracellularly by endo-5′-nucleotidase; The latter can leave the cell by means of an equilibrative adenosine transporter (Sawynok and Liu 2003).

Eventually adenosine is inactivated by adenosine-deaminase to inosine or by adenosine-kinase to AMP.

Nucleotides act at two receptor-types called P2X (ligand-gated cationic channels; P2X1-7 subtypes) or P2Y (G protein-coupled receptors; P2Y1,2,4,6,11-14 subtypes) (Fig. 2; Abbracchio and Burnstock 1994; Köles and others 2007). Adenosine acts at its own receptor-types, which are all coupled to G proteins (A1, A2A, A2B, A3; Fredholm and others 2011). P2XRs occur as trimeric constructs of the same subunit (homomeric) or of different subunits (heteromeric) receptors (Köles and others 2008). Each P2X subunit has two transmembrane domains, a large extracellular loop, and intracellular N- and C-termini. P2Y and adenosine receptors have typical seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus. P2YRs may also constitute homomeric or heteromeric assemblies with other P2Y or adenosine receptors. The heteromeric assemblies add further diversity to purinergic receptors, by combining the original biophysical and pharmacological characteristics of their parent subunits.

Pharmacology of Purinergic Receptors and Blockers of Purine Degrading Enzymes Involved in Pain Sensation

A number of P2X- and P2YR-subtypes as well as all adenosine receptors-types are involved in pain sensation (Table 1). Homomeric P2X3Rs and heteromeric P2X2/3Rs are located at the terminals of Aδ and C fibers projecting from sensory neurons of dorsal root ganglia to the innervated tissues (Chen and others 1995; Lewis and others 1995). P2X3Rs respond to the ATP structural analogue α,β-methylene ATP (α,β-meATP) and desensitize rapidly during agonist application. P2X2/3Rs are also sensitive to ATP/α,β-meATP, but do not exhibit desensitizing properties. P2X3 and P2X2/3Rs participate in acute, inflammatory, neuropathic and cancer pain as proven for various experimental pain models (Burnstock 2006; Wirkner and others 2007). The subcutaneous or intraplantar application of ATP and α,β-meATP causes nociceptive responses and hyperalgesia/allodynia as characteristics of acute and neuropathic pain, respectively. Further, non-selective (sursamin) and P2X3R-selective pharmacological antagonists (A317491) as well as antisense oligonucleotides all improve the mentioned pain states in animal models (Donnelly-Roberts and others 2008; Kennedy and others 2003). Accordingly, P2X3R-deficient mice exhibit decreased nocifensive behavior in comparison with their wild-type backgrounds in experimental pain states.

P2X4Rs at spinal microglia mediate neuropathic pain (Coull and others 2005; Inoue and others 2007). ATP-induced activation of microglial P2X4Rs leads to the
secretion of brain-derived neurotropic factor (BDNF), which was implicated in the hypersensitivity of dorsal horn neurons that follows sensitization and inflammation. BDNF causes a depolarizing shift in the E\textsubscript{anion} of spinal lamina I neurons at the dorsal spinal horn, resulting in conversion of GABA\textsubscript{A} and glycin receptor-mediated inhibition to excitation. Indeed intrathecal administration of recombinant BDNF-sequestering fusion protein (TrkB-Fc) acutely inhibited allodynia and the shift of E\textsubscript{anion} of lamina I neurons (Coull and others 2005). P2X7Rs are also involved in both inflammatory and neuropathic pain (Carroll and others 2009; Inoue and others 2007). P2X7R subunits possess a particularly long intracellular C-terminus which has been implicated in regulating receptor function, including signaling pathway activation, cellular localization, protein-protein interactions, and post-translational modification (Costa-Junior and others 2011). In addition, P2X7Rs function as non-selective cationic channels after activation by relatively low concentrations of ATP, but form large diameter pores on their long-lasting activation by high ATP concentrations or alternatively open pores formed by an accessory protein (e.g., Pannexin-1 hemichannels) (Sperlágh and others 2006; Sperlágh and Illes 2014). P2X7Rs also activate the apoptotic caspase enzyme cascade and induce rapid maturation and release of the proinflammatory cytokine interleukin-1β (IL-1β). All these properties as well as the localization of P2X7Rs at peripheral and central immunocytes (lymphocytes, monocytes/macrophages, microglia, astrocytes) enable them to become key players at the neuroimmune interface (Burnstock and others 2011; Carroll and others 2009).

Systemic application of P2X7R antagonists such as A438079 produced dose-dependent antinociceptive effects in models of neuropathic (Nelson and others 2006) and inflammatory pain (Honore and others 2006). In the case of neuropathic pain, P2X7Rs located at satellite glial cells (astrocyte-like cells in sensory ganglia) initiate an inflammatory reaction (Villa and others 2010). Accordingly, deletion of the p2rx7 gene not only altered inflammatory pain but also reduced pain associated with nerve injury (Chessell and others 2005). Other genetic manipulations of the IL-1 system, including targeted gene disruption of the IL-1 type I receptor or the IL-1 accessory protein (IL-1acp) have generated mice that show reduced nociceptive responses relative to

### Table 1. Purines Involved in Pain Sensation.

<table>
<thead>
<tr>
<th>Purines</th>
<th>Receptor Types</th>
<th>Receptor Subtypes</th>
<th>Modulation of Inflammatory/Neuropathic Pain</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Adenosine</td>
<td>P1</td>
<td>A1</td>
<td>↓</td>
<td>Lima and others (2010); Maione and others (2007); Sawynok (2015)</td>
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<tr>
<td></td>
<td></td>
<td>A2A</td>
<td>↑ or ↓</td>
<td>Sawynok (2015); Zylka (2011)</td>
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<td></td>
<td></td>
<td>A2B</td>
<td>↑ or ↓</td>
<td>Sawynok (2015)</td>
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<tr>
<td></td>
<td></td>
<td>A3</td>
<td>↓ or ↑</td>
<td>Little and others (2015); Sawynok (2015)</td>
</tr>
<tr>
<td>ATP</td>
<td>P2X</td>
<td>P2X1</td>
<td>–</td>
<td>Burnstock (2006); Donnelly-Roberts and others (2008); Kennedy and others (2003); Wirkner and others (2007)</td>
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<tr>
<td></td>
<td></td>
<td>P2X2</td>
<td>–</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>P2X3, P2X2/3</td>
<td>↑</td>
<td>Coull and others (2005); Inoue and others (2007)</td>
</tr>
<tr>
<td>ATP, ADP, UTP,</td>
<td>P2Y</td>
<td>P2Y1</td>
<td>↑ or ↓</td>
<td>Barragán-Iglesias and others (2014, 2015, 2016); Gerevich and others (2007)</td>
</tr>
<tr>
<td>UDP</td>
<td></td>
<td>P2Y2</td>
<td>↑</td>
<td>Moriyama and others (2003)</td>
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<tr>
<td></td>
<td></td>
<td>P2Y4</td>
<td>–</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>P2Y6</td>
<td>↑</td>
<td>Barragán-Iglesias and others (2014, 2015); Okada and others (2002); Syhr and others (2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P2Y11</td>
<td>↑</td>
<td>Barragán-Iglesias and others (2014, 2015); Okada and others (2002); Syhr and others (2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P2Y12</td>
<td>↑</td>
<td>Kobayashi and others (2008); Tozaki-Saitoh and others (2008)</td>
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<tr>
<td></td>
<td></td>
<td>P2Y13</td>
<td>–</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>P2Y14</td>
<td>–</td>
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</table>

↑, potentiation of pain; ↓, inhibition of pain; –, no relevance to pain.
The contribution of the metabotropic P2YRs to normal and pathological pain have been less well examined than that of P2XRs (Burnstock 2009a). P2Y1Rs have been described to cause analgesia by blocking via G proteins N-type Ca\textsuperscript{2+} channels responsible for the release of nociceptive transmitters in the spinal cord (Fig. 3A; Gerevich and others 2004; Gerevich and Illes 2004) or via P2X3Rs (Fig. 3B; Gerevich and others 2007). The analgesic effect of the endogenous P2Y1R agonist ADP has been demonstrated in acute thermal and neuropathic pain (Andó and others 2010; Barragán-Iglesias and others 2016). The transient receptor potential vanilloid 1 (TRPV1) is a polymodal detector of pain-producing chemical and physical stimuli. It has been reported that extracellular ATP potentiates the TRPV1 currents evoked by capsaicin or protons and reduces the temperature threshold for its activation through metabotropic P2Y2Rs (Moriyama and others 2003). Co-expression of TRPV1 mRNA with P2Y2 mRNA was demonstrated in the rat lumbar DRG using in situ hybridization histochemistry.

Increasing evidence indicates that UDP-sensitive P2Y6Rs are involved in pain, even though it has also been reported that the P2Y6R antagonist (MRS2578) has no effect on neuropathic pain behavior in mice (Syhr and others 2014). In the neuropathic pain model induced by partial ligation of the sciatic nerve of rats, intrathecal administration of UDP produced significant antiallodynic effects (Okada and others 2002). In these rats, the expression of P2Y6,11Rs was increased in the ipsilateral lumbar spinal cord 7 to 21 days after injury (Barragán-Iglesias and others 2014). Intrathecal treatment with selective P2Y6 and P2Y11R antagonists reduced both the injury-induced allodynia and the accompanying receptor up-regulation. Spinal application of minocycline damaged microglia and in consequence, depressed both nerve injury-induced up-regulation of P2Y6,11Rs and the accompanying tactile allodynia.

In formalin-induced inflammatory pain, pre-treatment with P2Y1, P2Y6, and P2Y11R agonists increased the flinching behavior in the ipsilateral paw (Barragán-Iglesias and others 2015). At the same time pretreatment with selective P2Y6 and P2Y11R antagonists reduced both the injury-induced allodynia and the accompanying receptor up-regulation. Western blot analysis confirmed the presence of P2Y1, P2Y6 and P2Y11Rs in dorsal root ganglia and sciatic nerve. It has been concluded that these receptors have a pronociceptive role in formalin-induced pain.

Accumulating evidence implicates that microglial P2Y12Rs critically participate in the development of neuropathic pain via p38 MAPK activation (Kobayashi and others 2008). First, up-regulated expression of P2Y12Rs...
was observed on microglia after peripheral nerve injury in rats (Kobayashi and others 2008; Tozaki-Saitoh and others 2008). Secondly, inhibition of P2Y12Rs by intrathecal administration of the selective antagonists AR-C69931MX or MRS2395, or the oral administration of clopidogrel (a pro-drug of a P2Y12R antagonist intermediary metabolite), the antisense knockdown of P2Y12R expression, or deletion of the P2ry12 gene, all prevented the development of tactile allodynia and inhibited thermal hyperalgesia (Kobayashi and others 2008; Tozaki-Saitoh and others 2008).

The effects of adenosine on the nociceptive system are quite divergent, because of the existence of 4 different adenosine receptor-types and also because of the localization of these receptors at different hierarchic levels of the nervous system (Sawynok 2015; Sawynok and Liu 2003; Sawynok and Sweeney 1989). The general view is that A1R agonists produce pain-alleviating actions in inflammatory and neuropathic pain models that exhibit hyper-responsiveness (alldynia, hyperalgesia). A1Rs are located at peripheral sensory nerve endings (Lima and others 2010), within the superficial layers of the dorsal horn of the spinal cord (Ackley and others 2003) and at specific supraspinal sites (Maione and others 2007) within the pain signaling neuraxis. A2ARs are mostly neuronal but occur also at immunocytes; it appears that with more prolonged inflammation, the loss of inhibitory A2ARs on inflammatory cells would outweigh the loss of pronociceptive A2ARs on nerve endings, such that there was now an overall increase in nociceptive signaling (Sawynok 2015). A2B and A3Rs have a preferential location at immune cells, and their stimulation results in potentiation of many inflammatory responses (Haskó and others 2009). The peripheral and central effects may oppose each other and in consequence the systemic application of A2BR ligands can generate highly unpredictable actions; by contrast, recent data supply convincing evidence for antinociception by A3R agonists in neuropathic pain (Sawynok 2015). In fact, A3R activation generated selective alleviation of persistent neuropathic pain states and decreased spinal cord pain processing by reducing the excitability of spinal-wide dynamic range neurons and causing supraspinal inhibition of spinal nociception via activation of noradrenergic bulbo-spinal and serotonergic circuits (Little and others 2015). Eventually, it is important to note that adenosine itself has a higher affinity to A1 and A2ARs than to A2B and A3Rs and therefore the modulation of neuronal functions predominates when this purine is released endogenously.

Adenosine receptors may be activated by the application of adenosine itself or by drugs blocking the enzymatic degradation or tissue uptake of adenosine (adenosine deaminase, deoxycoformycin; adenosine kinase, 5′-iodotubercidin; equilibrative adenosine transporter, dipyridamole) thereby leading to higher endogenous concentrations of the agonist. Positive allosteric modulators are a further possibility to induce the receptor-mediated effects with possibly lower incidence of unwanted effects than observed in the case of the application of adenosine or its agonistic structure analogues (Jacobson and Müller 2016). The endogenous levels of adenosine can be also raised by applying recombinant ectonucleotidases facilitating the conversion of AMP to adenosine (Zylka 2011). A single intrathecal injection of secretory (non-membrane bound) versions of prostatic acid phosphatase or ecto-5′-nucleotidase has long-lasting (2-3 days) antinociceptive effect in naïve mice and in mouse models of inflammatory pain and neuropathic pain (Sowa and others 2010; Zylka and others 2008; see also next section). In all cases, these antinociceptive effects were dependent on A1R activation.

There are many selective antagonists for all 4 adenosine receptor types available allowing the use of these ligands according to necessity. It is interesting that caffeine, the stimulatory ingredient of black coffee or tea, which is regularly consumed by a majority of the adult population over the world, is a combined A2A/A2BR agonist (Sawynok 2011). In preclinical studies, caffeine produces intrinsic antinociceptive effects in several rodent models, and augments the action of non-steroidal anti-inflammatory drugs.

**Acupuncture-Induced Analgesia and Purinergic Signaling**

Since the first report on acupurines demonstrated that (1) applying weak EAP stimulation at acupoints Yanglingquan (GB34) and Xuanzhong (GB39) prolonged the latency of nociceptive hind limb withdrawal reflex and (2) intraperitoneal administration of the adenosine receptor antagonists theophylline and caffeine blocked the EAP-induced elevation of the nociceptive threshold (Liu and others 1994), interest on the role of purines in AP analgesia has continuously increased.

Acupuncture treatment releases ATP/ADP from keratinocytes, the major cell type of the skin and from subcutaneous mast cells both during AP and moxibustion (Fig. 4; Burnstock 2009b; Wang and others 2015; Yao and others 2014). The outflow of the whole range of ATP metabolites (ADP/AMP/adenosine) has been measured in the neighboring interstitium by a microdialysis probe after AP of the Zusanli point of mice (Goldman and others 2010). A very similar spectrum of purines was observed in the microdialysate after acupuncturing the Zusanli point of human subjects; the concentration of purines did not increase when the needle was only inserted but not rotated (Takano and others 2012).
Purines were identified by high-performance liquid chromatography. The extracellular concentration of ATP returned soon to baseline after AP, whereas adenosine, AMP and ADP remained significantly elevated (Goldman and others 2010). ATP/ADP triggers the increase of cytosolic Ca\textsuperscript{2+} signaling in a cultured human fibroblast cell line and this leads to the remodeling of its actin cytoskeleton (Goldman and others 2013). A similar situation may arise under in vivo conditions and could contribute transient changes in fibroblast cytoarchitecture that is probably related to the beneficial local effects of AP.

In addition to the activation of P2Rs at fibroblast, P1/P2Rs at intracutaneous sensory nerve terminals can also be stimulated by the released ATP or its enzymatic degradation products ADP and adenosine. In fact, free nerve endings of both A\textsubscript{δ} and C classes of sensory fibers are located in the dermis and epidermis extending to the stratum granulosum and are activated by ATP (Zhang and others 2006). Based on these findings, a hypothesis was forwarded on the crucial role of purines in AP-induced analgesia (Burnstock 2009b). It has been suggested that sensory nerve activity initiated in the skin by AP would exert an inhibitory modulating effect on the spinoparabrachial and spinothalamic tracts to the brain pain centers by a mechanism which has still to be elucidated. In the following passages we will report on experiments aimed at supporting this “purinergic hypothesis of acupuncture.”

An exceptionally large number of studies have dealt with the involvement of P2X3Rs (the structure of this receptor was only recently clarified after successful crystallization of the zebrafish (zf)P2X4R; Fig. 5A) in AP-mediated alleviation of neuropathic pain (Fig. 5B). Neuropathic pain in rats was induced by constricting one sciatic nerve with a ligature (chronic constriction injury; CCI). Then, the withdrawal threshold to pressure and the withdrawal latency to radiant heat, as two parameters of hyperalgesia and allodynia, respectively, have been measured for 14 days in total. During the first 7 days of observation, the two parameters gradually declined and then levelled off at a minimum value. Starting with the seventh day, EAP was applied to ipsilateral or contralateral acupoints (Zusanli, ST36; Yanglinquan, GB34) for 30 minutes daily and for 7 days in total (Cheng and others 2013; Tu and others 2012; Wang and others 2014). This treatment caused a gradual and moderate reversal of the neuropathy symptoms by about 40%; the recovery was almost identical for mechanical pain and temperature sensation, and was practically indistinguishable for the ipsi- and contralateral EAP. Intrathecal application of the selective P2X3R antagonist A317491 on the 10th day, immediately and dramatically facilitated the EAP-induced analgesia (Wang and others 2014). Unfortunately, A317491 was applied only in combination with EAP but never alone and thereby it could not be decided whether the effects of EAP and A317491 were additive or not. An additive interaction would indicate that the mechanism of analgesia is the same, mediated in both cases by the exclusion of P2X3R function. However, equal efficiency of ipsi- and contralateral EAP tentatively suggests that this treatment acts at the spinal or supraspinal level rather than at the peripheral terminals of DRG neurons.
Very similar results were obtained by another group of researchers who used von Frey filaments and a hot plate apparatus for measuring pain intensity after CCI and a somewhat different time-schedule; EAP to Zusanli-Yanglingquan acupoints 3 days after ligating the sciatic nerve again caused moderate analgesia (Yu and others 2013). A possible weakness of this study is that neuropathic pain may not reach its maximum at this early time point and therefore the effect of EAP cannot be reliably evaluated.

All studies equally demonstrated an increase of the αβ-meATP-induced current amplitudes in acutely dissociated DRG neurons prepared from rats which underwent CCI, when compared with their non-operated counterparts (Cheng and others 2013; Tu and others 2012; Wang and others 2014). When the DRG cells were prepared from operated rats which obtained also an ipsi- or contralateral EAP treatment, the αβ-meATP currents were depressed but still larger than the current amplitudes measured in non-operated DRGs. Quantitative reverse transcription–polymerase chain reaction (RT-PCR), in situ hybridization, quantitative immunohistochemistry, and Western blotting, all showed an increased appearance of the P2X3R mRNA/protein in DRG neurons and the dorsal horn of the spinal cord after CCI to the unilateral sciatic nerve (Tu and others 2012, Yu and others 2013; Wang and others 2014). EAP moderately counteracted this increase, without any side preference.

Visceral pain is also supposed to react to AP. A model of the painful irritable bowel syndrome was generated in few days old rats by inflating a balloon in their terminal colon/rectum twice daily for 14 days in total (Weng and others 2013, 2015). These rats developed hypersensitivity to bowel distension within a further period of 6 weeks, elapsing without any pressure stimulation. An arbitrary withdrawal reflex score was used to determine the intensity of pain reaction caused by subsequent colorectal distension. In pressure-treated rats, the pain withdrawal score increased in an EAP reversible manner. The expression levels of P2X3R mRNA (real-time RT-PCR) and protein (quantitative immunohistochemistry) in DRGs also responded with increase and decrease to balloon distension and EAP, respectively. These changes in P2X3R expression were reflected by similar changes in the spinal cord, prefrontal cortex and anterior cingulate cortex. Thus, P2X3Rs appeared to become upregulated along the neuronal pathways mediating pain to higher brain centers. Quantitative RT-PCR measurements were performed in L4-L6 DRGs innervating the colon and rectum. Nonetheless, we are left with some justified doubt, because the criteria to prepare the spinal cord for RT-PCR were not reported in the discussed papers. Of course the spinal cord segments corresponding to the L4-L6 DRGs should be prepared (optimally only their dorsal parts containing the dorsal horn) and processed for RT-PCR.

In complete agreement with these findings, a rat neuropathic pain model showed up-regulated P2X3R protein levels in the midbrain periaqueductal gray (PAG), a crucial site in endogenous pain modulatory systems (Xiao and
others 2010). EAP at Zusanli and Sanyinjiao (SP6) ipsilateral to the side of the ligated nerve counteracted the increase of the P2X3R protein, whereas sham-acupuncture was ineffective. Interestingly, the down-regulated P2X3R expression in the PAG with an antisense oligonucleotide for the p2rx3 gene significantly attenuated the antinociceptive effect of EAP. At the same time a mismatch antisense oligonucleotide had no comparable action.

In conclusion, CCI as a model of neuropathic pain in rats increased P2X3R expression and function in DRGs as well as in pain-relevant areas of the spinal cord and the brain. Both ipsi- and contralateral EAP invariably counteracted these effects. It is reassuring that sham CCI (the same operation but without nerve constriction) was routinely performed as a control. A visceral pain model caused by balloon inflation in the colon/rectum delivered similar data with respect to EAP. Hence, peripheral and central P2X3Rs were decided to participate in the attenuation of pain by EAP, although, P2X4 and P2X7Rs could also contribute (see below). Unfortunately, sham EAP is almost always missing from the experimental designs. This is a major deficit, because the electrical stimulation of acupuncture needles inserted at non-acupoints is a necessary control procedure.

As mentioned earlier, ATP-mediated stimulation of microglial P2X4Rs causes the secretion of BDNF onto lamina I neurons of the spinal cord dorsal horn and is thereby one of the causal factors of neuropathic pain (Inoue and others 2007). Interferon-γ (IFN-γ) facilitated the activation of microglia endowed with P2X4Rs (Tsudo and others 2009). In consequence, excessive release of IFN-γ after nerve injury may transform quiescent spinal microglia into an activated state associated with P2X4R expression. It has been reported that in rats with CCI, EAP significantly increased the paw withdrawal threshold relative to controls (Chen and others 2015). According to expectations, EAP down-regulated both P2X4R and IFN-γ expression in the spinal cord after a preceding up-regulation of these proteins by CCI.

EAP in rats at the Shangjuxu (ST37) and Tianshu (ST25) acupoints improved visceral hypersensitivity after colorectal distension (Guo and others 2013). Accordingly, P2X4R immunoreactivity in colon and spinal cord was also decreased after EAP. In another study, moxibustion at Dachangsu (BL25) normalized the abdominal reflex score after colorectal balloon distension of rats (Liu and others 2015). The up-regulation of P2X7Rs in L5-S1 DRGs caused by balloon distension was also prevented by moxibustion as documented by quantitative immunohistochemistry, real-time RT-PCR, and western blotting. However, it is unclear whether P2X4 or P2X7Rs rather than e.g. P2X3Rs are primarily involved in the analgesic effect of EAP. In other words, the inhibitory effect of pharmacological antagonists, antisense oligonucleotides or siRNA should document the effectiveness of the blockade of P2X4,7Rs on visceral pain.

The significance of endogenous adenosine, generated by enzymatic degradation of ATP, released locally after AP stimulation, is much more clear-cut than the importance of ATP itself for the induction of analgesia. It has been convincingly demonstrated by the Nedergaard group that, in mice, after AP at a Zusanli point, considerable amounts of ATP appeared in the microdialysate which became rapidly converted to ADP, AMP, and adenosine; the rise in the local concentration of adenosine was larger and longer lasting than that of ATP (see above; Goldman and others 2010). These authors modelled inflammatory pain by injecting complete Freund’s adjuvant into the right paw of mice and neuropathic pain by spared injury of the sciatic nerve. Injection of a selective A1R antagonist (2-chloro-N6-cyclopentyladenosine; CCPA) into the Zusanli point reduced both inflammatory and neuropathic pain with equal efficacy. AP at the Zusanli point also reduced both inflammatory and neuropathic pain, apparently through the accumulation of adenosine and the consecutive A1R stimulation. This causal relationship has been confirmed by experiments showing that AP failed to reduce pain in A1R-deficient mice. Adenosine is degraded to the pharmacologically inactive inosine by adenosine deaminase. Blockade of adenosine deaminase by deoxycoformycin led to increased and prolonged adenosine concentrations in the microdialysate; at the same time, deoxycoformycin considerably lengthened the duration of analgesia after AP.

Further support to this finding has been lent by the injection of the selective A1R agonist N6-cyclopentyladenosine (CPA) into the popliteal fossa (Weizhong acupoint; UB40) of mice, while monitoring thermal and mechanical hypersensitivity in neuropathic and inflammatory pain models (Hurt and Zylka 2012). CPA caused analgesia which was antagonized by the selective A1R antagonist 8-cyclopropyl-1,3-dipropylxanthine (CPX). In further experiments, the endogenous levels of adenosine were manipulated by injecting a secretory version of human prostatic acid phosphatase (hPAP) into the popliteal fossa of mice. As already mentioned previously, hPAP generates adenosine by dephosphorylating AMP and thereby largely increases the local concentration of adenosine. Interestingly, hPAP injected to the popliteal fossa inhibited thermal sensitivity for 3 days in the injected leg of wild-type but not A1R-deficient mice, demonstrating a critical requirement for A1R activation.

The results of the Nedergaard and Zylka groups unequivocally confirm that increases in the local adenosine concentration either by increased enzymatic production (hPAP) or decreased enzymatic degradation (deoxycoformycin) both boost analgesia at certain pain-relevant acupoints (Zusanli, Weizhong). These effects
ATP versus Adenosine Effects: Which One Is Responsible for Acupuncture-Induced Analgesia?

In the past couple of years purines have been suggested to mediate AP-induced analgesia. This is a most important issue, because it complements a theory according to which mainly central opioids are responsible for analgesia caused by AP, and in addition it provides a natural scientific explanation for the observed effectiveness of AP in human patients. It is hypothesized that the mechanical or electrical stimulation of acupoints by AP needles causes the release of ATP, which can be enzymatically degraded to a whole range of biologically active metabolites (ATP, ADP, AMP, adenosine). ATP causes pain by acting at P2X3,4,7Rs, while ADP and/or UTP cause analgesia by acting at P2Y1,12 or pain by acting at P2Y2Rs. Eventually, adenosine has an analgesic activity especially by A1R stimulation, although under special conditions (inflammatory pain, A2ARs; neuropathic pain, A3Rs) other adenosine receptor-types may also participate.

Around the turn of this decade, strong arguments were forwarded which confirm that A1Rs mediate AP effects for inflammatory and neuropathic pain. The concentration of endogenous adenosine at the site of AP could be
raised by a blockade of its metabolic elimination, or by an enhancement of its production from the precursor AMP; the intensity of AP-induced pain mirrored these manipulations. Moreover, A1R antagonistic methylxanthines counteracted the effect of AP.

It was only after establishing the causal relationship between AP and adenosine-induced analgesia that many scientists started to elucidate the possible participation of P2XRs in this process. Numerous authors demonstrated an increased expression and function of these P2XR-types during inflammatory, neuropathic and visceral pain at different levels of the peripheral and central nervous system. It has also been shown that AP on the one hand induced analgesia and on the other hand decreased the facilitated expression and function of P2X3,4,7R-types. However, the modulation of P2XRs might be the consequence rather than the reason for anti-nociception. In other words, AP may act via A1Rs, and as a result of the normalized pain sensation, P2XR up-regulation may return to quasi normal levels.

A real deficit of most studies is that a selective antagonist for P2X3Rs has been used only in some cases and P2X4 and P2X7R antagonists have not been utilized at all. Of course, the modulation of ATP degradation by ecto-ATPase inhibitors would not really help to differentiate between ATP and adenosine as executors of AP-induced analgesia, because an increased level of local ATP concentration would eventually result in a correspondingly higher level of adenosine.

**Perspectives and Open Questions**

Further interesting questions also wait for an unequivocal answer:

1. **Purines**: So far, the concentration of purines (ATP, ADP, AMP, adenosine) following AP have only been detected at the puncture site. Do any changes occur in the concentration of purines in response to AP in pain-relevant areas of the brain? This approach would help to differentiate between peripheral and central effects of purines by AP.

2. **Purinoceptors**: Apart from A1, A2A, P2X3, P2X4, and P2X7Rs, do also other pain-relevant purine receptors (Table 1) participate in AP-induced analgesia? In fact, it is still unclear, how P2XR stimulation by ATP released in response to AP can directly generate analgesia. There are two main possibilities: (a) The stimulation of sensory afferents activates the opioid system suppressing the nociceptive system. An attractive alternative is that (b) the stimulation of sensory afferents by AP excludes the excitation of pain neurons either in the dorsal horn spinal horn or in the pain centers of the brain (Burnstock 2009b). In addition, it is most likely that interactions between different purine receptors (e.g., P2X4 and P2X7 or P2Y1 and P2X3) (Fig. 6) or between pain-sensitive purinoceptors and non-purinoceptors (e.g., TRPV1 and P2X3) can fine-tune the analgesic effect of AP. Desensitization of P2X3Rs at the peripheral or central terminals of sensory ganglia neurons and the consequent interruption of pain signaling is an additional possibility. Eventually, single nucleotide polymorphisms and transcriptional regulation of the involved receptors might also modify the alleviation of pain by AP. In contrast to various P2X and ARs, the involvement of P2YRs in AP-induced analgesia is hitherto a black box. Such investigations are badly needed, especially because combination of AP with adenosine and ADP agonists acting as presynaptic inhibitors of transmitter release via A1 and P2Y1Rs at synapses involved in pain pathways might be a novel therapeutic strategy for the treatment of pain.

3. **New experimental tools**: It might be most helpful to introduce new experimental tools in order to elucidate the role of purinoceptors in AP-induced analgesia such as optogenetics, Designer Receptors Exclusively Activated by Designer Drugs (DREADD), and so on.

4. **Acupuncture in humans**: It is most likely, but by no way proven without any doubt that ATP/adenosine participate in AP-induced analgesia in humans. Thus, a rest of uncertainty remains about the translation of data from laboratory animals to humans. Almost all animal experiments were carried out by EAP, which is a more powerful stimulus than AP usually applied in clinics. Of course, it should also be kept in mind that higher concentrations of ATP cause pain, while comparable concentrations of adenosine relieve pain. The various mechanisms by which low, endogenously released concentrations of the excitatory ATP could produce analgesia have been discussed in the section “Clinical Effectiveness and Neurobiological Basis of Acupuncture Analgesia.”

Adenosine has been tested in clinical trials and showed analgesic effect on intravenous or intrathecal application (Hayashida and others 2005). Injection of prostatic acid phosphatase (termed as PA Puncture by Zylka and others 2008) into the acupoint Weizhong exhibited analgesia for an extended period of time. Along this line of thinking enzymatically stable analogs of ATP at low concentrations and adenosine could be injected into various acupoints. Finally, the observation that AP appears to be less efficacious in the Western population than in China could be due to
the regular consumption of coffee with a high content of the adenosine antagonistic caffeine by Europeans and Americans, whereas in China, tea is the favored stimulant with a much lower content of caffeine.

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