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$P2X_3$ Receptor Involvement in Pain States

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Abstract The understanding of how pain is processed at each stage in the peripheral and central nervous system is the precondition to develop new therapies for the selective treatment of pain. In the periphery, ATP can be released from various cells as a consequence of tissue injury or visceral distension and may stimulate the local nociceptors. The highly selective distribution of $P2X_3$ and $P2X_{2/3}$ receptors within the nociceptive system has inspired a variety of approaches to elucidate the potential role of ATP as a pain mediator. Depolarization by ATP of neurons in pain–relevant neuronal structures such as trigeminal ganglion, dorsal root ganglion, and spinal cord dorsal horn neurons are well investigated. P2X receptor-mediated afferent activation appears to have been implicated in visceral and neuropathic pain and even in migraine and cancer pain. This article reviews recently published research describing the role that ATP and P2X receptors may play in pain perception, highlighting the importance of the $P2X_3$ receptor in different states of pain.

Keywords $P2X_3$ receptor · Acute pain · Inflammation · Neurophatic pain

Abbreviations

BBG	Brilliant blue G
BzATP	2′- and 3′-*O*-(4-benzoyl-benzoyl)-ATP
CFA	complete Freund's adjuvant
CGRP	calcitonin gene related peptide
DRG	dorsal root ganglion
ERK	extracellular signal-regulated protein kinase
Ip_5I	di-inosine pentaphosphate
IBS	irritable bowel syndrome
α,β-meATP	α,β-methylene ATP
2-MeSATP	2-methylthio ATP
NK-1	neurokinin-1
PAR	proteinase-activated receptors
PPADS	pyridoxal-phosphate-6-azophenyl-2′, 4′-disulfonic acid
RB2	reactive blue 2
TG	trigeminal ganglion
TNP-ATP	trinitrophenyl-substituted ATP
TRPV1	transient receptor potential vanilloid 1

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Introduction

Two broad categories of pain, acute and chronic, are seen in clinical practice. Acute pain is of short duration, normally has an identifiable cause, and is focal to the side of injury. In contrast, chronic pain with the characteristics of a disease state has no identifiable cause, serves no biological function, and typically has a poor prognosis because of the lack of complex treatment strategies at present.

In recent years, the involvement of a variety of important mediators in pain transmission has been demonstrated. Certain well-investigated and important neurotransmitters, such as glutamate, serotonin, acetylcholine, and various other endogenous agents, such as prostaglandins, histamine, substance P, or bradykinin are involved in the molecular mechanisms of activation and sensitization of nociceptors that trigger pain. Moreover, ATP is a further strong

candidate implicated in peripheral pain mediation. Early indication for the initiation of pain by ATP resulted from experiments with human skin blisters by Collier et al. in 1966 [1] and Bleehen and Keele [2] a decade later. Local application of ATP onto human skin induced a persistent sensation of pain associated with vascular changes [3]. Combined application of ATP with other algogenic substances such as capsaicin [4] or additional local ultraviolet irradiation enhanced the sensation of pain [5]. The infusion of ATP into the musculus trapezius evoked pain [6] and tenderness [7]. During the last 15 years, with the development of improved immunohistochemical and molecular biological techniques, a plethora of data approved the involvement of ATP in pain perception.

The potential importance of ATP in nociception results last, but not the least, from its ubiquitous presence at millimolar concentrations in living cells. On the one hand, ATP can be released from various cells as a consequence of tissue injury or visceral distension and may stimulate the local nociceptors [8–12]. Depolarization by ATP of neurons in pain relevant neuronal structures such as trigeminal ganglion (TG), dorsal root ganglion (DRG), and spinal cord dorsal horn neurons are well investigated. Nociceptive sensory neurons selectively express the $P2X_3$ receptor at high levels (Fig. 1), suggesting the outstanding role of this receptor subtype in processing pain. The main purpose of this review was to outline the relevance of homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors in different pain states (Table 1). Because the involvement of other P2 receptor-types in nociception has recently been postulated, their involvement is also taken into consideration.

P2 Receptors Implicated in Pain

ATP exerts its effects via P2 receptors, which can be subdivided into two major families, the P2X and P2Y receptors. P2X receptors are ligand-gated ion channels. Seven P2X subunits ($P2X_{1–7}$) have been identified and cloned so far [13–15].

The topology of a P2X receptor subunit exhibits two transmembrane domains, connected by an extracellular loop containing regulatory N-glycosylation sites [16–18]. The N- and C-termini reside intracellularly [19]. At present, the crystal structure of P2X receptors is unknown; it is thought, however, that the channels are formed by a composition of homomeric or heteromeric trimers of subunits [17]. The biophysical and pharmacological properties of P2X receptors have been reviewed recently in detail [20, 21].

P2Y receptors belong to the superfamily of G protein coupled heptahelical receptors. Eight different mammalian P2Y subtypes are currently distinguished, namely, $P2Y_{1,2,4,6,11,12,13,14}$ [22]. Negative coupling to $G_{i/o}$ proteins, and thereby, inhibition of adenylate cyclase was shown for $P2Y_{12,13,14}$ receptors, whereas positive coupling to $G_{q/11}$ proteins followed by activation of phospholipase C was reported for the $P2Y_{1,2,4,6,11}$ subtypes. The $P2Y_{11}$ receptor

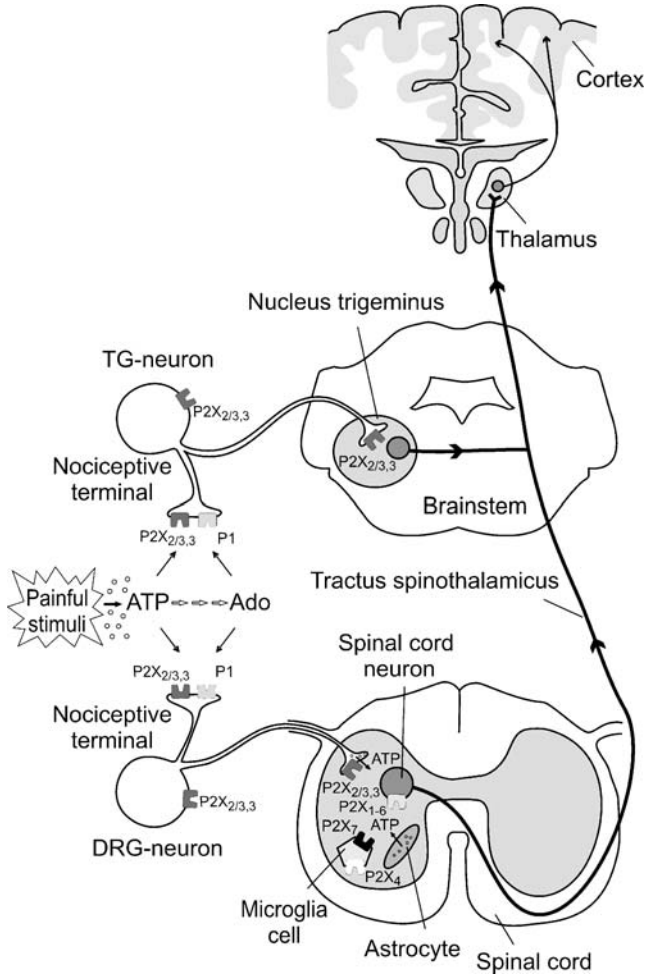

Fig. 1 Ascending control of pain and localization of pain relevant P2X receptors in both neuronal and non-neuronal cells along the neuraxis. The receptor expression in dorsal root ganglion (DRG) and trigeminal ganglion (TG) neurons that is depicted in this schematic drawing is representative also for other sensory ganglia (e.g., nodose ganglia) neurons. High concentrations of ATP are released by nociceptive stimuli from the synaptic vesicles of primary afferent neurons or from damaged neuronal or non-neuronal cells in peripheral tissue. Before its rapid degradation to adenosine, ATP may stimulate $P2X_{2/3,3}$ receptors localized on nociceptive terminals of sensory ganglia neurons. DRG neurons project to the dorsal horn of the spinal cord. On dorsal horn neurons, the $P2X_{1–6}$ subtypes were detected, but the highest expression levels have been demonstrated for $P2X_{2,4}$ and $P2X_6$ receptors. Microglia cells of the dorsal horn possess $P2X_4$ and $P2X_7$ receptors. After activation of these receptors, a host of diffusible factors such as cytokines and chemokines are released, which then act at adjacent neurons to promote hypersensitivity responses. $P2X_3$ receptors situated on the terminals of DRG neurons have been shown to positively modulate the release of glutamate and possibly ATP. Dorsal horn astrocytes can release ATP, which is, in turn, able to activate neighboring cells *via* several types of P2Y receptors. *Ado* Adenosine, *ATP* adenosine 5′-triphosphate, *DRG* dorsal root ganglion, *TG* trigeminal ganglion

Table 1 Involvement of $P2X_3$ and $P2X_{2/3}$ receptors in different pain states

Model	Species	Response (increase, ↑; no effect, −; decrease, ↓)	Agonist or allosteric modulator (route of application)	Antagonist	Reference
Acute pain					
Pain sensation rating	Human	Painful sensation (↑)	ATP (onto blister base of skin)		[2]
Pain sensation rating	Human	Painful sensation (↑)	ATP, ADP, AMP (but not adenosine) (injection into the back)	Diphenhydramine; not effective were: cimetidine, doxantrazole, indomethazine	[3]
Pain sensation rating	Human	Modest burning pain	ATP (iontophoresis onto skin)		[72]
Formalin test	Rat	Early phase of nociceptive behavior (−)	α,β-meATP (pre-treatment, intraplantar)		[35]
Formalin test	Rat	Early phase of nociceptive behavior (−)		Suramin (intraplantar)	[82]
Formalin test	Rat	Late phase of nociceptive behavior (↑)	ATP, α,β-meATP (intraplantar)	PPADS	[82]
Formalin test	$P2X_3^{-/-}$ mouse	Early and late phases of nociceptive behavior (↓ compared to wild-type mice)			[41]
Formalin test	Rat	Early phase of nociceptive behavior (↓)		TNP-ATP (intraplantar)	[83]
Formalin test	Rat	Late phase of nociceptive behavior (↑)	Cibacron blue (intraplantar)		[83]
Formalin test	Rat	Late phase of nociceptive behavior (↓)		A-317491 (s.c.) A- 317344 (*R*-enantiomer was ineffective)	[77]
Formalin Test	Rat	Early phase of nociceptive behavior (↓)		Tetramethyl-pyrazine (intraplantar)	[79]
Behavioral observation	Rat	Paw lifting and licking (↑)	ATP, α,β-meATP (intraplantar)	Desensitization by pretreatment with α,β-meATP and capsaicin; local anesthetic bupivacaine (intraplantar)	[35]
Behavioral observation and thermal threshold testing	Rat	Paw lifting and thermal hyperalgesia (↑)	ATP, α,β-meATP, 2-MeSATP (intraplantar)		[89]
Behavioral observation	Mouse	Paw lifting, licking, biting (dose-dependent ↑)	ATP		[40]
Behavioral observation	$P2X_3^{-/-}$ mouse	Paw lifting, licking, biting (lower ↑ compared to wild-type mice)	ATP	PPADS (additionally inhibition of the response)	[40]
Behavioral observation	Rat	Paw flinching (↑)	BzATP (intraplantar)	TNP-ATP (intraplantar)	[83]
Behavioral observation	Rat	BzATP-induced paw flinching (↑)	Cibacron blue (intraplantar)		[83]
Behavioral observation	Rat	Flinching behaviors (↑)	α,β-meATP, formalin (intraplantar)	$P2X_3$ antisenseoligo-nucleotides (pretreatment, intrathecal)	[109]
Behavioral observation	Rat	Paw flinching, licking, guarding	ATP, α,β-meATP, BzATP	MK-801 (NMDA receptor antagonist) L-703,606 (NK1 receptor antagonist) (ineffective *vs.* α,β-meATP effects) (intrathecal)	[88]
Behavioral observation	Rat	Flinching behaviors (↑)	ATP, α,β-meATP (intraplantar)	Tetramethyl-pyrazine (intraplantar)	[79]
Mechanical threshold testing	Mouse	Mechanical allodynia (↑)	ATP, α,β-meATP (intraplantar)	PPADS (intraplantar)	[4]
Electrophysiological recordings	Anesthetized rat	Firing of articular afferents (↑)	ATP, α,β-meATP (i.a. or intra-articular)	PPADS (i.a.)	[108]

Table 1 (continued)

Model	Species	Response (increase, ↑; no effect, −; decrease, ↓)	Agonist or allosteric modulator (route of application)	Antagonist	Reference
Electrophysiological recordings	Anesthetized rat	Firing of spinal dorsal horn neurons (↑)	α,β-meATP, β,γ-meATP (intraplantar)		[104]
Inflammatory pain					
Pain sensation rating	Human	ATP-induced pain after UV-light-induced skin inflammation vs non-inflamed skin (↑)	ATP (iontophoresis onto skin)		[72]
Formalin test	Rat	Late phase of nociceptive behavior (−)	α,β-meATP pre-treatment (intraplantar)		[35]
Formalin test	Rat	Late phase of nociceptive behavior (−)		Suramin (intraplantar)	[82]
Formalin test	Rat	Late phase of nociceptive behavior (↓)		TNP-ATP (intraplantar)	[83]
Carrageenan- and UV-light-induced inflammation	Rat	Agonist-induced paw lifting and thermal hyperalgesia (↑)	ATP, α,β-meATP, 2-MeSATP (intraplantar)		[89]
Carrageenan- induced inflammation	$P2X_3^{-/-}$ mouse	Thermal hyperalgesia (↑ compared to wild-type mice)			[41]
Complete Freund's adjuvant (CFA) model	Rat	Thermical hyperalgesia (↓)		A-317491 (s.c.) A- 317344 (R-enantiomer was ineffective)	[77]
Complete Freund's adjuvant (CFA) model	Rat	Mechanical hyperalgesia and allodynia (↑)	α,β-meATP (intraplantar)	$P2X_3$ antisense oligonucleotides (intrathecal)	[80]
Complete Freund's adjuvant (CFA) model	Rat	Thermal hyperalgesia (↓)		$P2X_3$ antisense oligo-nucleotides (pre-treatment, intrathecal)	[109]
Neuropathic pain					
Chronic constriction sciatic nerve injury (Bennett model)	Anesthetized rat	Ectopic discharges in A fibers at the site of nerve injury (↑)	ATP (i.v.)	Reactive blue, but not aminophylline	[132]
Chronic constriction sciatic nerve injury (Bennett model)	Rat	Mechanical allodynia and thermal hyperalgesia (↓)		A-317491 (s.c.) A- 317344 (R-enantiomer was ineffective)	[77]
Chronic constriction sciatic nerve injury (Bennett model)	Rat	Mechanical hyperalgesia and allodynia (↑)	α,β-meATP (intraplantar)	$P2X_3$ antisense oligo-nucleotides (pre-treatment, intrathecal)	[80]
Chronic constriction sciatic nerve injury (Bennett model)	Rat	Mechanical hyperalgesia and tactile allodynia (↑)	α,β-meATP (intraplantar)	$P2X_3$ siRNA (pre-treatment, intrathecal)	[139]
Chronic constriction sciatic nerve injury (Bennett model)	Rat	Mechanical allodynia (↓)		TNP-ATP , PPADS (intraplantar)	[134]
Chronic constriction sciatic nerve injury (Bennett model)	Rat	Nocifensive flinch behavior (↑)	α,β-meATP (intraplantar)	TNP-ATP , (intraplantar)	[134]
Chronic constriction sciatic nerve injury (Bennett model)	Anesthetized rat	C-and A_δ-fiber- evoked responses (↓ and wind up)	α,β-meATP (intraplantar)	A-317491 (i.v.)	[140]
Mechanical sensitivity testing after lumbar 5/6 spinal nerve ligation	Rat	Mechanical allodynia (↓)		Suramin + phentolamine (i.p.)	[133]
Mechanical sensitivity testing after lumbar 5/6 spinal nerve ligation	Rat	Mechanical allodynia (−)		$P2X_3$ antisense oligo-nucleotides (pre-treatment, intrathecal)	[109]
Visceral pain					
Electrophysiological recordings	Anesthetized rat	Mesenteric afferent nerve discharge (↑)	ATP, α,β-meATP (i.a.)	PPADS, suramin (i.v.)	[157]

Electrophysiological recordings in i.a. perfused rat tongue preparation	Rat	Neuronal activity in capsaicin-sensitive tongue afferents (↑)	ATP, α,β-meATP (i.a.)	PPADS, suramin (i.a.)	[173]
Electrophysiological recordings in isolated bladder-pelvic nerve preparation	Rat	Afferent nerve discharge in response to bladder distension (↓)	α,β-meATP pretreatment (into bladder)		[147]
Electrophysiological recordings in isolated bladder-pelvic nerve preparation	Rat	Afferent nerve discharge in response to bladder distension (↓)		Suramin (into bladder)	[147]
Electrophysiological recordings in gastroesophageal vagal nerve preparation	Mouse	Afferent nerve discharge in response to agonist application	α,β-meATP		[160]
Electrophysiological recordings in lumbar splanchnic (LSN) and sacral pelvic nerve (PN) preparation	Rat	Afferent nerve discharge in response to agonist application (LSN: ↑) (PN: 1/14 cells responded to agonist)	α,β-meATP (LSN)	PPADS (LSN)	[161]
Acetic acid-induced abdominal constriction assay (ACA)	Mouse	Twisting of the trunk, extension of the hind limbs (↓)		A-317491 (s.c.) A-317344 (R-enantiomer was ineffective)	[77]
Acetic acid-induced abdominal constriction assay (ACA)	Mouse	Twisting of the trunk, extension of the hind limbs (↓)		TNP-ATP, TNP-AMP >> suramin, PPADS (i.p.)	[109]
Migraine pain					
CGRP-treatment of trigeminal neurons in vitro	Rat, cultured trigeminus neurons	$P2X_3$ receptor expression in tumour tissue (↑); accelerated recovery of $P2X_3$ receptor from desensitization			[102]
Cancer pain					
Tumour within calcaneus bone and surrounding tissue	Mouse	$P2X_3$ receptor expression in tumour tissue (↑)			[170]
Gastric sensory neurons from rats with acetic acid-induced gastric ulcers (Kissing ulcers)	Rat, cultured DRG and NG neurons	Current responses to purinergic agonists DRG neuron (↑); NG neuron (−)	ATP, α,β-meATP		[168]
Adenoid squamous cell carcinoma	Rat	Mechanical and thermical hypersensitivity; $P2X_3$ receptor expression in tumour tissue (↑)			[172]

ACA Acetic acid-induced abdominal constriction assay, *BBG* Brilliant blue G, *BzATP* 2′- and 3′-*O*-(4-benzoyl-benzoyl)-ATP, *CGRP* calcitonin gene related peptide, *DRG* dorsal root ganglion, *LSN* lumbar splanchnic nerve, *α,β-meATP* α,β-methylene ATP, *2-MeSATP* 2-methylthio ATP, *NG* ganglion nodosum, *PN* pelvic nerve, *PPADS* pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid, *RB2* reactive blue 2, *TG* trigeminus ganglion, *TNP-ATP* trinitrophenyl-substituted ATP

can additionally activate adenylate cyclase via positive coupling to $G_{i/o}$. Excellent updates summarizing P2Y receptor pharmacology and intracellular signalling have been published recently [14, 23, 24].

P2X Receptors

In the past, the distribution of P2X receptors in pain relevant neuronal structures was investigated in a number of studies. In sensory ganglia, all seven cloned mammalian P2X receptors are present [11, 25, 26]. Six of them ($P2X_{1–6}$) were detected with distinct distribution patterns in primary afferent neurons of rats by means of immunohistochemistry and in situ hybridization [27–29, 36]. In contrast to the $P2X_2$ and $P2X_3$ receptors which were expressed in a comparably high magnitude, low mRNA and protein levels of the other subtypes, except for the nodose ganglion, were observed only.

However, in a recent study by Kobayashi and collaborators [30], a different expression pattern of P2X subunit mRNA was detected in rat DRG neurons. In conflict with former studies, the authors did not find $P2X_1$ receptor mRNA either in DRG neurons or in DRG glia cells. Furthermore, the extent of $P2X_3$ receptor expression was more than twice the extent of $P2X_2$ receptor expression. Whereas in rats and mice up to 90% of DRG neurons were $P2X_3$-immunoreactive, in the upper lumbosacral DRG of the cat, only 30% of the neurons expressed $P2X_3$ receptors [31]. While $P2X_7$ receptors could neither functionally nor immunohistochemically be found in small diameter neurons from both human [32] and rat DRG [26], both small and large diameter neurons of cat DRG were positively stained with $P2X_7$ polyclonal antibodies [31]. In addition, the occurrence of this receptor subtype on non-neuronal cells in rat DRG has been repeatedly demonstrated [26, 32]. Because of the well-known problems concerning the specificity of P2X receptor antibodies, further studies will be necessary to clarify the discrepancies between functional and immunohistochemical data.

Nociceptive neurons can be divided into two major populations, non-myelinated C-fiber and myelinated A-fiber neurons [33]. The group of C-fiber neurons is comprised of both peptidergic neurons, which possess a receptor tyrosine kinase (TrkA) for nerve growth factor (NGF) and of non-peptidergic neurons, which lacks TrkA. A subpopulation of non-peptidergic C-fiber neurons expresses the receptor complex for glial cell-derived neurotrophic factor (GDNF). This population of neurons can be labeled by isolectine B4 from *Griffonia simplicifolia* [34, 35]. The majority of $P2X_3$-positive neurons belongs to the GNDF sensitive population [36, 37] and project to inner lamina II of the dorsal horn of the spinal cord. The GDNF-sensitive population also expresses the transient receptor potential V1 (TRPV1), formerly known as vanilloid receptor 1 (VR1), which can be activated by capsaicin, the pungent agent of hot chili pepper. It is broadly accepted that TRPV1-positive neurons are nociceptive neurons.

The coexpression of TRPV1 and high levels of $P2X_3$ (and $P2X_2$) receptors in sensory neurons supports the involvement of the $P2X_3$ and $P2X_{2/3}$ receptors in pain [27, 38]. This assumption was confirmed by both in vivo experiments [39] and in vitro electrophysiological studies indicating the activation of primary afferents by ATP via these receptor-types [27, 40, 41, 42]. In capsaicin-sensitive neurons, ATP and its analog α,β-meATP, which selectively stimulates the $P2X_{1,3}$ subtypes [43], evoke inward currents with fast activation and inactivation kinetics [42] that were lacking in $P2X_3$-deficient mice [44, 45]. Medium-sized DRG neurons with fast activation but slow desensitization kinetics towards ATP and α,β-meATP were not sensitive to capsaicin and expressed mRNAs for $P2X_2$ and $P2X_3$ subunits, suggesting the presence of heteromeric $P2X_{2/3}$ receptors [42].

An interesting study by Vacca and collaborators [46] provided evidence for the constitutive localization of $P2X_3$ receptors in cholesterol and sphingolipid-rich membrane domains, the so-called lipid rafts, in rat DRG neurons. Lipid rafts are thought to be involved in many functions in neurons, including neurotrophic factor signaling, protein sorting in the *trans*-Golgi network and vesicular trafficking [47, 48]. Moreover, selective functional cross-talk of the $P2X_3$ receptor with other raft-resident receptors, such as $GABA_B$ receptors, could be explained by their coexpression in the same membrane domain [46].

Approximately 40% of rat DRG neurons in culture ([36, 37], but see also [30]) and 37–58% of rat TG neurons [49] show $P2X_3$ immunoreactivity. In DRG, the $P2X_3$ receptor is expressed almost exclusively on small- and medium-diameter neurons. However in TG, $P2X_3$ receptor immunoreactivity was detected also in the somata and processes of large-diameter neurons [29, 41, 50]. In nodose ganglion neurons, even a predominant expression of $P2X_2$ and $P2X_{2/3}$ receptors with sustained responses to ATP and α,β-me ATP has been found [51, 52]. These responses were modulated by both endogenous and synthetic cannabinoids suggesting an interplay between cannabinoid receptors and $P2X_2$ or $P2X_{2/3}$ receptors in the sensation of pain [53].

In addition to the fact that the expression of $P2X_2$, $P2X_{2/3}$, and $P2X_3$ receptors depends on the type of sensory neurons (TG, DRG, or nodose ganglion) studied, the duration of culturing may also be important, as a gradual decline of positive $P2X_3$ immunostaining during postnatal development has been reported in DRG [54]. Although nearly all sensory neurons in DRG, TG, and nodose ganglion of mice express $P2X_3$ receptors on embryonic day 14, only about 50% of both the small and medium-sized neurons possess this subtype on postnatal day 14.

Based on the onset and offset kinetics, three types of ATP-induced P2X currents were recorded in DRG neurons

from adult rats [55, 56]. Fast currents were predominantly observed in small IB4-positive DRG neurons and were found to be mediated by homomeric $P2X_3$ receptors. Both slow $P2X_2$ and mixed $P2X_{2/3}$ receptor-mediated currents occurred in small and medium-sized DRG neurons that were mostly IB4-positive. In $P2X_3$ receptor deficient mice, about 90% of DRG neurons did not respond to ATP or α,β-meATP, and only a small percentage of neurons exhibited slowly desensitizing currents after the application of ATP, but were not activated by α,β-meATP [57, 58]. Most of these cells did belong to the medium-sized subgroup of DRG neurons, which are endowed with the $P2X_2$ receptor subtype [12].

While the involvement of homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors in nociception is broadly accepted, homomeric $P2X_2$ receptors have probably no major role in the transmission of pain. In fact, $P2X_2$ knockout mice did not exhibit alterations in acute nocifensive behavior in response to intraplantar injection of P2X receptor agonists and react to acute thermal nociceptive stimulation with unchanged intensity [58]. These data suggest that $P2X_2$ and also $P2X_{2/3}$ receptors are probably not essential in mediating acute pain stimuli. By contrast, in the second phase of their responses to formalin, $P2X_2$ knockout mice showed deficits in pain-related behaviors [58] that may be attributed to the loss of heteromeric $P2X_{2/3}$ receptors.

The first indication for the functional expression of P2X receptors in spinal dorsal horn neurons was provided by investigations of Jahr and Jessell [59] who observed an excitation of a subpopulation of cultured spinal dorsal horn neurons by ATP. In rat spinal cord slice preparations, the application of ATP and ATPγS to lamina II neurons [60] and lamina V neurons [61], respectively, induced inward currents, suggesting the occurrence of functional P2X receptors. In the dorsal horn of the spinal cord, both mRNA and receptor protein for the $P2X_{1–6}$ subtypes were detected, indicating their possible presence on second-order sensory neurons [28, 36, 62, 63]. In dorsal horn neurons, the highest expression levels have been shown for $P2X_2$, $P2X_4$, and $P2X_6$ receptors; the $P2X_3$ receptor was also present but at a lower level [36, 50]. In addition, $P2X_1$, $P2X_2$, and $P2X_3$ receptors have been found to be localized at the terminals of primary afferent fibers that may belong to the most important sites involved in sensory signal regulation [64]. It is suggested that the activation of presynaptically localized P2X receptors may directly depolarize primary afferent fibers to initiate nociceptive signals, which are then transmitted to lamina neurons [65]. In this respect, $P2X_3$ receptor-expressing terminals have been described to be restricted to the inner layer of lamina II [66]. Patch-clamp recordings from lamina I neurons in a spinal cord slice preparation also provided evidence for the expression of P2X receptors on many capsaicin-sensitive afferent fibers innervating lamina I neurons [65], whereas P2X receptor-expressing afferent fibers projecting onto lamina V neurons were capsaicin-insensitive Aδ-fibers [67]. Regrettably, due to the lack of selective agonists and antagonists, the respective P2X receptor subtypes could not be functionally identified.

An increasing body of evidence suggests the involvement of $P2X_4$ receptors expressed at high levels by activated spinal microglia cells in neuropathic pain sensation [68, 69]. After stimulation by ATP, diffusible factors, such as tumor necrosis factor-α (TNF-α) and cytokines, released from microglia, may modulate neuronal pain processing within the spinal dorsal horn.

P2Y Receptors

In contrast to the plethora of data related to the role of $P2X_3$ receptors in pain, only little is known about the involvement of P2Y receptors in sensory transmission and even this limited amount of data is somewhat controversial. However, a few studies have suggested that P2Y receptors contribute to pain transmission in an antinociceptive rather than nociceptive way [70, 71]. Recently, UTP-/UDP-sensitive P2Y receptors were shown to be involved in producing antiallodynic effects after ligation of the sciatic nerve in rats [70]. In contrast, both, ATP and α,β-meATP induced a gradually developing and long-lasting hyperalgesic effect in the same model, indicating the opposing effects of pain-relevant P2Y and P2X receptor subtypes in nociception [72].

Whereas in the rat spinal cord, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor mRNA was detected by means of reverse transcription-polymerase chain reaction, the presence of $P2Y_2$ and $P2Y_4$ receptor mRNA was found in DRG, only [70]. This finding disagrees with a recent study reporting the existence of $P2Y_1$ and $P2Y_2$ mRNA, but not of $P2Y_4$ and $P2Y_6$ mRNA, in a subset of DRG neurons [73]. Moreover, in non-neuronal DRG cells, mRNAs for $P2Y_2$, $P2Y_{12}$, and $P2Y_{14}$ receptors were also found.

The observation that UTP, but not UDP, evokes sustained action potential firing in a subset of C fibers suggested the occurrence of one or more UTP sensitive P2Y receptors [70]. Furthermore, the majority of the UTP-sensitive fibers also responded to α,β-meATP demonstrating the coexpression of P2Y and P2X receptor subtypes [74].

Intrathecal administration of UTP and UDP, respectively, dose-dependently elevated the mechanical nociceptive threshold in the paw pressure test and prolonged the thermal nociceptive latency in the tail-flick test of rats [70]. Because UTP preferentially activates the $P2Y_2$ and $P2Y_4$ subtypes and UDP has some specificity for the $P2Y_6$ subtype, the antinociceptive effect could be mediated by one of these receptors. Unfortunately, because of the lack of

specific P2Y receptor antagonists, the receptor subtype involved could not be identified in this study. However, the involvement of a $P2Y_4$ receptor is possible because of the existence of $P2Y_1$ and $P2Y_4$ mRNA in some of the dorsal horn neurons in the spinal cord [73].

Moreover, ATP and UTP, but not α,β-meATP, inhibited a slow depolarization caused by repetitive dorsal root stimulation in a subset of substantia gelatinosa neurons by activation of spinal P2Y receptors [75]. Similarly, the C-fiber-induced population of polysynaptic excitatory postsynaptic potentials (EPSPs) in the hemisected spinal cord was inhibited by ADP-β-S [71]. However, neither the non-selective P2 receptor antagonist suramin, nor the $P2Y_1$ receptor antagonists PPADS and MRS 2179 interacted with the $P2Y_{1,12,13}$ receptor agonist ADP-β-S, suggesting the involvement of a hitherto unclassified P2Y receptor subtype [71].

$P2X_3$ Receptors in Acute Pain

Exogenously applied P2X receptor agonists induce acute pain in humans and animals. Intraplantar injection of the $P2X_{1,2,3}$ and $P2X_{2/3}$ receptor agonist α,β-meATP resulted in spontaneous, short-lasting licking, biting, and lifting of the injured hindpaw [39]. After local administration into the hindpaw, BzATP, which preferentially activates $P2X_1$, $P2X_3$, and $P2X_7$ subtypes, also produced an acute paw flinching response [76] that was dose-dependently attenuated by the $P2X_1$, $P2X_3$, and $P2X_{2/3}$ receptor antagonist TNP-ATP [77]. Moreover, the effect of BzATP was potentiated by coadministration of Cibacron blue, a selective allosteric enhancer of $P2X_3$ and $P2X_{2/3}$ receptor activation. Cibacron blue enhanced both agonist-induced receptor activity and recovery from desensitization of the human $P2X_3$ receptor [76].

In human volunteers, application of low concentrations of ATP (0.2–0.6 nmol) into blister bases produced pain with a delayed onset and typically lasting up to 100 s [2]. In another study, only concentrations higher than 250 nmol ATP caused pain after intradermal injection [3]. In humans, iontophoretically applied ATP onto normal skin induced modest pain during 0–20 s of delivery, but was potentiated more than twice in hyperalgesic skin that was generated by UV inflammation and in combination with acute capsaicin treatment, respectively [78]. Usually, the pain produced by ATP diminished after several minutes, despite continuous iontophoretic administration of ATP, indicating the involvement of a fast desensitizing $P2X_3$ receptor subtype. Considering their fast desensitization kinetics and slow recovery, the importance of homomeric $P2X_3$ receptors in acute pain seems to be rather limited. Already, agonist concentrations far below the threshold concentration noteworthy to activate the receptor have been shown to desensitize $P2X_3$ receptors in cultured mouse or rat sensory neurons [79]. Because nanomolar concentrations of endogenous ATP are normally found in the extracellular space [80, 81], a significant fraction of homomeric $P2X_3$ receptors on nociceptive neurons should be desensitized, and thus, unable to sense changes in ATP concentrations produced after tissue damage [79]. However, a recent study contradicts this view. A/J mice exhibit a reduced magnitude of acute nocifensive behavior after intraplantar injection of α,β-meATP in comparison with other strains. In cultured capsaicin-sensitive DRG neurons, $P2X_3$ protein and mRNA levels as well as an increase in intracellular calcium concentration, which were evoked by α,β-meATP, indicated a lower expression of $P2X_3$ receptors in the A/J mouse strain compared to C57BL/6 J mice [82]. Hence, downregulation of sensory $P2X_3$ receptors was postulated to be one reason for low sensitivity to tissue injury pain.

In several models of acute nociception in rats and mice, the administration of the selective non-nucleotide $P2X_3/P2X_{2/3}$ receptor antagonist A-317491 was not effective [83, 84]. Neither acute thermal nor mechanical nociceptive stimuli were antagonized by subcutaneous application of this antagonist. A marginal suppression of pain-related behaviors was found after intraplantar capsaicin administration, only. Surprisingly, tetramethylpyrazine that has been used in traditional Chinese medicine as an analgesic, decreased acute paw flinching responses induced by α,β-meATP injection into the hindpaw and reduced current responses mediated by $P2X_3$ receptors in isolated DRG neurons [85].

Indication for the relevance of $P2X_3$ receptors in mediating acute pain results also from observations made in $P2X_3$ receptor null-mutant mice [44, 45] or after continuous intrathecal delivery of $P2X_3$ receptor antisense oligonucleotides [86]. In $P2X_3$-null mice injection of ATP into the hindpaw evoked a significantly decreased nociceptive behavior compared to wild-type mice [44]. In contrast, administration of capsaicin instead of ATP evoked normal pain responses. It is notable that in this study, responses to noxious thermal and mechanical stimuli were similar in $P2X_3^{-/-}$ and wild-type mice suggesting the lack of $P2X_3$ receptor involvement in the mediation of both sensations, whereas another group has reported that $P2X_3^{-/-}$ mice display altered neural processing of mild "warming" thermal stimuli [45].

The formalin test is considered as one of the widely used standard animal models of nociception [87]. After injection of formalin into the hindpaw of mice, two distinct phases of nociceptive behaviors can be distinguished. The first, acute phase can be observed at about 5 min after formalin application and may result from a direct stimulation of C-fibers. The second, persistent phase develops after about 15–30 min and appears to depend on an inflammatory reaction in the peripheral tissue combined with functional changes in the dorsal horn of the spinal cord [87]. Whereas there is, in principle, agreement on the involvement of

$P2X_3$ receptors in the late phase of nociceptive behavior [39, 45, 88, 89], some controversial data exist regarding the role of spinal $P2X_3$ receptors in the first, transient phase of formalin-induced nociception. Intraplantar pretreatment with α,β-meATP and subsequent subplantar formalin injection or coadministration of ATP and formalin did not enhance hindpaw licking and lifting [39, 88]. In contrast, intrathecal administration of the nonselective P2 receptor antagonist PPADS or the $P2X_1$, $P2X_3$, $P2X_{2/3}$ receptor antagonist TNP-ATP, respectively, reduced the first phase without any effect on the second phase of the formalin test [90]. Because both phases of formalin-evoked nociceptive behaviors were potentiated by Cibacron blue, a selective allosteric enhancer of $P2X_3$ receptor function [89], the involvement of $P2X_3$ receptors in acute and inflammatory pain (see below) is of strong likelihood.

This assumption is supported by a recent study investigating pain behaviors of $P2X_2$ receptor null mice in the formalin model [58]. Interestingly, $P2X_2^{-/-}$ mice did not show alterations in nocifensive responses in the acute phase, but revealed deficits in nocifensive responses in the second phase. Moreover, intraplantar injection of ATP or α,β-meATP resulted in a dose-dependent increase in typical pain behaviors like lifting and biting the treated hindpaw in both $P2X_2^{+/+}$ and $P2X_2^{-/-}$ mice excluding an involvement of $P2X_2$ receptors in acute nocifensive behaviors. These data suggest that $P2X_2$ and $P2X_{2/3}$ are probably not important for acute pain sensation. Hence, homomeric $P2X_3$ receptors may be sufficient for mediation of ATP or α,β-meATP induced acute pain-related behaviors.

The potential role of the spinal glutamate-NMDA receptor system in acute nociceptive signaling induced by α,β-meATP has repeatedly been demonstrated [91–93]. Presynaptically localized $P2X_3$ receptors may increase glutamate release from the first sensory synapse in the spinal cord [92]. Nocifensive behavior which was evoked by hindpaw administration of ATP, α,β-meATP, or BzATP appeared to involve spinal release of excitatory amino acids, as the intrathecally delivered NMDA receptor antagonist MK-801 reduced the number of nociceptive events after the injection of the P2 receptor agonists [94]. The nocifensive behavior triggered by ATP and BzATP, but not by α,β-meATP and formalin, was also reduced by intrathecally injection of a neurokinin-1 (NK-1) receptor antagonist. Thus, two sets of fibers that were activated by ATP and BzATP or by α,β-meATP may contribute to the spontaneous, short lasting nocifensive behaviors.

$P2X_3$ Receptors in Inflammatory Pain

Pain sensation as a result of P2X receptor activation is greatly increased in inflamed tissue. In normal rats, injection of α,β-meATP at ≥5 nmolar concentrations into the hindpaw is required to induce nocifensive behaviors like paw lifting and licking [95]. However, a comparable pain-related behavior was caused in rats by the application of a 100-fold lower α,β-meATP concentration after the injection of intraplantar carageenan into the hindpaw or irradiation with ultraviolet.

During acute inflammation, high concentrations of extracellular ATP have been measured on the sites of tissue injury in experimental animals and in arthritic patients [9, 10, 96, 97]. High extracellular levels of ATP originated not only from damaged cells, but also from non-damaged endothelial cells [98], and in consequence, ATP may modulate vascular perfusion of the damaged tissue and stimulate immune response [99] or may produce nociceptive responses and hyperalgesia. The already mentioned algogenic action of ATP, after its application into blister base preparations of human volunteers, was potentiated under conditions of inflammation [2, 100]. In inflamed tissue, pH values as low as 5.5 have been observed [101, 102]. Hence, an increase in P2X receptor conductance under acidic conditions (for $P2X_2$ and $P2X_{2/3}$ receptors see [103, 104]) could contribute to the algogenic effect of ATP.

An elevated P2X receptor activity can also result from the enhanced expression of this receptor in inflamed tissue and can contribute to abnormal pain responses associated with inflammatory injuries. In the CFA model, a potentiation of the current responses to ATP was observed in isolated DRG neurons due to an enhanced expression of the $P2X_2$ and $P2X_3$ receptor protein [105]. Similarly, in TG neurons, the $P2X_3$ receptor expression was increased already 1 day after induction of masseter muscle inflammation by CFA [106]. Interestingly, in contrast to the results with DRG neurons, the $P2X_3$ receptor expression in TG neurons was not limited to small-sized (muscle afferent) neurons but was also observed in the medium-sized (cutaneous afferent) neuronal population. TG neurons co-expressed $P2X_3$ with either calcitonin gene-related peptide or substance P, and $P2X_3$ receptor expression was increased in both neuronal populations after CFA treatment [106]. Recently, it was demonstrated that under the same conditions the levels of CGRP and substance P were also elevated [107]. This might be of particular importance because CGRP can up-regulate $P2X_3$ receptor mRNA levels in TG neurons as it was shown in migraine pain ([108], see below).

There are numerous reports demonstrating antinociceptive effects of $P2X_3$ receptor antagonists in different models of inflammatory pain in vivo providing strong evidence for the involvement of $P2X_3$ receptors in acute and chronic inflammatory pain. Moderate decrease in nociceptive behavior in the tail-flick assay was observed after spinal instillation of various nonselective P2 receptor antagonists

like suramin, Evans blue, trypan blue, or reactive blue 2, but not after application of PPADS [109]. Intrathecal administration of suramin caused significant antinociception in the formalin test [109] and inhibited the noxious electrically evoked responses of dorsal horn neurons 3 h after carageenan injection into the rat hindpaw but not in control animals [110]. In the same model, the nonselective PPADS was ineffective. During the second phase of formalin-evoked nociceptive behavior in the rat, the $P2X_{1,3}$ receptor antagonistic PPADS [90] and TNP-ATP [89] were both effective in suppressing pain-relevant behavioral changes. In experimental pain models of both the CFA-induced inflamed paw [111, 112] and CFA-induced monoarthritis of the temperomandibular joint [111], TNP-ATP dose-dependently reduced the mechanical hypersensitivity.

ATP and α,β-meATP elevated the responsiveness of nociceptors in skin which had been inflamed with carrageenan treatment [113] or ultraviolet irradiation [78]. In rats with monoarthritis, α,β-meATP but not the selective $P2X_1$ receptor agonist β,γ-meATP decreased pressure pain threshold, confirming the immunohistochemically documented enhancement of $P2X_3$ receptor expression in trigeminal ganglion neurons [111]. In contrast, the injection of α,β-meATP into the knee joint of anesthetized rats suffering from chronic arthritis did not change C- and Aδ-fiber responses [114]. Although primary afferent terminals in the dorsal horn of the spinal cord show $P2X_3$ receptor immunoreactivity [36, 66], intrathecal instillation of α,β-meATP did not influence C-fiber evoked responses of dorsal horn neurons in carrageenan-inflamed rats [110]. In addition, in in vivo recordings of dorsal horn neurons, both threshold and suprathreshold responses evoked by electrical stimulation of C- and Aδ-fiber primary afferents were comparable in wild-type and $P2X_3$ knockout mice [45]. These results are somewhat puzzling, because nociceptive behaviors as a result of inflammatory pain after injection of CFA or formalin into the hindpaw of rats was significantly reduced by continuous intrathecal administration of $P2X_3$ receptor antisense oligonucleotides [115]. Moreover, $P2X_3$ knockout mice showed a reduced formalin-induced flinching behavior in both acute and persisting phase [44], but surprisingly, an enhanced thermal hyperalgesia in chronic inflammation compared to the wild-type animals [45]. Finally, $P2X_3$ receptor knockdown after treatment with $P2X_3$ antisense oligonucleotides dose-dependently decreased CFA-induced thermal hyperalgesia [115].

Hence, it is quite conceivable that spinal $P2X_3$ receptors are not essentially involved in every pain state produced by the different experimental models of inflammation, and moreover, the involvement of other receptors or ion channels should also be considered. In this context, the involvement of P2X receptor-subtypes other than $P2X_3$ in chronic inflammatory pain has recently been postulated. In tissue sections of injured nerves of patients suffering from persistent neuropathic pain and hypersensitivity after trauma, an elevated $P2X_7$ receptor immunoreactivity was observed [32]. However, $P2X_7$ receptor immunostaining was not detected in sensory neurons of human dorsal root ganglia, but was present in satellite cells surrounding these neurons [32].

Local administration of oxidized ATP, a selective $P2X_7$ receptor antagonist, relieves inflammatory pain in arthritic rats [116, 117]. In mice lacking the $P2X_7$ receptor, inflammatory hypersensitivity was completely absent in the CFA model [32], and the development of arthritis after injection of collagen into their joints was reduced [118]. Thus, inflammation may cause $P2X_7$ receptor-dependent release of interleukin-1β, which in turn stimulates the production of other algogens, such as nerve growth factor, cyclooxygenase 2, and superoxide products [119–121]. Thereby, a vicious circle may develop because activation of the inflammatory cascade initiates degenerative and pathological changes, which in turn may elevate inflammatory and/or neuropathic insults. Hence, drugs that specifically inhibit $P2X_7$ receptor function may have analgesic properties and may reduce inflammatory or neuropathic pain [122].

Furthermore, pain processing may also be influenced by activated microglia cells which have been shown to express high levels of $P2X_7$ receptors [62, 123, 124]. Both peripheral nerve injury and inflammation leads to hypertrophy, proliferation, and activation of microglia [125]. In addition to $P2X_7$ receptors, microglia cells express receptors for a host of mediators released during inflammation, such as substance P, calcitonin gene-related peptide (CGRP), and excitatory amino acids [126]. Interactions between the receptors for these agonists and the $P2X_7$ receptor may have consequences for the functioning of pain transmission pathways.

Inflammation results in the production of a multiplicity of cytokines, growth factors, and inflammatory mediators, which can directly activate the nociceptors or may interact with ligand-gated ion channels [112]. Inflammatory mediators, such as substance P and bradykinin, potentiated currents through ATP receptor channels containing the $P2X_3$ subunit [127]. However, the regulatory effect did not result from a direct modulatory action of substance P or bradykinin on the $P2X_3$ or $PX_{2/3}$ receptor, like it has been shown for the recombinant $P2X_2$ receptor [128], but rather on an interaction between the receptors for substance P and bradykinin with the P2X receptor, which was mediated by a protein kinase-dependent mechanism.

Pain hypersensitivity elicited by intraplantar administration of multiple inflammatory mediators, such as prostaglandins of the E type and bradykinin, or $P2X_3$ receptor agonists,

is reduced or absent in mice with mutation of the SCN11A gene that encodes the voltage-gated sodium channel $Na_v1.9$ [129]. In DRG neurons of the mouse, $Na_v1.9$ was highly coexpressed with $P2X_3$ receptors, which suggests that this sodium channel is a downstream effector of the increased pain sensitivity produced by α,β-meATP [129].

Members of the MAPK cascade such as nerve growth factor and extracellular signal-regulated protein kinase (ERK) have been shown to be involved in pain transmission and sensitization in DRG neurons and spinal dorsal horn [16, 130, 131]. Intrathecally applied nerve growth factor induced novel expression of $P2X_3$ receptors at cervical and lumbar DRG neurons and at axons projecting to lamina I and outer lamina II of the spinal dorsal horn and to the ventromedial afferent bundle beneath the central canal [132].

ERK, in turn, induces prodynorphin and NK-1 up-regulation and contributes to persistent inflammation and hyperalgesia [130]. In CFA inflamed rats, both α,β-meATP injection into the damaged paw and mechanical stimulation of the hindpaw increased the phosphorylated extracellular signal-regulated protein kinase (pERK) immunoreactivity in primary afferent neurons [112]. It has been shown that about 75% of the pERK-labeled DRG neurons coexpress $P2X_3$ receptors [112]. Most of the neurons labeled for pERK belonged to the small-sized subgroup of DRG neurons, but some of them were medium-sized A-fiber neurons, which may possess heteromeric $P2X_{2/3}$ receptors [4]. The rise in pERK immunoreactivity occurred already several minutes after α,β-meATP treatment and was significantly, but not completely, decreased in the presence of PPADS or TNP-ATP. This and the ineffectiveness of the selective $P2X_1$ receptor antagonist IP_5I [133], strongly indicated the inclusion of a $P2X_3$ receptor subtype in the induction of hypersensitivity towards mechanical stimulation during peripheral inflammation.

Furthermore, $P2X_3$ receptors localized on sensory neurons interact with proteinase-activated receptors (PAR) that are supposedly involved in tissue inflammation and repair [134]. PARs belong to the family of G protein-coupled receptors and are expressed by small-sized DRG neurons, and after activation, may facilitate the release of substance P and CGRP in peripheral tissues [135]. Activation of PAR reduced the threshold of pain-like behavior after administration of α,β-meATP into the hindpaw of rats and augmented the α,β-meATP induced Fos expression in laminae I and II of the spinal dorsal horn [136]. In contrast, Shimizu and coworkers [137] reported that enhanced cFos expression after the exposure of one hindpaw to heat persisted in mice, in spite of the genetic deletion of their $P2X_3$ receptors.

However, not only peripheral and spinal, but also supraspinal $P2X_3$ and $P2X_{2/3}$ receptors may be involved in the transmission of inflammatory pain. Antinociceptive effects in peripheral tissues were registered after bilateral microinjecton of α,β-meATP into the nucleus locus coeruleus [138] and intracerebroventricular instillation of α,β-meATP [139]. Furthermore, intracerebroventricular administration of the selective $P2X_3$ receptor antagonist A-317491 or pretreatment with antisense oligonucleotides for the $P2X_3$ gene decreased inflammatory nociceptive behaviors induced by intraplantar injection of formalin and intraperitoneal injection of acetic acid [139]. The authors postulated the release of endogenous ATP that may inhibit pain transmission via activation of supraspinal $P2X_3$ and/or $P2X_{2/3}$ receptors, and in this way, may relief pain sensation.

Altogether, these findings support an outstanding role of $P2X_3$ receptors in inflammatory pain processing, although many details and inconsistencies still expect further clarification.

$P2X_3$ Receptors in Chronic Neuropathic Pain

Neuropathic pain syndromes are clinically characterized by spontaneous and evoked types of pain, which arise from damage or disease within the nervous system [140]. In the absence of stimulation, nociceptors are normally silent. However, these neurons may become abnormally sensitive and may develop pathological spontaneous activity after peripheral nerve lesion. Moreover, inflammatory reactions of the damaged nerve trunk can induce ectopic nociceptor activity. There is a great deal of evidence indicating the involvement of $P2X_3$ receptors in painful peripheral neuropathies [83, 141–143]. It is widely accepted that the expression of peripheral $P2X_3$ receptors is regulated by peripheral nerve injury. However, between single studies are discrepancies regarding the direction of the change, even though most investigators report an up-regulation of $P2X_3$/$P2X_{2/3}$ receptor expression or function in different models of neuropathic pain.

An increased $P2X_3$ receptor expression in DRG and spinal cord was observed after chronic constriction injury of the sciatic nerve [144] and in trigeminal ganglia after mandibular inferior alveolar nerve injury [49]. An enhanced expression of $P2X_3$ receptors was only observed on intraspinal terminals of small and medium diameter primary sensory neurons and in the ipsilateral spinal dorsal horn [144]. In contralateral, uninjured nerves, no ectopic sensitivity to ATP was observed [141]. Using in situ hybridization, reduced levels of $P2X_3$ receptor mRNA in rat sensory ganglia were detected in axotomized neurons, but increased levels in adjacent intact neurons [145]. These data suggest an elevated synthesis of the $P2X_3$ receptor protein in pain-relevant neurons. Interestingly, whereas the total amount of $P2X_3$ receptor protein did not change, the membrane fraction of this protein increased in DRG several

days after spared nerve injury, suggesting that nerve injury promotes trafficking of the $P2X_3$ receptor to the cell surface but does not elevate total expression of the $P2X_3$ receptor in the cytoplasm [143]. Furthermore, the elevated membrane expression of the $P2X_3$ receptor was accompanied by an increase in receptor sensitivity without any change in extracellular endogenous ATP levels.

In contrast, after sciatic nerve axotomy, down-regulation by more than 50% of $P2X_3$ receptor expression was found in DRGs of the lumbar segments 4 and 5, which was completely reversed after intrathecally delivered glial cell line-derived neurotrophic factor (GDNF) [37]. A decrease in $P2X_3$ receptor expression was accompanied by reduced current responses towards α,β-meATP in acutely isolated DRG neurons 2 weeks after L5/L6 spinal nerve ligation [146]. However, subsets of small and large DRG neurons maintained unchanged $P2X_3$ receptor expression and function. These neurons possibly contribute to neuropathic pain [146]. Similar to the decrease in $P2X_3$ receptor immunoreactivity in rat DRG after peripheral axotomy, there was a significant decrease in numbers of $P2X_3$-like immunoreactive neurons in intact post mortem human DRG after central axotomy [147].

The local administration of P2X receptor agonists has repeatedly been shown to increase thermal and mechanical hyperalgesia and allodynia in chronic constriction sciatic nerve injury [86, 143, 148]. After nerve injury, the usefulness of nonselective or selective P2 receptor antagonists or $P2X_3$ antisense oligonucleotides in reducing neuropathic pain was also demonstrated. In the spared nerve injury model, allodynia and α,β-meATP-induced flinching were effectively reversed by PPADS and TNP-ATP (an antagonist of the $P2X_1$, $P2X_3$, and $P2X_{2/3}$ subtypes, [143]). Furthermore, treatment with the more specific $P2X_3$/$P2X_{2/3}$ receptor antagonist A-317491 reduced the excitability of dorsal horn neurons in the chronic constriction injury model of neuropathic pain, compared with sham-operated rats, suggesting an antinociceptive effect after the blockade of $P2X_3$ receptors [149]. After subcutaneous injection, the active S- but not the inactive R-enantiomer of A-317491 dose-dependently reduced thermal hyperalgesia and mechanical allodynia in both the Bennett model and the CFA-induced thermal hyperalgesia [83].

Evidence for the involvement of $P2X_3$ receptors in neuropathic pain also results from the observation that down-regulation of $P2X_3$ receptors reduces hyperalgesic and allodynic responses of rats with ligated nerves [115, 148]. A distinct decrease of $P2X_3$ mRNA in the DRG and $P2X_3$ receptor protein in inner lamina II of the dorsal horn of the spinal cord, combined with reduced mechanical hyperalgesia, was reported [150, 151]. The effects of antisense oligonucleotides on nociceptive indices were observed in the ipsilateral, but not in the contralateral, paw in both models. Moreover, intrathecal injection of $P2X_3$ antisense oligonucleotides attenuated the hyperalgesia and allodynia after the nerve injury [82, 86].

Recently, $P2X_4$ receptors with no previously known connection to nociception have been described as participants in neuropathic pain. Tsuda and co-workers [68] have demonstrated that ligation of rat peripheral sensory nerves stimulates $P2X_4$ receptor expression in microglia on the ipsilateral side of the spinal cord. Significant $P2X_4$ receptor accumulation was detected as early as 1 day after lesion [152]. Both, pharmacological blocking by TNP-ATP and the intrathecal administration of antisense oligonucleotides targeting $P2X_4$ receptors reduced the resulting neuropathic pain [68]. Vice versa, stimulation of $P2X_4$ receptors in microglia caused and maintained allodynia. It is conceivable that processing of neuropathic pain may involve diffusible factors like plasminogen or TNF-α, which were released by microglia after $P2X_4$ receptor activation and may modulate neuronal pain signaling [69]. Interestingly, in contrast to the nerve injury model, chronic inflammation after administration of complete Freund's adjuvant was not associated with $P2X_4$ receptor up-regulation [68] and microglia activation [153].

$P2X_3$ Receptors in Visceral Pain

ATP can be released from epithelial cells upon distension of hollow visceral organs and was, for the first time, identified as a motor neurotransmitter in the urinary bladder of the guinea pig in 1972 [154]. More recently, the involvement of ATP in sensory neurotransmission in the rabbit bladder was detected [8, 44]. This sensory function may be important for the physiological action of bladder filling and emptying cycles but also for nociception in pathological states [11, 44]. Birder and coworkers [155] have suggested that urothelial cells share a number of similarities with sensory neurons and may have "neuron-like" properties.

In dependence on the magnitude of distension, different amounts of ATP are released from epithelial cells of hollow organs [8, 156]. After moderate distension, low extracellular ATP concentrations would stimulate P2X receptors on intrinsic sensory fibers and contribute to peristalsis. In the bladder, ATP was suggested to act on $P2X_1$ receptors of the detrusor muscle mediating contraction. Excessive distension would release large amounts of ATP that may activate P2X receptors situated on extrinsic sensory nerves, which then relay pain signals to the central nervous system, finally resulting in colic pain. Evidence has been accumulated supporting this hypothesis. In rats, enhancement of nerve discharges has been observed during distension of the urinary bladder and application of α,β-meATP to a bladder–pelvic preparation, which was prevented by the

unselective P2 receptor antagonist suramin [157]. Moreover, with increasing postnatal age, the expression of $P2X_3$ receptors rose in rat urothelial cells and suburothelial plexus, but did not change in detrusor and serosa [158]. The peak expression was reached at postnatal days 14–21, followed by a decline to values that were observed after birth. Interestingly, in the same study, the expression of the $P2X_2$ receptor subunit decreased with age in urothelial cells.

$P2X_3$ knockout mice have been found to have not only a loss in the rapidly desensitizing currents in sensory neurons, but also a marked urinary bladder hyporeflexia, characterized by an impaired voiding frequency and an increased bladder capacity [44, 159]. Because the loss of $P2X_3$ receptors might decrease sensory neuron activity during bladder filling, and in consequence, disturb micturition reflex, selective modulation of $P2X_3$ receptors may provide new treatment strategies for many bladder storage disorders.

Pain is the most troubling symptom to patients with interstitial cystitis [160]. Several studies have shown that bladder urothelial cells from patients with interstitial cystitis release more ATP after stretching than those from control patients. The released ATP may activate $P2X_3$ receptors, which are expressed in the urothelium of patients but also, to a lower degree, of healthy persons [160, 161]. Moreover, $P2X_2$ receptors were detected in human urothelium, also suggesting the expression of $P2X_{2/3}$ heteromeric receptors [160]. Similarly, an increase in $P2X_3$ immunoreactivity was detected in suburothelial nerve fibers in bladder biopsies of patients with refractory neurogenic detrusor overactivity. The up-regulation of $P2X_2$ and $P2X_3$ receptors in interstitial cystitis was accompanied by an elevated protein expression, and a decrease in $P2X_3$ gene expression, whereas the expression of the $P2X_2$ gene remained unchanged [160, 161]. In contrast to the up-regulation of $P2X_3$ receptors in urothelium in interstitial cystitis, $P2X_3$ (and/or $P2X_{2/3}$) receptor activity in lumbrosacral dorsal root ganglion neurons innervating the urinary bladder was decreased in the rat model of cyclophosphamide-induced cystitis [162].

ATP is suggested to be also an enteric transmitter because the $P2X_3$ receptor is expressed by specific functional groups of enteric neurons. $P2X_3$ receptors were localized on nerve cells of the mesenteric ganglia of the stomach, small and large intestines, and on nerve cells of the submucosal ganglia in the small and large intestine of the guinea pig [163]. These neurons were shown to be excitatory and inhibitory muscle motor neurons, ascending interneurons and cholinergic secretomotoric neurons, suggesting an involvement of $P2X_3$ receptors in enteric mechano- and chemosensitivity. In most cases, the $P2X_3$ receptor was not co-localized with the $P2X_2$ subunit on the same neuron, with the exception of some nitric oxide synthase-immunoreactive neurons where both subunits form heteromers [163].

Retrograde labeling combined with immunocytochemistry indicated that small- to medium-sized neurons from the DRG at levels T8–L1 and L6–S1 innervate the descending colon of mice [164]. More than one-third of the retrogradely labeled cells expressed the $P2X_3$ receptor subtype. This value corresponds well with the $P2X_3$ receptor endowment of the whole DRG population, where about 40% of the neurons were $P2X_3$ positive. Hence, the $P2X_3$ receptor might have an important role in the transmission of noxious signals from the colon.

One of the most common abdominal pain syndromes is the irritable bowel syndrome (IBS), which is characterized by enhanced visceral sensation and abdominal pain [165]. Comparable to interstitial cystitis, $P2X_3$ receptor expression is up-regulated in colonic nerve fibers of patients suffering from IBS [166]. Several studies have shown that α,β-meATP may excite gastrointestinal afferents [167–169]. α,β-meATP induced a reproducible, concentration-dependent excitation of lumbar splanchnic nerve afferents that was reversed by the P2 receptor antagonist PPADS [170]. Recently, it has been demonstrated that lumbar splanchnic nerves, which terminate in the thoracolumbal spinal cord, are more likely to express $P2X_3$ receptors than paired sacral pelvic nerves, which terminate in the lumbrosacral spinal cord [171].

Intraparenteral injection of acetic acid provoked visceral pain in mice that was attenuated by the $P2X_{1-3}$ receptor antagonist TNP-ATP and the nonselective antagonists PPADS and suramin [172]. IP_5I, the selective $P2X_1$ receptor antagonist, was ineffective. Thus, $P2X_3$ and/or $P2X_{2/3}$ receptors, which are activated by endogenously released ATP appear, to play a role in acetic acid-induced abdominal pain. Interestingly, the antinociceptive potency of TNP-ATP was comparable to that of morphine in blocking acetic acid-induced abdominal constriction [172].

$P2X_3$ Receptors in Migraine Pain

The involvement of ATP in migraine was first suspected in conjunction with the vascular theory of this disorder [108, 174]. Currently and largely based on animal models, migraine is also hypothesized to involve neuronal dysfunction in brain areas that mediate nociception, particularly, the trigeminal nerve, spinal trigeminal nucleus, and thalamus [106, 108]. Conceivably, ATP, released in pain attack, may activate $P2X_3$ receptors, which are endogenously expressed by a considerable portion of trigeminal nociceptive neurons.

In the last years, evidence has accumulated that the neuropeptide CGRP, which is a potent vasodilator and pro-inflammatory agent, may play a major role in migraine [173]. Activation of trigeminal nerves enhances the release of CGRP and other peptides that, in turn, cause the release

of pro-inflammatory mediators [174]. These mediators may additionally stimulate CGRP synthesis and release over hours to days. CGRP did not only elevate the concentration of pro-inflammatory molecules, but also enhanced $P2X_3$ receptor conductance in an in vitro model of cultured trigeminal nociceptive neurons [108]. This effect peaked 5 h after CGRP treatment and was accompanied by an up-regulation of $P2X_3$ mRNA and protein synthesis. Furthermore, CGRP did not increase agonist sensitivity but accelerated recovery of the $P2X_3$ receptor from desensitization, which possibly contributed to the elevated $P2X_3$ receptor function. Interestingly, the effect of CGRP was selective and limited to the $P2X_3$ receptor because TRPV1 receptor function has not been changed.

Whereas most trigeminal neurons coexpress $P2X_3$ receptors and CGRP binding sites [106], in DRG neurons, only a low expression of CGRP binding sites, and moreover, an infrequent coexpression with $P2X_3$ receptors was found [175]. In contrast to the effects on trigeminal neurons, in DRG neurons, no increase in $P2X_3$ receptor function by CGRP was observed [108]. Hence, coexpression of both $P2X_3$ receptors and CGRP binding sites in the same neuron seems to be a precondition for the increase in $P2X_3$ receptor activity by CGRP. It is possible that $P2X_3$ receptor conductance was stimulated through second messenger mechanisms, which were activated by CGRP binding.

$P2X_3$ Receptors in Cancer Pain

Until now, a wide variety of P2X and P2Y receptors has been detected in numerous cancer tissues. An excellent review summarizing the present knowledge about the role of P2 receptors in cancer has been published recently [176]. Whereas most studies focus upon involvement of different P2 receptor subtypes in tumor growth and apoptosis, only sparse and if available, discrepant information exists concerning the role of ATP in cancer pain. Interestingly, tumor tissues contain ATP in exceptionally high concentrations, which may be released during growth of the tumor [177], and moreover, may activate nociceptive nerve endings.

An elevated $P2X_3$ receptor function compared to control animals was observed in DRG neurons, which were prepared from rats with gastric ulcers induced by acidic acid injection into the wall of the distal stomach [178]. Whereas in saline-treated controls, 38 and 29% of the cells did respond to ATP and α,β-meATP with inward currents, respectively; the number of responsive cells was enhanced in rats with gastric ulcer. Furthermore, $P2X_3$ receptor currents showed decelerated desensitization kinetics in DRG neurons prepared from tumor-expressing rats compared to their controls. In contrast, in the same model, virtually all nodose ganglion neurons prepared from the saline-treated group produced inward currents after application of ATP and α,β-meATP, suggesting the involvement of specific gastric sensory neurons in gastric ulcer pain.

In a murine model of bone-cancer pain, in which a tumor develops within the calcaneus bone and in the surrounding subcutaneous tissue, about one-third of C-fiber nociceptors that are located in the skin overlying the tumor exhibited spontaneous activity [179]. This effect was accompanied by a decrease in the number of protein gene product 9.5 (PGP 9.5) labeled epidermal nerve fibers, a phenomenon, which is characteristic in cancer-evoked hyperalgesia [180] and also in a variety of neuropathic conditions in humans [181]. Hence, the decline in PGP 9.5-immunoreactive epidermal nerve fibers suggested the involvement of a neuropathic component in this model of bone-cancer pain. After implantation of osteolytic fibrosarcoma cells in and around the calcaneus bone, an increased $P2X_3$ receptor expression was found in a subset of nociceptive epidermal nerve fibers during tumor growth, although the total number of nociceptive epidermal nerve fibers decreased [180]. In this subpopulation of nociceptive fibers, $P2X_3$ receptor immunoreactivity was co-localized with immunoreactivity for CGRP. In another study investigating migraine pain, CGRP has stimulated the up-regulation of $P2X_3$ receptors in trigeminal sensory neurons [108], implying an interplay between $P2X_3$ and CGRP (see above). Hence, CGRP may enhance trafficking of $P2X_3$ receptors to the cell-surface membrane in the calcaneus bone tumor model, as it was already shown for trigeminal sensory neurons [108].

The close association between the levels of CGRP and $P2X_3$ receptors was also demonstrated in a new rat model of adenoid squamous cell carcinoma [182]. In this model, an elevated expression of CGRP and $P2X_3$ receptors, but also of substance P and TRPV1 receptors has been found in trigeminal ganglia. The over-expression of these molecules was supposed to be involved in the mechanical and thermal hypersensitivity observed in rats with oral tumors via a facilitation of nociceptive transmission.

Concluding Remarks

The understanding of how pain is processed at each stage in the peripheral and central nervous system is the precondition to develop new therapies for the selective treatment of different states of pain. Especially, the specific expression of $P2X_3$ receptors on pain relevant sensory afferent neurons favors this receptor subtype as a potential target for the development of new analgesic drugs. Despite the plethora of data dealing with the role of P2X receptors, primarily $P2X_3$ and $P2X_{2/3}$ receptors, in pain transmission, a final evaluation seems to be far away. In particular, the degree of

involvement of this receptor subtype in migraine and tumor pain has to be elucidated. The recently described interactions between $P2X_3$ receptors and neuropeptides and the discovery of other P2 receptor subtypes like $P2X_4$ and $P2X_7$ that are supposed to be involved in the mediation of painful stimuli make clear that the mechanisms of the algogenic action of ATP are more complex than originally assumed.

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