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Molecular Mechanisms of Sensitization of Pain-transducing $P2X_3$ Receptors by the Migraine Mediators CGRP and NGF

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Abstract Migraine headache originates from the stimulation of nerve terminals of trigeminal ganglion neurons that innervate meninges. Characteristic features of migraine pain are not only its delayed onset but also its persistent duration. Current theories propose that endogenous substances released during a migraine attack (the neuropeptide calcitonin gene-related peptide [CGRP] and the neurotrophin nerve growth factor [NGF]) sensitize trigeminal neurons to transmit nociceptive signals to the brainstem, though the mechanisms remain poorly understood. Recent studies indicate that acute, long-lasting sensitization of trigeminal nociceptive neurons occurs via distinct processes involving enhanced expression and function of adenosine triphosphate (ATP)-gated $P2X_3$ receptors known to play a role in chronic pain. In particular, on cultured trigeminal neurons, CGRP (via protein kinase A-dependent signaling) induces a slowly developing upregulation of the ionic currents mediated by $P2X_3$ receptors by enhancing receptor trafficking to the neuronal membrane and activating their gene transcription. Such upregulated receptors acquire the ability to respond repeatedly to extracellular ATP, thus enabling long-lasting signaling of painful stimuli. In contrast, NGF induces rapid, reversible upregulation of $P2X_3$ receptor function via protein kinase C phosphorylation, an effect counteracted by anti-NGF antibodies. The diverse intracellular signaling pathways used by CGRP and NGF show that the sensitization of $P2X_3$ receptor function persists if the action of only one of these migraine mediators is blocked. These findings imply that inhibiting a migraine attack might be most efficient by a combinatorial approach. The different time domains of $P2X_3$ receptor modulation by NGF and CGRP suggest that the therapeutic efficacy of novel antimigraine drugs depends on the time of administration.

Keywords ATP · Trigeminal neurons · Purinergic receptors · Nociception · Headache

Abbreviations

α,β-meATP	α,β-methylene ATP
BDNF	brain-derived nerve factor
CGRP	calcitonin gene-related peptide
DRG	dorsal root ganglia
NGF	nerve growth factor
NO	nitric oxide
PMA	phorbol 12-myristate 13-acetate
$5\text{-HT}_{1B,1D,1F}$	5-hydroxytryptamine (serotonin) receptor subtypes
TG	trigeminal ganglia
TrkA	tyrosine receptor kinase
TRPV1	transient receptor potential vanilloid 1

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How Can ATP-gated $P2X_3$ Receptors be Involved in Chronic Pain?

Extracellular adenosine triphosphate (ATP) is an important mediator of intercellular communication in the peripheral

and central nervous systems operating via the activation of metabotropic P2Y or ionotropic P2X receptors [1]. One target for these ATP effects is the modulation of transmitter release at central [2] and peripheral synapses [3] either directly on neurons or indirectly via receptors expressed by glial [4] or inflammatory cells [5]. Among the P2X receptor class, the $P2X_3$ subtype is very largely expressed by sensory nociceptive neurons [6, 7] and, therefore, is thought to be strongly implicated in the large galaxy of pain states [8]. It should be noted that under standard physiological conditions, the ATP concentration in the extracellular milieu is usually very low because of rapid enzymatic hydrolysis. This process should restrict the action of ATP to the receptors close to the ATP release sites and prevent systematic desensitization of P2X receptors [1, 2, 7]. Nevertheless, as a consequence of strong painful stimuli, transient large release of ATP is likely to occur and may contribute to painful syndromes [2]. This notion is confirmed by recent studies indicating that the selective pharmacological block of $P2X_3$ receptors [9] and downregulation of $P2X_3$ subunits using silencing ribonucleic acid (RNA) or antisense oligonucleotides [10–12] significantly reduces inflammatory and neuropathic pain. During this phenomenon, nociceptive sensory neurons become "sensitized" to stimuli that may, otherwise, be subthreshold under standard conditions. $P2X_3$ knockout mice also show reduced inflammatory pain [13–15].

Figure 1 depicts a scheme to exemplify how, following nerve injury and/or inflammation, various stimuli including extracellular ATP can more readily activate nociceptive neurons [7] to enhance discharge of action potentials propagated to the spinal cord and brain and perceived as pain signals.

One basic property of $P2X_3$ receptors (usually made by homomeric assembly of three subunits) is fast and long-lasting desensitization, namely receptor inactivation in the continuous agonist presence [16–18]. Desensitization could, thus, be a natural limiting factor for ATP-mediated excitation of nociceptive neurons [7]. Conversely, decreasing desensitization of $P2X_3$ receptors or accelerating recovery from it may represent an efficient mechanism for sensitization of nociceptive neurons to ATP in chronic pain [9, 16].

Another mechanism to ensure persistent sensory stimulation via P2X receptors is based on the heteromeric assembly of $P2X_3$ and $P2X_2$ subunits [7, 19, 20]. Owing to the presence of $P2X_2$ subunits intrinsically slow to desensitize, heteromeric $P2X_{2/3}$ channels generate long-lasting (biphasic) membrane currents [21], which might shape the activation of sensory neurons. It is, however, unclear what signals are responsible for assembling $P2X_{2/3}$ heteromeric channels in vivo.

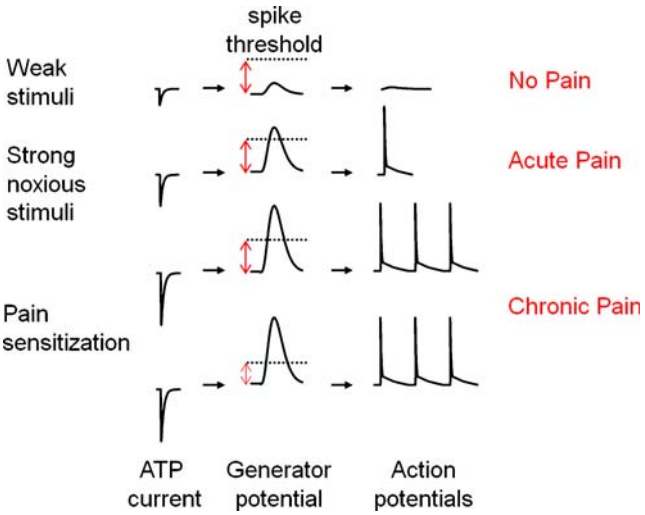

Fig. 1 Scheme to summarize how ATP-mediated membrane currents produced by activated nociceptive sensory neurons can trigger pain signals. If the noxious stimulus is weak (*top row*), the ATP membrane current is small and cannot produce a generator potential large enough to reach the threshold for spike firing and pain signal. A strong stimulus (*middle row*) can, however, evoke an ATP receptor current large enough to induce generator potential that reaches firing threshold and thus elicits pain. When there is pain sensitization (*bottom rows*) due to pathological processes like, for instance, migraine, the ATP receptor current is proposed to be very large even for modest stimuli: The resulting increase in generator potential may allow repetitive firing and stronger pain signals. Furthermore, pain sensitization may also arise because of pathological lowering of the firing threshold for facilitation of spike activity

It is noteworthy that, during chronic pain, sensory neuron sensitization can additionally develop because of a decrease in the threshold for firing action potentials so that neurons may be more readily excited even by minimal stimuli [8]: This condition may be accompanied by a change in $P2X_3$ receptor structure and function, though this is not a mandatory requirement.

The present review is focused on the potential role of $P2X_3$ receptors in trigeminal chronic pain, taking as one example the severe, long-lasting headache of migraine. Because migraine pain is typically associated with hypersensitivity to most sensory stimuli (mechanical, acoustic, visual), current theories propose that during a migraine attack, release of endogenous substances ("migraine mediators") can sensitize trigeminal neurons to normally subthreshold stimuli to increase the nociceptive signal flow to the brainstem [22, 23].

As far as $P2X_3$ receptors are concerned, even if they normally undergo fast desensitization, their contribution to migraine pain may be substantial [1, 8] because certain migraine mediators (see below) decrease receptor desensitization and, in particular, accelerate their recovery, thus ensuring more efficient transmission of nociceptive signals. Furthermore, enhanced responses of trigeminal neurons mediated by $P2X_3$ receptors in the presence of migraine

mediators suggests that the phenotype of such cells has become a more effective transducer of ATP-mediated signaling.

CGRP as a Migraine Mediator

Migraine mediators are a heterogeneous group of substances that comprise nitric oxide (NO), the neuropeptides calcitonin gene-related peptide (CGRP) and substance P, the neurotrophins brain-derived nerve factor (BDNF) and nerve growth factor (NGF), serotonin, and cytokines released from the trigeminal neurovascular system [24–26]. Sensitization of meningeal afferents by endogenous mediators such as NO, inflammatory mediators, and low pH has previously been demonstrated [27–29]. Recently, the role of CGRP has been considered of primary importance to the pathophysiology of migraine pain (Table 1). In fact, during an acute attack of migraine, perivascular afferents innervating the cranial dura mater and intracerebral arteries release large concentrations of CGRP that contributes to the development and to the maintenance of neurogenic inflammation [30]. In accordance with these experimental data, high concentrations of CGRP, NGF, and BDNF have been found in plasma and cerebrospinal fluid of patients [31–33], suggesting their use as potential biomarkers of the disease evolution. Since CGRP and neurotrophins activate several complex intracellular signaling cascades downstream of their receptor activation, it seems likely that their ultimate target ought to include membrane channels such as $P2X_3$ or transient receptor potential vanilloid 1 (TRPV1) [31]. High coexpression of $P2X_3$ and CGRP receptors by trigeminal neurons observed in culture [34, 35] provide a rationale for the potential action by CGRP on $P2X_3$ receptors.

CGRP Triggers Delayed Sensitization of $P2X_3$ Receptors

Even though the activation of CGRP G-protein-coupled receptors is usually followed by cyclic adenosine monophosphate (cAMP)-dependent intracellular Ca^{2+} rise [36], this phenomenon is rarely observed on trigeminal neurons [34, 37]. Furthermore, acute administration of CGRP does not produce immediate changes in $P2X_3$ receptor function of mouse trigeminal sensory neurons in vitro [34] or activation of meningeal nociceptors of anesthetized rats [38]. Nevertheless, when the application of CGRP to isolated trigeminal neurons lasts for 1 h, strong facilitation of $P2X_3$ receptor activity is manifested with an increased peak amplitude of the membrane current [34], presumably allowing the generator potential to produce stronger action potential signaling (Fig. 1). This effect is accompanied by accelerated recovery of $P2X_3$ receptors from desensitization [34], outlining a new mechanism for overcoming the low ability by sensory neurons to respond to ATP (Fig. 1), without changing agonist receptor affinity. Delayed sensitization of $P2X_3$ receptors requires prior activation of CGRP receptors [34, 39] because it is prevented by the specific antagonist $CGRP_{8–37}$ [40, 41]. While CGRP may be expected to be coreleased with substance P [42], sustained application of substance P does not change $P2X_3$ responses of trigeminal neurons (Giniatullin, unpublished), indicating a different target for substance P activity. $P2X_3$ receptor sensitization continues for many hours after CGRP washout [34] in close similarity to the long-term time profile of migraine pain [43].

Molecular Mechanisms of $P2X_3$ Receptor Sensitization by CGRP

Previous studies of trigeminal neurons have identified various mechanisms responsible for controlling the activity of CGRP on trigeminal ganglia. In fact, CGRP can directly activate glial cells to release NO, which in turn strongly increases CGRP release to amplify the peptide effects. This phenomenon proceeds together with upregulation of CGRP gene expression by the activation of mitogen-activated protein kinase pathways [44]. It is noteworthy that cell ability to respond to CGRP is determined by the expression of the receptor activity-modifying protein-1 (RAMP1), an obligatory subunit of the CGRP receptor, thought to be functionally rate limiting [36]. Hence, it has been suggested that elevated RAMP1

Table 1 Studies supporting the role of CGRP in migraine pain

Data	Species	References
CGRP receptor are found in trigeminal neurons, non neuronal cells and meninges	Rat, mouse	[34, 35, 45, 74, 75]
Increased CGRP level in plasma of migraneurs	Man	[76–79]
Headache after intravenous infusion of CGRP	Man	[80, 81]
Anti-migraine triptans normalize CGRP level	Man	[82, 83]
CGRP antagonists BIBN4096BS or MK-0974 are effective in the treatment of migraine pain	Man	[84–86]

might sensitize certain individuals to the CGRP action and predispose them to migraine attacks.

Within the meningeal trigeminovascular system [45], the widespread expression of CGRP receptors should ensure amplification of the peptide effects via release of endogenous substances with their own potential algogenic ability. In keeping with this notion, a part of the CGRP effects on $P2X_3$ receptors upregulation is mediated by the release of BDNF [37, 46].

These are important actions to understand the potent role of this migraine mediator at the start of a headache attack and raise the question of how the action of this peptide can be translated downstream into stronger effectiveness of pain-sensing $P2X_3$ receptors. Recent investigations have shown that larger membrane expression of $P2X_3$ receptors on trigeminal neurons underlies the CGRP-evoked increase in the maximum $P2X_3$ response amplitude [34]. As summarized in Fig. 2B, C, the CGRP-mediated enhancement (dependent on protein kinase A [PKA] and protein kinase C [PKC]) of $P2X_3$ receptor trafficking from intracellular stores to the cell surface leads to the insertion of new $P2X_3$ channels into the neuronal membrane [34].

The long-lasting nature of $P2X_3$ upregulation suggests additional mechanisms to support larger membrane delivery of $P2X_3$ subunits; otherwise, receptor supply might soon be exhausted. In accordance with this view, recent experiments with in vitro trigeminal neurons indicate that CGRP increases $P2X_3$ gene expression via cAMP-dependent cAMP response element-binding [CREB] protein activation mediated by calcium/calmodulin-dependent protein kinase II [CaMKII] [46]. This effect is as a potential process to support the hour- and day-long headache of migraine.

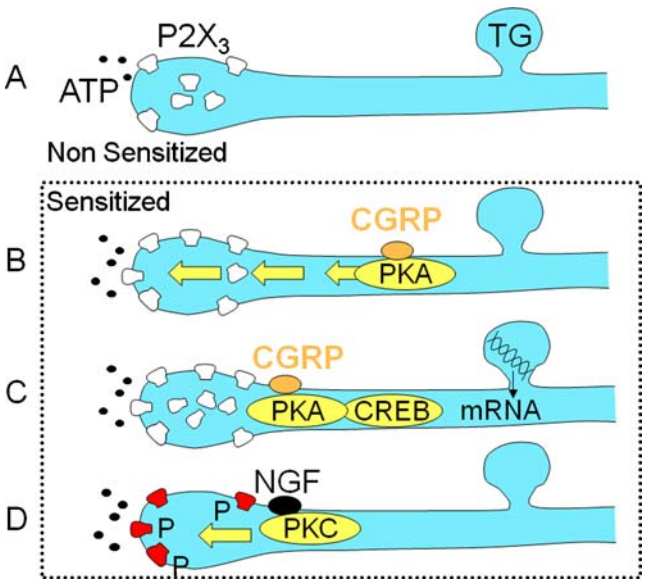

Fig. 2 Molecular mechanisms regulating $P2X_3$ receptors of trigeminal neurons (*TG*) in basal conditions (nonsensitized, **A**) and in a migraine pain model (sensitized, **B**–**D**), when pain mediators such as CGRP (**B**, **C**) or NGF (**D**) are released. **A** ATP-gated $P2X_3$ receptors are expressed intracellularly and on the neuronal membrane of trigeminal neurons at rest. **B** CGRP stimulates trafficking of $P2X_3$ receptors from intracellular stores to the cell membrane via PKA- and PKC-dependent mechanisms. **C** CGRP triggers new $P2X_3$ gene expression to support long-lasting upregulation of $P2X_3$ receptor function. **D** NGF induces rapid and reversible upregulation of $P2X_3$ receptor function through their PKC-dependent phosphorylation

Selectivity of CGRP Action

While TRPV1 channels play a major role in pain transduction [47], they are expressed only by a subpopulation of mouse and human trigeminal neurons [48, 49] and are not modified by 1-h administration of CGRP [34]. This observation accords with the rather limited coexpression of TRPV1 channels and CGRP-binding sites in trigeminal neurons in culture [34] and reinforces the notion that the effects of CGRP are preferentially directed to $P2X_3$ receptors.

CGRP upregulation of $P2X_3$ receptors seems to have topographic specificity because, unlike trigeminal ganglion sensory neurons, which have a strong coexpression of CGRP receptors and $P2X_3$ receptors [34], dorsal root ganglion (DRG) neurons show a low coexpression of $P2X_3$ and CGRP receptors and thus lack the substrate for direct interaction between these signaling systems as confirmed by electrophysiological studies [34]. It is likely that on DRG neurons, the mechanism of action by CGRP is different (though still mediated via PKA and PKC activity), and it involves the enhancement of Na^+ (tetrodotoxin-resistant) and Ca^{2+} membrane currents that allow repeated discharges of action potential [50].

Sensitization of $P2X_3$ Receptors by NGF

NGF binding to tyrosine receptor kinase (TrkA) receptors of sensory nociceptive neurons can evoke potent algogenic effects via multiple signal transduction mechanisms impinging upon the $P2X_3$ and TRPV1 channels [51–53]. In particular, on trigeminal neurons, NGF produces rapid upregulation of $P2X_3$ receptor function through a molecular pathway essentially different from CGRP [39] even if NGF can stimulate CGRP neosynthesis [54]. As shown in Fig. 2D, the NGF-mediated action on $P2X_3$ receptors is associated with increased threonine subunit phosphorylation via PKC activation [39], a process known to enhance $P2X_2$ or $P2X_3$ receptor-mediated currents [55, 56].

On trigeminal neurons treated with NGF for 24 h, the enhancement in the amplitude of $P2X_3$ receptor current persists and is supported by increased $P2X_3$ messenger ribonucleic acid (mRNA) neosynthesis, without changing the overall level of $P2X_3$ protein [39].

In view of the variable ambient concentration of NGF in trigeminal ganglion cultures [49] and even in vivo, it is difficult to understand the effects of the application of exogenous NGF. To better understand the action of NGF in a simplified model and to obtain a stable baseline condition, it is useful to investigate any neurotrophin-mediated activity after endogenous NGF deprivation (with a neutralizing antibody). In fact, culturing trigeminal neurons under NGF-neutralizing conditions drastically reduces the peak amplitude of $P2X_3$ receptor-mediated currents and enhances membrane coexpression of $P2X_2$ subunits in association with $P2X_3$ subunits [39]. Anti-NGF treatment also induces the expression of a significant (yet small) population of heteromeric $P2X_{2/3}$ receptors [39]. Though capable of mediating a more persistent current, such heteromeric receptors generate responses of very small amplitude, probably inadequate for long-lasting excitation of nociceptors [39]. Furthermore, the functional impact of heteromeric $P2X_{2/3}$ receptors, formerly studied in recombinant expression systems [57], may depend on the expression of $P2X_2$ subunit splice variants [58]. In accordance with this view, neutralization of NGF in trigeminal neurons in culture induces the expression of the $P2X_{2/e}$ splicing variant subtype [39], known to confer reduced receptor function [59] and thus contributing to lower neuronal excitability. These observations also suggest that, under standard conditions, endogenous NGF may repress the formation of these heteromeric receptors to facilitate $P2X_3$ receptor signaling.

The more general question then arises about the gene expression and function of $P2X_2$ subunits in mammalian neurons. In trigeminal ganglia, their expression is species dependent because they are much more abundant in rat than in mouse sensory neurons [49], thus contributing to the presence of the slowly decaying currents evoked by ATP with the biophysical characteristics of heteromeric $P2X_{2/3}$ receptors. Since there is no reliable in vivo rodent model of migraine pain, it is difficult to ascertain the actual role of $P2X_2$ subunits in the generation of headache. We can hypothesize that $P2X_2$ subunits (assembled as homomeric or heteromeric receptors) are involved in neuronal signaling including perhaps migraine headache because they are expressed by the frontal cerebral cortex in man [60] and by sensory neurons in monkey [61]. This proposal accords with the view that peripheral sensory neurons express mRNA and protein for multiple P2X receptor subtypes, comprising functional $P2X_2$ and $P2X_3$ subunits [62].

Multiple Targets of NGF Action Concur to Amplify its Algogenic Effects

$P2X_3$ receptors are not the unique target for the direct action by NGF, especially because not all trigeminal neurons express high-affinity TrkA receptors to bind this neurotrophin [39]. Thus, it is well established that NGF facilitates acute sensitization of TRPV1 channels [51, 52, 63, 64] and activation of voltage-gated Na^+ channels [65, 66]. Both effects would be powerful mechanisms to augment and prolong the algesic action of NGF.

The different molecular mechanisms of action by NGF and CGRP on $P2X_3$ receptors suggest that the sensitization of trigeminal neurons is a combinatorial phenomenon with a synergy of action of (rather than occlusion between) distinct migraine mediators. This prediction is supported by the finding that anti-NGF treatment does not block the potentiation by CGRP of $P2X_3$ receptors, as well as CGRP receptor antagonism does not prevent the NGF-dependent enhancement [39]. The multiplicity of migraine mediators (including serotonin, prostaglandins, and interleukins) raises the possibility that they also modulate $P2X_3$ receptors via intracellular pathways that may be partly shared with NGF or CGRP. In the future, it will be interesting to clarify if the selectivity of action depends on cell expression of certain receptors for migraine mediators, whether different mediators use intracellular cascades that converge upon common final gateways, and whether the presence of multiple receptor systems sensitive to nociceptive substances may ensure the persistence of signaling typical of chronic pain.

Clinical Implications

As neuronal sensitization underlying trigeminal pain remains unsatisfactorily treated by current pharmacological agents, the recent advances in molecular mechanisms described in this review might help to design new pharmacological tools. Sensitization by CGRP of nociceptive $P2X_3$ receptor signaling may be blocked by the antimigraine triptans (agonists on metabotropic $5HT_{1B/D/F}$ receptors) that operate, at least in part, via the inhibition of CGRP release [67]. However, triptans might produce adverse cardiovascular effects and require administration at the beginning of the migraine attack to be effective. Recently, CGRP receptor antagonists have been used to block migraine pain and prevent long-lasting sensitization of trigeminal neurons [23, 68–70]. Clinical studies demonstrate the effectiveness and apparent safety of the CGRP antagonists, BIBN4096BS or MK-0974, as antimigraine drugs [71]. Experimental studies showing how the action of CGRP may persist long after its washout [34] suggest the early use of these antagonists during a migraine attack.

Some patients remain, however, unsatisfactorily treated with currently available drugs and even with the new antagonists BIBN4096BS or MK-0974. One may question whether this condition originates from the interplay of

various migraine mediators that are inadequately controlled by blocking only one of them. In particular, the independence of NGF or CGRP potentiation of $P2X_3$ receptors suggests that inhibiting the algogenic action of this neurotrophin might expand the effectiveness of antimigraine treatments. Unfortunately, there are no drugs to selectively and reversibly block NGF release or its receptors. Hence, the suggestion that NGF functional antagonism is a promising tool to treat headache [32, 72] requires clinical validation, even though anti-NGF antibodies prevent trigeminal pain caused by in vivo activation of $P2X_3$ receptors [39]. Finally, as $P2X_3$ receptors are a target for multiple migraine mediators, it might be preferable to block their sensitization with novel $P2X_3$ receptor antagonists [73] rather than inhibiting processes upstream of them.

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