Characterization of ATP release, its fast actions on sensory neurons and their pharmacological modulation

Thesis submitted for the degree of
"Doctor Philosophiae"

S.I.S.S.A.
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NOTE

The data reported in the present thesis have been published in the enclosed articles, which are listed below, in chronological order of publication. In all cases the candidate performed the experimental work (concerning electrophysiology and calcium imaging) and data analysis, and contributed to paper writing.

Fabbro A, Nistri A. Chronic NGF treatment of rat nociceptive DRG neurons in culture facilitates desensitization and deactivation of GABA(A) receptor-mediated currents. *British Journal of Pharmacology* (2004), 142, 425-434.

Fabbro A, Skorinkin A, Grandolfo M, Nistri A, Giniatullin R. Quantal release of ATP from clusters of PC12 cells. *Journal of Physiology* (2004), 560, 505-517.

Fabbretti E, Sokolova E, Masten L, D'Arco M, **Fabbro A**, Nistri A, Giniatullin R. Identification of negative residues in the $P2X_3$ ATP receptor ectodomain as structural determinants for desensitization and the Ca^{2+}-sensing modulatory sites. *The Journal of Biological Chemistry* (2004), 279, 53109-53115.

Simonetti M, **Fabbro A**, D'Arco M, Zweyer M, Nistri A, Giniatullin R, Fabbretti E. Comparison of P2X and TRPV1 receptors in ganglia or primary culture of trigeminal neurons and their modulation by NGF or serotonin. *Molecular Pain* (2006), 2, 11.

Fabbretti E, D'Arco M, **Fabbro A**, Simonetti M, Nistri A, Giniatullin R. Delayed upregulation of ATP $P2X_3$ receptors of trigeminal sensory neurons by calcitonin gene-related peptide. *The Journal of Neuroscience* (2006), 26, 6163-6171.

The following publication, arising from a collaboration project, is not included in the present thesis:

Fabbro A, Pastore B, Nistri A, Ballerini L. Activity-independent intracellular Ca^{2+} oscillations are spontaneously generated by ventral spinal neurons during development *in vitro*. *Cell Calcium* (2006), doi:10.1016/j.ceca.2006.07.006 (in Press).

ABSTRACT

ATP is a neurotransmitter at several sites in the central and peripheral nervous systems, particularly in pathways concerned with nociceptive input processing. This realization has prompted a number of studies to investigate how manipulating the action of ATP might lead to new forms of pain treatment. There is, however, a dearth of information about the mechanisms regulating the release of ATP in response to depolarization and how its effect on target neurons can be pharmacologically modulated. The present work was, therefore, focussed on two main aspects concerning the action of ATP. First, a new method, based on the use of PC12 cells in culture, was set up to investigate the characteristics of quantal release of endogenous ATP. Second, the characteristics of activation, desensitization and pharmacological modulation of the main class of ionotropic $P2X_3$ receptors on sensory ganglia were studied. All these results were obtained with whole-cell patch clamping and calcium imaging experiments.

PC12 cells contain ATP in dense core vesicles in which it is co-stored with catecholamines. By applying depolarizing pulses to PC12 cells, transient inward currents (STICs) were detected only from PC12 cells that formed clusters with their closely juxta-apposed membranes, thus creating a restricted extracellular space for the local action of ATP. STICs were Ca^{2+} dependent events due to quantal release of endogenous ATP (acting on $P2X_2$ receptors). The action of ATP was readily facilitated by extracellular Zn^{2+} and blocked by the selective ATP receptor antagonist PPADS. STICs originating from clusters of PC12 cells may therefore be a useful, cheap and simple model to study ATP-mediated quantal currents.

$P2X_3$ receptors underlying the algogenic effects of ATP were characterized on trigeminal ganglion (TG) neurons in culture, and compared with other nociception-related receptors like the TRPV1 ones. Under the present experimental conditions, TG neurons remained functional for many days. Their $P2X_3$ and TRPV1 receptors were differentially upregulated by chronic application (24 h) of NGF and 5-HT, respectively. Such an effect of NGF was selective for $P2X_3$ receptors, because it spared $GABA_A$ receptors that actually acquired accelerated receptor desensitization and deactivation.

Because strong desensitization rapidly curtails the effects of $P2X_3$ receptors, we considered this process as an important target to up or down regulate their activity. To identify the sites involved in desensitization, single point mutations in the extracellular loop of $P2X_3$ receptors (heterologously expressed in HEK cells) were introduced to specifically affect the onset of

desensitization and/or the recovery from it, indicating that these two properties were structurally distinct. Could the prototypical migraine mediator CGRP induce pain by affecting $P2X_3$ receptors, especially their desensitization properties? This question was examined by using cultured TG neurons as a validated model to study $P2X_3$ receptors in the presence of CGRP. CGRP largely and powerfully potentiated the amplitude of responses mediated by $P2X_3$ receptors. This phenomenon was attained not only *via* increasing their membrane expression (due to accelerated trafficking), but also by decreasing their desensitization characteristics. The action of CGRP was dependent on activation of intracellular PKA and PKC, and did not involve modulation of TRPV1 receptors. These data provide a novel molecular substrate for the algogenic action of CGRP.

INTRODUCTION

1. ATP as a neurotransmitter

Adenosine triphosphate (ATP) is one of the most widespread compounds in living cells. It is now well established that it plays an important role not only in energy supply but also in intercellular communication.

Non-neuronal cells, like those of the vascular system after mechanical stimulation (red blood cells, endothelial cells, smooth muscle cells, platelets during aggregation), epithelial cells lining tubes and sacs (e.g. intestine and bladder), adrenal chromaffin cells, astrocytes and glial cells are important stores of ATP ready for release (Burnstock, 2000; Bodin and Burnstock, 2001). ATP is indeed physiologically released by a wide variety of sources (mainly *via* vesicular release or ATP-binding cassette transporters; Lazarowski et al., 2003). The concentration of ATP in such vesicles can be particularly high: for example, dense core vesicles of chromaffin cells (and of their relatives, the PC12 cells) contain ATP at a concentration exceeding 100 mM (Njus et al., 1986). Remarkably, large amounts of ATP can be released from damaged tissues and during tissue inflammation, thus pointing to this substance as one of the major contributors to nociceptive input transmission (see section 3; Burnstock, 2001; Cook and McCleskey, 2002).

In addition to non-neuronal cells, neurons represent a notable source of ATP, as this molecule can act as a neurotransmitter, which is widely used in both peripheral and central nervous systems (Dunn et al., 2001; North and Verkhratsky, 2006; Pankratov et al., 2006; see below). In neurons, ATP can be released from dedicated vesicle pools or it can be co-stored and/or co-released together with classical neurotransmitters. For example, ATP is released in association with acetylcholine at neuromuscular junctions (Silinsky and Redman, 1996), with noradrenalin and neuropeptide Y from sympathetic neurons (Huidobro-Toro and Donoso, 2004), with GABA from spinal dorsal horn neurons (Jo and Schlichter, 1999), with catecholamines from peripheral and central noradrenergic neurons (von Kugelgen and Starke, 1985; Vizi and Burnstock, 1988; Poelchen et al., 2001), and with calcitonin gene-related peptide (CGRP) and substance P (SP) from sensory neurons (Sweeney et al., 1989; Bodin and Burnstock, 2001).

On the basis of the frequent co-release of ATP with other neurotransmitters (and depending on the expression and localization of ATP receptors at the correspondent sites; see section 2) there is potential for a double role for ATP, namely as a neurotransmitter itself (or modulator of the

effects of other neurotransmitters) at postsynaptic level, or as a modulator of neurotransmitter release at presynaptic sites. In this context, the characterization of the mechanisms involved in the elementary release of ATP and the functional responses consequent to it assume particular interest.

ATP release

Many studies focussed on ATP-mediated transmission have been carried out by using *exogenous* ATP (for reviews, see Dunn et al., 2001; North, 2002) or heterologously expressed ATP receptors (Hollins and Ikeda, 1997; North, 2002; see section 2). As a consequence, our knowledge about ATP-mediated transmission between neurons and, in particular, about *endogenous* ATP release and the functional consequences of this release is quite limited. Actually, ATP has been shown to be a fast (non-metabotropic) synaptic transmitter in several parts of the brain, as it mediates a substantial fraction of both miniature and evoked excitatory post-synaptic currents in medial habenula (Edwards et al., 1992), as well as in hippocampus (Pankratov et al., 2006), thus making ATP release an important subject in view of the physiological role of ATP in the central nervous system.

The release of endogenous ATP can be analyzed by several currently available methods, enabling quantification of its extracellular levels. The most widely used are the luciferin-luciferase assay, which is a simple method based on the light-emitting reaction of luciferin with the ATP molecule, and the HPLC (high performance liquid chromatography), which can be used to monitor the release of radiolabelled purines (Lazarowski et al., 2003). These techniques are highly sensitive as they can detect ATP levels in the nanomolar range; nevertheless, they have a poor time resolution and, by measuring the ATP concentration of the bulk solution under examination, they are likely to severely underestimate the local ATP concentration near the cell surface. Recently, specific probes have been made available for measuring extracellular ATP, although their size is uncompatible with direct assays at synaptic sites (Dale et al., 2005; Llaudet et al., 2005).

In addition to the direct study of ATP release, the physiological responses consequent to it should also be monitored for a better understanding of the mechanisms of ATP-mediated synaptic transmission, thus pointing to the need for a simple experimental model to study unitary events based on *endogenously* released ATP. To this aim, a reliable method to study endogenous ATP release and its effects at the level of the post-synaptic membrane could be the

use of a simple *in vitro* cell system, where the spontaneous (or evoked) ATP release could activate natively expressed ATP receptors on neighbour cells. In this respect, a suitable example of "ATP biosensor" with the above mentioned characteristics might be chromaffin cells or their relatives PC12 cells, because they contain ATP in their dense core vesicles (together with catecholamines; Njus et al., 1986) and also natively express ionotropic $P2X_2$ ATP receptors (Brake et al., 1994; see section 2). This issue was therefore investigated and the outcome is described in the first part of the results reported in the present thesis.

2. ATP receptors

Once released into the intercellular space, the action of ATP is mediated by ionotropic (P2X) or metabotropic (P2Y) receptors. These receptors are further classified into various subtypes, the P2X 1,2,3,4,5,6,7 (Ralevic and Burnstock, 1998; North, 2002) and the P2Y 1,2,4,6,11,12,13,14 (Abbracchio et al., 2003; Boehm, 2003) receptors, operating via differential and specific mechanisms.

P2Y receptors have a widespread distribution in both non-neuronal (gastrointestinal tract, heart, lung, liver, skeletal muscle) and neuronal tissues (spinal cord, brain, sensory neurons). They are activated by purine and pyrimidine nucleotides and are coupled to G proteins; their intracellular transduction mechanisms are extremely complex and involve several specific signalling pathways, including protein kinase C (PKC), phospholipase A_2 (PLA_2) and phospholipase C (PLC), depending on the specific P2Y receptor subtype involved (Ralevic and Burnstock, 1998; Burnstock, 2001; Ruan and Burnstock, 2003; Erb et al., 2006).

P2X receptors

In contrast to the relatively delayed, metabotropic response to ATP mediated by P2Y receptors, ionotropic P2X receptors account for the fast-developing response to ATP (Ralevic and Burnstock, 1998). P2X receptors are ATP-gated channels permeable to Na^+, K^+ and Ca^{2+}, whose expression and distribution varies considerably in different tissues. For example, while $P2X_1$ subunits are predominantly localized to platelets and smooth muscle cells, $P2X_5$ to differentiating cells of muscle and skin, and $P2X_7$ to glia and immune system cells, the other subunits are normally expressed by both neuronal and non-neuronal cells, implying an involvement of ionotropic ATP receptors in a large variety of physiological processes (Gever et al., 2006). The gene encoding the $P2X_2$ subunit has been first cloned in the neuronally-

derived PC12 cells: this protein has been then shown to have a widespread distribution, with different levels of expression, in both central and peripheral nervous systems (including neurons of the sensory and autonomic ganglia and of the dorsal horn of the spinal cord) in addition to many non-neuronal cell types (Gever et al., 2006). On the other hand, $P2X_3$ subunits are predominantly located in neurons, and they are specifically and abundantly expressed by small- and medium-sized cutaneous and visceral afferents from dorsal root ganglia (DRG) and trigeminal ganglia (TG), where these receptors are likely to play a fundamental role in nociceptive transmission and mechanosensory transduction (Burnstock, 2000; Chizh and Illes, 2001; Petruska et al., 2000a; Ichikawa and Sugimoto, 2004; see also section 3). Primary afferents also express quite a high level of the $P2X_2$ subunit; in this respect, it is interesting to note that these neurons have an embryological origin common to that of adrenomedullary chromaffin cells (from which PC12 cells are derived; Dunn et al., 2001; Tischler, 2002) and indeed share a relatively high level of expression of the $P2X_2$ ATP receptor subunit (North, 2002).

Structure and function of P2X receptors

As already mentioned above, 7 different members of the P2X subunit protein family have been cloned, and they share 40-50% homology in aminoacid sequence (North, 2002). As exemplified in Figure 1, and in contrast to the "classical" four-transmembrane domain subunits building up ligand-gated ion channels, the P2X one has only two membrane-spanning hydrophobic domains separated by a large extracellular loop, while both the N- and C-terminals are cytoplasmic (Vial et al., 2004). The extracellular loop has several conserved cysteines, which are likely to be implicated in disulfide bond formation, and specific aminoacid residues involved not only in agonist binding, but also in the receptor gating and deactivation (Vial et al., 2004; Zemkova et al., 2004). The intracellular portions of the P2X subunit show specific sites involved in receptor trafficking and phosphorylation by protein kinase C, which seems to be effectively involved in the functional modulation of this receptor (North, 2002; Vial et al., 2004; Stojilkovic et al., 2005).

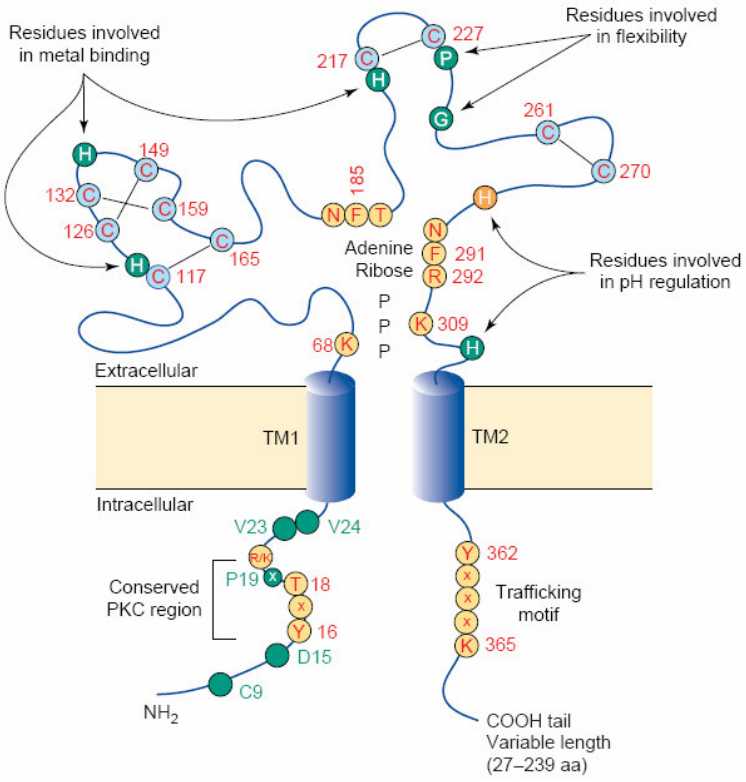

Figure 1.

Model of a representative P2X subunit (human $P2X_1$). The subunit comprises two transmembrane domains, a large extracellular loop and cytoplasmic N- and C-terminal regions. Conserved aminoacids are indicated in red (residues identified from $P2X_2$ and $P2X_4$ subunits are highlighted in green and orange background). The N-terminal domain comprises a consensus site for protein kinase C (PKC) phosphorylation, and residues variably involved in ATP-evoked responses modulation (in green). On the extracellular loop, several positively charged aminoacid residues are thought to be involved in the binding of the negatively charged ATP phosphates, while other residues are likely to interact with the adenine ring. The conserved cysteine residues are likely to form the disulfide bonds shown in the figure, whereas some non-conserved histidine residues are implicated in the regulation of some of the P2X subunits by cations. The C-terminal region show variable length among the different P2X subunits and includes a conserved motif involved in receptor trafficking (from Vial et al., 2004).

The functional form of a P2X receptor is made up by the co-assembly of usually three subunits, leading to the formation of dimers or multimers (North, 2002). The kinetic properties of the whole-cell ionic current mediated by these receptors during ATP application vary considerably

on the basis of the receptor subunit composition. Figure 2 A shows typical examples of the whole cell currents mediated by heterologously expressed homomers of cloned P2X subunits, and reveal one of the most striking features of the different subtypes of P2X receptors, namely their distinct desensitization properties (see below). While $P2X_1$ and $P2X_3$ receptor-mediated currents promptly decay during agonist application (that is, they desensitize rapidly), the other P2X receptors show little or no response decrease within the same time frame.

Both homomeric and heteromeric P2X receptors have been found in neurons from different areas of central and peripheral nervous system and can be reproduced in heterologous expression systems (North, 2002). One of the most commonly found example of such a heteromerization is the $P2X_{2/3}$ receptor, natively found in nodose ganglia neurons and primary afferents of DRG and TG (Dunn et al., 2001; North, 2002; Figure 2 B).

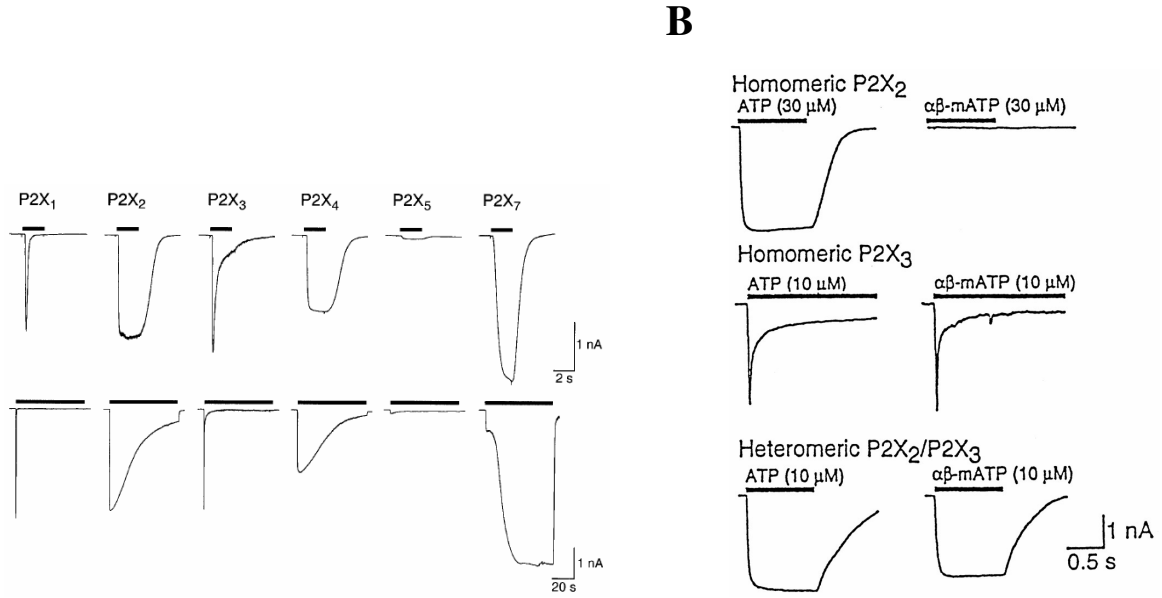

Figure 2.

A. *Top, examples of whole cell currents mediated by P2X receptors heterologously expressed in transfected HEK cells. Note the fast current fading (desensitization) during a 2 second-long ATP application (black bar, 30 µM; 1 mM only for $P2X_7$) in $P2X_1$ and $P2X_3$ receptors. Bottom, longer ATP application induces slow desensitization also in $P2X_2$ and $P2X_4$ receptors (from North, 2002).*

B. *Examples of ATP-evoked currents recorded from HEK cells expressing homomeric $P2X_2$, homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors. Note that only $P2X_3$ subunit-containing receptors are activated by the ATP analogue α,β-meATP (see text; from Ralevic and Burnstock, 1998).*

Even if their distinct desensitization properties may help to distinguish the various P2X receptor types, the functional differentiation of these receptors is made difficult by the relative lack of agonists highly specific for each receptor type. Nevertheless, it is possible to some extent to discriminate between different P2X receptors on the basis of their differential pattern of responsiveness to agonists, sensitivity to antagonists and modulation by extracellular cations (Dunn et al., 2001; North, 2002; Roberts et al., 2006). For example, only $P2X_1$ and $P2X_3$ subunit-containing receptors are activated by the stable ATP analogue α,β-meATP (with a potency similar to that of ATP), while homomeric $P2X_2$ receptors are almost insensitive to this agonist (Figure 2 B, top). The lack of subunit-specific antagonists was improved only recently, with the availability of a selective antagonist for $P2X_3$ subunit-containing receptors (5-[[[(3-Phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid: A-317491; Jarvis et al., 2002), but highly selective agonists and antagonists for the other P2X subtypes remain to be developed.

As mentioned above, primary sensory neurons represent the site where the expression of a specific subtype of ATP receptor subunit mediating the fast action of ATP (namely the $P2X_3$ one, as either a homomer or heteromerized with the $P2X_2$ one) is specific and particularly strong. In view of this fact, and because of the involvement of $P2X_3$ receptors in nociceptive input transmission (see section 3), the characterization of the structural and functional properties of $P2X_3$ receptors is of particular interest and will be the focus of large part of the experimental work reported in the present thesis.

$P2X_3$ receptor-mediated currents

As already mentioned above, P2X receptors mediate a cationic current and are markedly permeable to Ca^{2+}. The evaluation of the channel unitary conductance has been done by means of single channel recordings for several homomeric P2X receptor subtypes (North, 2002) and the one of $P2X_3$ receptors seems to be close to 7 pS (Grote et al., 2005). Macroscopic currents obtained with whole cell patch clamp recordings have shown that activation of P2X receptors by ATP induces an inwardly directed, fast-developing current (see Figure 2 A). For instance, as far as native $P2X_3$ receptors expressed on primary nociceptors are concerned, the peak current response to ATP can be achieved in about 10 ms (notwithstanding the considerable influence of agonist application speed; Burgard et al., 1999). In addition, inward rectification of currents

mediated by P2X receptors, including the $P2X_3$ ones natively expressed by sensory neurons, has been observed (North, 2002; Giniatullin et al., 2003).

Desensitization of P2X receptors

Desensitization is classically defined as decrease or loss of biological activity following prolonged or repetitive stimulation (Katz and Thesleff, 1957), that is, in the case of $P2X_3$ (and $P2X_1$) receptors, the decline in the receptor-mediated current response during continuous exposure to the agonist. Receptor desensitization is, thus, an important "use-dependent" property, which controls receptor activity and modulates the physiological consequences of receptor activation.

As already mentioned above, P2X receptors show different properties with respect to desensitization onset during agonist application (onset being measured as the speed at which the receptor-mediated current declines in the presence of the agonist; see Figure 2 and Figure 3). Homomeric $P2X_3$ receptors are particularly prone to this phenomenon, as they are readily desensitized by both ATP and its analogue α,β-meATP to a similar extent (North, 2002; Sokolova et al., 2004), while heteromeric $P2X_{2/3}$ receptors show little or no desensitization in the presence of the agonist (Figure 2 B). Like for other ligand-gated ion channels, desensitization onset of $P2X_3$ receptors depends on agonist concentration and even very low, subthreshold concentrations of extracellular ATP can induce receptor desensitization, *via* the process of "high-affinity desensitization". In addition, desensitization onset of $P2X_3$ receptors strongly depends on the nature of the agonist, as it varies considerably in the presence of different ATP analogues (Sokolova et al., 2004; Sokolova et al., 2006; Figure 3).

Specific regions of the P2X receptor have been demonstrated to be involved in desensitization, including the extracellular loop, the transmembrane domains and the intracellular portions susceptible of phosphorylation by protein kinase C (PKC; Stojilkovic et al., 2005) or dephosphorylation by the phosphatase calcineurin (the last being particularly important for $P2X_3$ receptors, *via* Ca^{2+} entry; King et al., 1997).

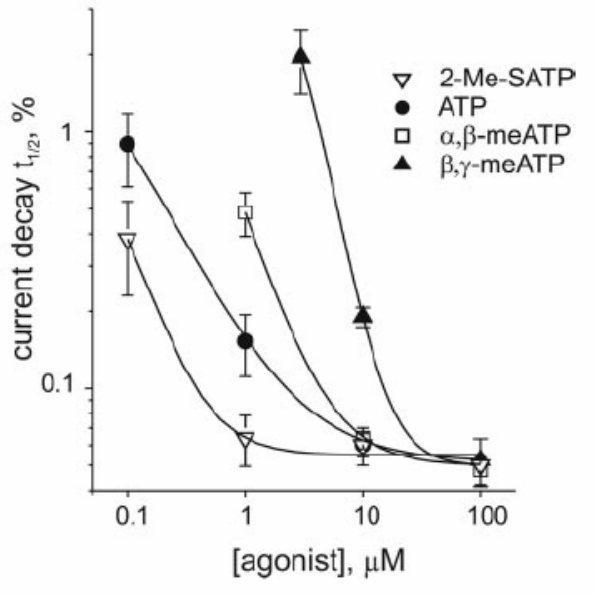

Figure 3.

The half-time ($t_{1/2}$) value of current decay during agonist application is an indication of desensitization onset. This value is a function of agonist concentration (desensitization onset stronger for higher agonist concentrations) and depends on the nature of the agonist. Figure shows the values of $t_{1/2}$ for currents induced by ATP and its analogues 2-Me-SATP, α,β-meATP and β,γ-meATP on cultured rat DRG neurons (from Sokolova et al., 2006).

Desensitization of P2X receptors is thought to correspond to an agonist-bound, closed state of the receptor; after agonist removal, receptors recover and are again available for activation (Rettinger and Schmalzing, 2003; Sokolova et al., 2006). In addition to desensitization onset, recovery from desensitization is an important functional feature of the receptor and could be considered as a component of a receptor "short term memory" (Cook et al., 1998).

A characteristic functional feature of $P2X_3$ receptors, in addition to their rapid desensitization onset, is their extremely slow recovery from desensitization, which is complete only after several minutes. Like desensitization onset, also recovery from desensitization is strongly influenced by the agonist nature because, for example, recovery of responses induced by the $P2X_3$ receptor agonist α,β-meATP is faster than the one by ATP (Sokolova et al., 2004; Sokolova et al., 2006; Figure 4).

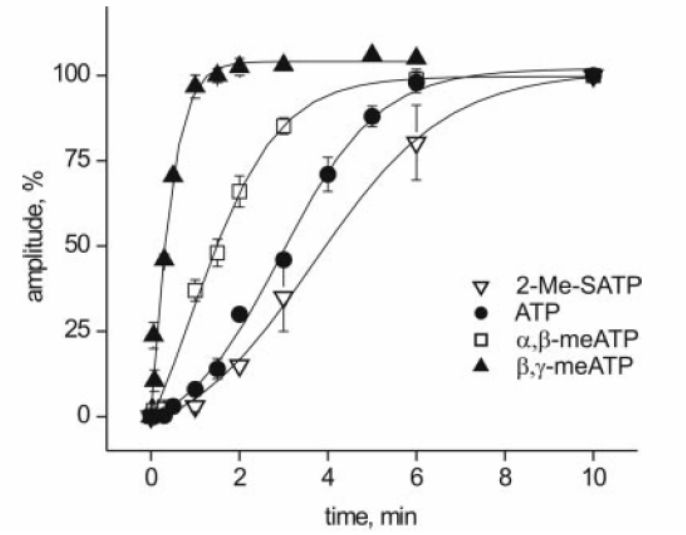

Figure 4.

Recovery from desensitization can be quantified by a paired-pulse protocol (Sokolova et al., 2004), in which two agonist pulses are spaced by different time intervals and the amplitude of the second pulse is expressed as a percentage of the first one. Recovery from desensitization strongly depends on the type of the agonist used. Plots are obtained from cultured DRG neurons (from Sokolova et al., 2006).

$P2X_3$ receptor recovery from desensitization is thus a complex phenomenon, influenced by a variety of factors, including receptor agonists themselves and, remarkably, extracellular polyvalent cations. Indeed, increase in extracellular Ca^{2+} levels accelerates $P2X_3$ recovery in a dose- and time-dependent fashion, an effect also mimicked by Gd^{3+} (Cook et al., 1998), while elevation of Mg^{2+} and extracellular Ca^{2+} depletion have the opposite effect (Giniatullin et al., 2003). These properties imply that there should be specific sites on the $P2X_3$ molecule (more likely in the extracellular portion) accounting for such a modulation, disclosing the possibility of identifying residues involved in the exogenous modulation of $P2X_3$ receptors activity. In this respect, it is noteworthy that significant fluctuations of Ca^{2+} or Mg^{2+} levels take place during specific physiological or pathological conditions. Indeed, local Ca^{2+} levels can fluctuate at sites of elevated neuronal activity (Pumain et al., 1987; Stabel et al., 1990; Borst and Sakmann, 1999). In addition, as far as pain-related conditions are concerned, tissue damage can dissipate the extracellular Ca^{2+} gradient existing in skin, thus exposing $P2X_3$-expressing free endings of sensory neurons to altered Ca^{2+} levels (Menon et al., 1985). Conversely, a decrease in the level of cerebrospinal fluid free Mg^{2+} is present in patients suffering for migraine attacks and,

furthermore, the lowest Mg^{2+} levels are correlated with the most severe pain states (Lodi et al., 2001). Taking into account the negative and positive effects of Ca^{2+} and Mg^{2+}, respectively, on $P2X_3$ receptor desensitization, the above mentioned observations could well fit with an involvement of $P2X_3$ receptor desensitization in the transmission of nociceptive inputs related with these pathological states.

Receptor desensitization is a fundamental property, which limits the receptor-mediated responses in situations when the agonist persists for a relatively long period of time. This is likely to be the case for ATP, when it is released in considerable amounts after tissue damage and could then act on $P2X_3$ receptors on the peripheral processes of primary afferents for a long time (see section 3). Taking into account that $P2X_3$ receptors have a relatively high permeability to Ca^{2+}, that the increase in intracellular Ca^{2+} concentration is amplified by voltage-activated Ca^{2+} channels as a consequence of membrane depolarization, and that an excessive increase in intracellular Ca^{2+} levels might have toxic effects (Paschen, 2000), $P2X_3$ receptor desensitization may be considered a protective mechanism against the possibly toxic consequences of an intense and long-lasting exposure of these receptors to their physiological agonist. At the same time, since desensitization is a process having strong impact on the level of operation of $P2X_3$ receptors, it could be considered as a suitable potential target for an efficient shaping of the activity of these receptors by exogenous modulators.

3. P2X receptors in sensory neurons and the role of ATP in pain transmission

The mRNA for diverse P2X subunits has been localised in sensory ganglion neurons, even if at different levels of expression (Vulchanova et al., 1998; Xiang et al., 1998; Dunn et al., 2001; Ruan et al., 2004). As already mentioned above, $P2X_3$ receptors seem to be expressed almost exclusively by primary sensory afferents and in particular, as far as dorsal root ganglion (DRG) neurons are concerned, by the afferents of small- and medium-sized neurons from which small calibre fibres (Aδ and C) mediate the responses to noxious stimuli detected in the periphery (Millan, 1999; Hunt and Mantyh, 2001; Petruska et al., 2000a; Burnstock, 2001; North, 2002; Petruska et al., 2002; Figure 5). In addition to homomeric $P2X_3$ receptors, also $P2X_2$ subunits are expressed at a significant level by primary sensory neurons, and are supposed to be mainly part of heteromeric $P2X_{2/3}$ receptors localized at peripheral and central terminals (Dunn et al., 2001; North, 2002). Activation of peripheral P2X receptors triggers the excitation of

nociceptors by locally high levels of ATP, which can come from a wide variety of sources. In addition to being released from the cytoplasm of damaged cells, ATP can derive also from Merkel cells in the skin and tumour cells, as a consequence of tissue/skin injury or mechanical friction. Endothelial cells and platelets may be a source of ATP in several types of "vascular pain", like migraine, angina and ischemic muscle pain. Sympathetic neurons also release ATP together with noradrenalin and are thought to be implicated in sympathetically-maintained chronic pain (Burnstock, 2001; North, 2004; Figure 5). As a consequence of stimulation, primary afferents themselves can release ATP at their central terminals. At this level, the endogenous, vesicular ATP release can influence both pre- and the post-synaptic sites, that is, it can act as a positive feedback mechanism to further enhance transmitter release from central terminals of primary afferents (Gu and MacDermott, 1997); in addition, it contributes to depolarization of second order neurons in the dorsal horn of the spinal cord, where functional P2X ATP receptors are expressed (Bardoni et al., 1997; Burnstock, 2001; Figure 5). Stimulus-evoked ATP release and its action on P2X receptors at both pre-and post-synaptic sites could therefore be one of the sites where the modulation of transmission of nociceptive inputs from primary afferents to the central nervous system could take place.

As indicated in Figure 5, the action of ATP continues also after its breakdown, when adenosine activates excitatory or inhibitory adenosine receptors in the peripheral and/or central terminals of primary afferents, accounting for multiple feedback mechanisms regulating neuronal excitability.

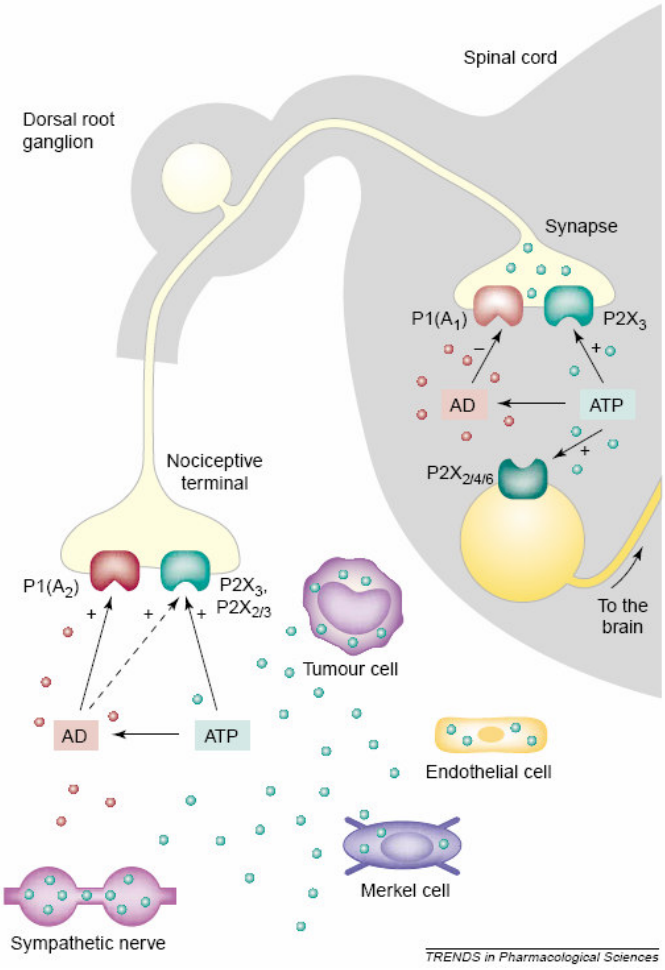

Figure 5.

P2X ATP receptors play an important role in processing nociceptive information from the periphery to the second order neurons in the spinal cord (or in brainstem nuclei, in the case of TG neurons). Homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors expressed on the peripheral afferents of sensory neurons mediate the neuronal response to ATP released not only from damaged cells, but also by other peripheral sources. Activation of P2X receptors leads to the depolarization of primary afferents and the consequent glutamate release on second order neurons. ATP itself is also released by central terminals of sensory neurons and acts at both pre- and post-synaptic sites. At the post-synaptic site it activates P2X receptors (containing the $P2X_2$, $P2X_4$ and $P2X_6$ subunits) on second order neurons, thus contributing to their excitation, while at pre-synaptic level it acts as a positive feedback mechanism to enhance glutamate release via activation of $P2X_3$ receptors. The breakdown product of ATP, adenosine, can act on its own receptors at both peripheral and central terminals of sensory neurons, inhibiting or

potentiating neuronal excitation depending on the receptor present (A1 or A2; from Burnstock, 2001).

At a higher system level, the specific expression of ionotropic P2X ATP receptors in primary sensory neurons is documented by many reports directly demonstrating the *acute* pain-inducing action of ATP. Indeed, ATP elicits pain when applied to the skin (Coutts et al., 1981) as well as after intracutaneous or intramuscular injection in humans (Hilliges et al., 2002; Mork et al., 2003). Similarly, animal studies have shown dose-dependent nocifensive behavior induced by administration of ATP or its analogues (Bland-Ward and Humphrey, 1997; Hamilton et al., 1999; Jarvis et al., 2001). ATP also directly excites primary afferent neurons in both *in vivo* and *in vitro* preparations: for example, application of ATP or its analogues to joint nociceptors (Dowd et al., 1998) or skin nerves (Hamilton et al., 2001) causes excitation of a subset of Aδ and C afferents. Further confirmation of the algogenic action of ATP and of the specific involvement of $P2X_3$ receptors in pain transmission comes from the observation that intrathecal administration of the $P2X_3$ receptor agonist α,β-meATP has pro-nociceptive effects (Driessen et al., 1994) and that $P2X_3$ knockout mice show a markedly reduced sensitivity to certain noxious stimuli (Cockayne et al., 2000).

In addition to their role in acute pain, $P2X_3$ receptors undergo plastic changes during *chronic* painful conditions, as their expression is strongly enhanced in DRG neurons during inflammation or neuropathic pain (Novakovic et al., 1999; Xu and Huang, 2002): this provides the rationale for investigating the possible modulation of $P2X_3$ receptor function by different factors over a wide range of time frames.

The induction/transmission of several features of nociception *via* $P2X_3$ receptors on DRG neurons is well established. Nevertheless, relatively little is known about the pain-related mechanisms involving $P2X_3$-mediated excitation of primary afferents of the trigeminal ganglion (TG) neurons, especially in chronic pain states. For instance, during headache attacks, pain is believed to be due to repetitive firing of nociceptive trigeminal ganglion neurons. Trigeminal nerve terminals possess high density of $P2X_3$ receptors that mediate sensitization of trigeminal sensory neurons (for example during persistent dental pain; Hu et al. 2002); such cells may be activated by locally released ATP and may also release ATP as excitatory transmitter on central neurons within the trigeminal nucleus of the mesencephalon (Khakh and Henderson, 1998). It seems thus feasible that several pain states of cranial origin, including

headache, are at least in part mediated by $P2X_3$ receptors, and this represents an interesting topic for further investigation.

4. Modulation of $P2X_3$ receptors activity

In addition to extracellular cations (like Ca^{2+}; see above), P2X receptor activity can also be modulated by intracellular factors, some of which have been identified. The N-terminal region of all P2X receptors contains a consensus site for protein kinase C (PKC), but it is feasible that receptors may also be directly phosphorylated at other intracellular sites, or regulated indirectly *via* phosphorylation of accessory proteins (Vial et al., 2004; Roberts et al., 2006). For instance, as far as $P2X_3$ receptors are concerned, ATP-evoked currents mediated by heterologously expressed $P2X_3$ or $P2X_{2/3}$ receptors can be potentiated by *acutely*-applied substance P and bradykinin, with an action dependent on the PKC-mediated phosphorylation of this conserved consensus site (Paukert et al., 2001).

In addition to phosphorylation, P2X receptors can be regulated by the interaction with other receptor types as, for example, $P2X_3$ receptors show occlusion with GABA currents and the $P2X_2$ ones can directly interact with serotonin (5-HT) receptors (Sokolova et al., 2001; Boue-Grabot et al., 2003). Most probably other, not yet identified intracellular regulatory mechanisms are likely to be involved in the modulation of P2X receptor activity. In relation to this, one fundamental topic of interest concerns the identification of the extracelluar substances triggering this modulation, namely the endogenous (or exogenous) factors which could influence the physiological response mediated by P2X receptors. One attractive hypothesis is that specific nociception-modulating substances, whose levels are elevated during pathological states (like, for example, inflammatory reactions), could potentiate the responses mediated by ATP receptors expressed by nociceptors. For instance, *long-term* inflammatory conditions, like those induced by complete Freund's adjuvant injection, induce a markedly increased response of DRG neurons to ATP application (Xu and Huang, 2002), a phenomenon in accordance with the enhancement of nocifensive behavior by ATP injection in rats after carrageneenan-induced inflammation (Hamilton et al., 1999). It seems likely that some of the soluble factors produced during such pathological states could contribute to the modulation of the activity of $P2X_3$ receptors and of other pain-related receptors endogenously expressed by sensory neurons (see below).

Inflammation and Nerve Growth Factor (NGF)

It is now well established that Nerve Growth Factor (NGF), in addition to being a survival factor for peripheral neurons during development, subserves different functions in the adult (Sofroniew et al., 2001). In particular, NGF is among the substances whose levels are significantly increased for a relatively long time in a number of pathological states, particularly in inflammation (Sah et al., 2003; Pezet and McMahon, 2006). *In vivo*, NGF has a pro-algesic action as its administration induces pain and hyperalgesia, an effect which can be reversed by anti-NGF antibodies. Accordingly, *in vitro* studies have demonstrated that acute or chronic administration of NGF to DRG neurons increases their sensitivity to capsaicin, noxious heat and acidic pH, it increases the expression of TTX-sensitive and -insensitive Na^+ channels (thus increasing neuronal excitability), and also enhances the production of neuropeptides like substance P (SP) and calcitonin gene-related peptide (CGRP; Pezet and McMahon, 2006).

Even if the basal levels of NFG are quite low, various cell types, including epithelial cells, fibroblasts, keratinocytes, Schwann cells and macrophages are significant sources of NGF in inflammatory conditions (Pezet and McMahon, 2006); sensory ganglion neurons might also contribute to NGF synthesis, although this issue is controversial (Wetmore and Olson, 1995; Jacobs and Miller, 1999).

Both low- and high-affinity receptors for NGF exist (p75 and TrkA) and they are expressed by a specific subtype of nociceptive primary sensory neurons in a developmentally-regulated fashion. Activation of TrkA receptors triggers several signalling pathways, including the MAPK pathway, phosphatidyl inositol-3 kinase and phospholypase C; this can lead, in turn, to the sensitization of nociceptor responses to noxious stimuli cited above *via* both transcriptional and post-translational mechanisms (Pezet and McMahon, 2006).

Despite the quite well established role of NGF in inflammatory pain, information about the *long-term* effects of NGF as an "inflammatory mediator" specifically on ionotropic ATP receptors of sensory neurons is relatively scanty. In particular, there are only few studies which analyzed in detail the possible modulatory action of NGF on $P2X_3$ receptors, with basically different experimental protocols for NGF administration (chronic exposure of cultured DRG neurons or osmotic minipumps fitted to *in vivo* animals; Bevan and Winter, 1995; Ramer et al., 2001). No effect has been found in receptor function after exposure to NGF in the former case, while an enhancement of $P2X_3$ expression in primary sensory neurons has been observed in the latter.

One interesting point in the action of NGF is its time-course. Indeed, while acutely applied NGF *via* TrkA activation might directly phosphorylate pre-existing intracellular targets, over the longer term NGF could exert a more global influence on neuronal function, possibly by regulating the expression of releasable substances (SP, CGRP), but also, and more interestingly, nociceptor-specific ion channels like $P2X_3$ receptors. In this respect, for example, NGF has been suggested quite a long time ago to be a hyperalgesia-provoking factor in migraine (Sandler, 1995), thus implying its action on TG nociceptors. Indeed, NGF levels have been found to be increased in the cerebrospinal fluid of subjects experiencing headache attacks (Sarchielli et al., 2001), thus raising the need to investigate the effects of NGF on $P2X_3$ receptors not only on DRG nociceptors, but also on the TG ones.

Serotonin (5-HT)

Serotonin (5-hydroxytryptamine; 5-HT) is a common neurotransmitter in the central nervous system and it contributes to processing and modulating pain transmission at the central level, *via* inhibition of dorsal horn neurons in the spinal cord through descending inhibitory pathways. In contrast to its central effects, the peripheral action of 5-HT is often pro-algesic, as this substance is considered to be an inflammatory mediator in the periphery (Millan, 1999; Sommer, 2004). Indeed, 5-HT has been reported to elicit pain in certain conditions, but especially, and more consistently, to enhance the pro-nociceptive action of other inflammatory mediators (Sommer, 2004).

5-HT can be released in considerable amounts by platelets, mast cells, endothelial cells and serotonergic neurons surrounding the microcirculation and its levels are increased in several models of inflammatory pain and tissue injury; increased serum levels of 5-HT during migraine attacks have also been reported by several clinical studies (Ferrari et al., 1989; Ribeiro et al. 1990; Sommer, 2004).

A number of 5-HT receptor types have been detected in both DRG and TG primary sensory neurons, at the level of protein or mRNA (Lazarov, 2002; Sommer, 2004). Among them, the activation of ionotropic $5\text{-}HT_3$ receptors leads to direct excitation of primary afferents, while an excitatory or inhibitory effect on neuronal activity can be obtained by activating the other 5-HT receptors subtypes, which are metabotropic and operate *via* a multiplicity of signal transduction mechanisms (Raymond et al., 2001). For example, while $5\text{-}HT_3$ receptor activation directly excites TG neurons by evoking an inward current (Hu et al., 2004), $5\text{-}HT_{1B/D}$

receptor subtypes have an inhibitory action (*via* inhibition of adenylate cyclase) and are indeed the target of 5-HT_1 receptor agonists developed for migraine treatment (triptans; Goadsby et al., 2002).

5-HT modulates the activity of several receptors on primary afferent neurons. Application of 5-HT to visceral nociceptors acutely and robustly enhances the neuronal responsiveness mediated by TRPV1 receptors, which are among the main pain transducers (see section 5; Sugiura et al., 2004). As far as P2X receptors are concerned, an inhibitory cross-talk between the ionotropic 5-HT_3 and $P2X_2$ receptors has been found in enteric neurons (Boué-Grabot et al., 2003), while no *acute* effects of 5-HT on the activity of $P2X_3$ receptors has been found (Nakazawa and Ohno, 1997). These findings thus suggest the need for elucidating the possible role of 5-HT in shaping the responses of primary afferents, and in particular of nociceptors, to several excitatory stimuli, particularly in relation to long-lasting exposure to 5-HT. Within this framework, the possibility that such a modulation could take place also on $P2X_3$ receptors seems plausible at the level of both DRG and TG neurons, in view of the high level of expression of these receptors and the likelihood of marked fluctuations in the local levels of 5-HT in certain physiological or pathological conditions.

Calcitonin Gene-Relate Peptide (CGRP)

A substantial fraction of nociceptors of DRG and TG synthesizes and releases the neuropeptides substance P (SP) and calcitonin gene-relate peptide (CGRP; Hunt and Mantyh, 2001; Durham and Russo, 2002). CGRP is a 37 aminoacid peptide generated by the alternative splicing of calcitonin gene transcripts; two different CGRP isoforms exist, α and β, and the α one is primarily expressed by sensory neurons (Arulmani et al., 2004). The receptors for CGRP have been identified in various tissues, including the cardiovascular system (where CGRP has a vasodilatory effect) and, notably, in several regions of the central and peripheral nervous system, including TG and DRG neurons themselves (Van Rossum et al., 1997; Segond von Banchet et al., 2002). CGRP binds to metabotropic receptors belonging to a family of proteins in which a seven-transmembrane domain, G protein-coupled receptor binds to two accessory proteins (RAMP -receptor activity modifying protein- and RCP -receptor component protein-) in order to form functional receptors. The resulting CGRP-activated receptor works *via* an intracellular pathway involving the activation of adenylate cyclase and leading ultimately to the accumulation of cyclic AMP (Poyner et al., 2002; Arulmani et al., 2004).

CGRP seems to be not directly algogenic, since peripheral administration of CGRP does not produce hyperalgesia (Chu et al., 2000; Weidner et al, 2000). Nevertheless, the synthesis and release of CGRP from TG neurons has been found to be significantly increased by depolarization or by treatment with a mixture of substances involved in inflammation and, in full agreement with these findings, CGRP levels are increased during inflammation and in various forms of headache, including migraine. This increase is probably a consequence of enhanced release by activated TG afferents innervating the brain microcirculation at the meningeal level. In this respect, it is worth noting that during a migraine attack the levels of CGRP are significantly augmented, while those of SP are not (Durham and Russo, 2002; Edvinsson and Uddman, 2005). Furthermore, CGRP levels often correlate with the time course and the intensity of pain, suggesting a direct relationship between CGRP and migraine (Juhasz et al., 2003; Juhasz et al., 2005). A further confirmation of the pro-nociceptive role of CGRP in specific pathological states comes from the growing amount of data demonstrating the effectiveness of CGRP receptor antagonists in the treatment of migraine pain (Olesen et al., 2004; Edvinsson, 2005).

CGRP seems to have no direct, acute effect on the activity of meningeal TG nociceptors (Levy et al, 2005); however, it can enhance the release of excitatory aminoacids from primary afferent neurons (Kangrga et al., 1990) and can also cause a slight increase in the intracellular calcium level in a subset of DRG neurons (Segond von Banchet et al., 2002). In view of the fact that CGRP can be synthesized and released by peripheral and, probably, also central afferents of sensory TG neurons (Storer et al., 2004; Fischer et al., 2005; Strassman and Levy, 2006), and that the same neurons can express receptors for CGRP, it seems likely that sensory afferents might be exposed to relatively large local amounts of this neuropeptide and could then be important targets for the CGRP action. Within this framework, the highly expressed $P2X_3$ receptors might be among the favorite candidates for CGRP-mediated modulation.

Notwithstanding the role of the above discussed substances, there is, of course, plenty of other substances whose levels fluctuate significantly in specific physiological or pathological conditions and which could then be tested for their ability to modulate $P2X_3$ receptors on primary nociceptors (Millan, 1999). A detailed examination of the effects of these compounds is outside the scope of the present thesis.

5. TRPV1 receptors as "prototypical" pain receptors

The expression of transient receptor potential vanilloid 1 (TRPV1, also known as VR1) receptors is widespread in several areas of the nervous system, but it is particularly strong in both DRG and TG nociceptors, in which they are considered to be among the "prototypic" detectors of noxious inputs (Julius and Basbaum, 2001), in addition to $P2X_3$ receptors. It is interesting to note that the relation between TRPV1 and $P2X_3$ receptors expression may considerably vary. Indeed, while $P2X_3$ receptor-expressing DRG neurons are usually capsaicin-responding (that is, they have functional TRPV1 receptors; Petruska et al., 2000b; Petruska et al., 2002), the colocalization of $P2X_3$ and TRPV1 receptors in TG neurons innervating skin and tooth pulp is significantly lower (Ichikawa and Sugimoto, 2004).

TRPV1 receptors are activated by a wide variety of stimuli, both exogenous (noxious heat, capsaicin) and endogenous (protons, anandamide, lipoxygenase products, N-arachidonoyl dopamine) and they mediate a non-selective cationic current with particularly high Ca^{2+} permeability (Tominaga and Caterina, 2004).

The role of TRPV1 receptors in the transmission of selective nociception modalities comes from studies of TRPV1-deficient mice, whose behavioral reaction to capsaicin and acute heat is strongly attenuated and the response of primary sensory neurons to the same stimuli is severely impaired (Caterina et al., 2000). In addition, TRPV1 receptors have been shown to be particularly prone to *acute* sensitization by a number of substances known to be key inflammatory mediators, like NGF, bradykinin and lipoxygenase products (Julius and Basbaum, 2001; Tominaga and Caterina, 2004). In view of this fact, TRPV1 receptor expression and activity might be considered as markers for a specific subtype of sensory neurons, and their activation by exogenous stimuli (e.g., capsaicin) could be a useful tool to test the possible modulatory effects of pain-related substances.

6. Counteracting excitation: $GABA_A$ receptors as possible targets for modulation

Primary sensory neurons express not only receptors which physiologically mediate excitation (e.g. $P2X_3$ and TRPV1), but also receptors exerting an opposite function in physiological conditions, among which ionotropic $GABA_A$ receptors are representative elements (White, 1990; Ma et al., 1993; Lazarov, 2002). Indeed, a fundamental check-point in the processing of peripheral inputs *in vivo* is related to the activity of GABAergic interneurons located in the dorsal horn of the spinal cord. Sustained and relatively long-lasting GABA release activates

$GABA_A$ receptors on the central terminals of sensory neurons and might be responsible for presynaptic inhibition due to primary afferent depolarization (PAD; Frank and Fuortes, 1955; Dickenson et al., 1997; Rudomin and Schmidt, 1999). Actually, $GABA_A$ receptors expressed by primary afferent neurons show the classical molecular structure of these receptors, that is, a developmentally-regulated combination of specific $GABA_A$ subunits (Ma et al., 1993). Nevertheless, in contrast to neurons of the central nervous system, $GABA_A$ receptors expressed by sensory neurons mediate *efflux* of chloride ions and, therefore, their activation has a depolarizing effect; depolarization of central terminals can then either block the invasion of afferent terminals by action potentials or reduce action potentials amplitude, thus leading to a decrease in neurotransmitter release (MacDermott et al., 1999; Rudomin and Schmidt, 1999).

From the functional point of view, there are several indications of an involvement of GABAergic transmission in the modulation of nociceptive inputs. Among them, it is interesting to note that GABA ionotropic (and metabotropic) receptors seem to contribute to the inhibitory control of nociceptive transmission directly at the level of the spinal cord (Dickenson et al., 1997). In addition, GABA receptors seem to directly interact with "excitatory" receptors, since a quite strong, mutually inhibitory interaction takes place between GABA receptors and ionotropic, "pain-mediating" $P2X_3$ receptors (Sokolova et al. 2001; Sokolova et al., 2003). These findings suggest that $GABA_A$ and $P2X_3$ receptors might be differentially regulated in certain conditions, as the activity of $P2X_3$ receptors could facilitate, while that of the $GABA_A$ ones could inhibit, the transmission of sensory input by primary afferent neurons. In this context, the same modulatory factors which could potentially influence the activity of $P2X_3$ receptors (see above) might also exert an effect on $GABA_A$ receptors. In this respect, both the ability of the receptor to respond to its agonist and the desensitization of this response are key factors characterizing receptor activity. In particular, desensitization of GABA-induced responses could provide an indication of the *persistence* of the GABA-mediated action and could then be as important as $P2X_3$ receptors desensitization for shaping the transmission of nociceptive inputs.

7. Cell cultures as a model to study ligand-gated ion channels

The functional properties of primary sensory neurons and their receptors are usually investigated by means of a wide variety of experimental models, both *in vivo* and *in vitro*. Several *in vivo* animal models have been developed and characterized for modelling specific

sensory modalities, including nociception, particularly in rodents (see, for a few representative examples, Pezet and McMahon, 2006). The use of these models offers the advantage of a situation closer to the human physiological/pathological condition, and it is therefore a fundamental step in the comprehension of the basic mechanisms underlying the function of sensory systems and for the development and validation of new possible strategies for their modulation. As far as the modulation of receptors involved in nociceptive input transmission is concerned, it is indeed possible to reproduce in rodents various painful states observed in humans (e.g. inflammatory or neuropathic pain states; see Pezet and McMahon, 2006).

The lack of suitable animal models for certain pain syndromes requires the use of strictly controlled *in vitro* paradigms in order to dissect the fundamental properties of the basic processes. A particular case, in which this situation is of special importance, is represented by migraine pain syndromes, since no reliable test for detecting and measuring the intensity of migrainous pain in *in vivo* animal models are available, and an *in vitro* system based on isolated TG neurons seems to be an appropriate tool to study some aspects of the pathophysiological mechanisms underlying nociception.

Therefore, notwithstanding the importance of *in vivo* animal models, simpler systems are useful, in which the intrinsic nature of the basic elements building up more complex pathways can be analyzed without the interference of the intricate regulatory connections operating in the intact animal.

One possible approach to the study of the functional aspects of neurotransmitter release and receptor-operated ion channels activity is the direct recording of the receptor response to its agonist on isolated cells expressing the receptor, by means of patch clamp recordings or calcium imaging techniques. One simple experimental model to do this is the study of the responses mediated by receptors endogenously expressed by established cell lines. For instance, PC12 cells are known to express a high amount of $P2X_2$ receptors (Brake et al., 1994) and can be used for this purpose. Alternatively, the receptor under investigation can be expressed in an appropriate heterologous system: for example, as far as ionotropic ATP receptors are concerned, they can be efficiently expressed in Human Embryonic Kidney (HEK) cells, that lack endogenous $P2X_3$ receptors and show good expression ability (Vulchanova et al., 1997; North, 2002). Nevertheless, care should be taken to avoid concurrent activation of metabotropic P2Y ATP receptors (expressed by these cells; Fischer et al., 2003) by ATP

application, a result attainable by means of ATP analogues selective for $P2X_3$ receptors (α,β-meATP; North, 2002).

The next step in the study of receptor activity would be its characterization in the native system, where it is physiologically expressed. In order to systematically dissect the basic properties of primary sensory neurons in terms of receptor expression, function and modulation, several *in vitro* preparations are used as model systems, including intact DRGs (e.g. Stebbing et al., 1998), skin-nerve preparations (e.g. Caterina et al., 2000; Hamilton et al., 2001), acutely dissociated neurons (e.g. Petruska et al., 2000a, 2000b; Petruska et al., 2002) and primary cultures of sensory neurons (e.g. Cook et al., 1997; Caterina et al., 2000; Durham and Russo, 2003).

One important aspect related to the use of *in vitro* systems and, in particular, neuronal cultures, is the assumption that the receptors expressed on the neuronal soma mirror the receptor population on the terminals of the same cells. Indeed, functional and histological studies indicated that the key receptors involved in the transmission and modulation of nociceptive inputs are expressed at the level of the fine peripheral and/or central terminals of DRG and TG neurons (e.g. $GABA_A$, $P2X_3$ and TRPV1, receptors; Rudomin and Schmidt, 1999; Dunn et al., 2001; Tominaga and Caterina, 2004). The study of these receptors at the somatic level can overcome the difficulty of direct access to these fine terminals. The assumption of a receptor expression pattern on the neuronal soma mirroring the receptor populations on the primary afferent terminals seems to be correct, since, for example, functional $P2X_3$, TRPV1 and $GABA_A$ receptors are expressed also on the soma of primary sensory neurons in intact ganglia or in acutely dissociated neurons (e.g. Petruska et al, 2000a; Labrakakis et al., 2003; Ichikawa and Sugimoto, 2004). Hence, somatic receptors expressed by sensory neurons in culture have been extensively used (e.g. Caterina et al., 2000; Sokolova et al., 2001) and sensory neurons in culture are a suitable model for studying the properties of a variety of receptors and channels and their modulation. In this respect, one fundamental point is that the experimental model selected to explore a specific topic should be first fully characterized in its basal, *naive* condition. In particular, if neuronal cell cultures are chosen for investigating the properties of a particular receptor, possible differences with respect to the native tissue condition should be taken into account and characterized. Additionally, if the interest is in the possible long-term

effects of a modulatory factor, the intrinsic temporal development of the model culture in terms of cell viability and patterns of receptor expression/function should not be neglected.

AIMS

The findings reported in the previous sections illustrate that various features concerning the mechanisms of action of ATP and its receptors, the physiological actions of pain-signalling TRPV1 receptors and that of the inhibitory $GABA_A$ receptors on sensory neurons have been well characterized. Nevertheless, many aspects of the activity of these receptors and the factors contributing to their modulation are still not completely understood. The work reported in this thesis aims at contributing to the understanding of the functional features of the above mentioned receptors. In particular, the experimental work addresses the following issues:

1- ATP is physiologically released by neuronal terminals and can act both pre-and post-synaptically: could PC12 cells (containing ATP in their vesicles and endogenously expressing $P2X_2$ receptors) serve as a simple and cheap *in vitro* model system to study endogenous ATP release and the electrophysiological responses consequent to it?

2- How do P2X and TRPV1 receptors in trigeminal ganglia relate to those expressed in culture? Are they functional and stable in culture? Are rat or mouse TG neurons in culture equivalent models to study these receptors? Can P2X and TRPV1 receptors be positively or negatively modulated in culture, in particular by NGF or 5-HT?

3- $P2X_3$ receptors of primary afferent, nociceptive neurons are among the main pain-transducer receptors and their activity is potently influenced by their strong desensitization: what molecular determinants of $P2X_3$ receptors contribute to their desensitization properties?

4- TG neurons in culture could be used to mimic the conditions which they are expected to meet during an attack of migraine: what are the effects of calcitonin gene-related peptide (CGRP) on their $P2X_3$ and TRPV1 receptors?

5- NGF has been implicated in the pathogenesis of inflammatory pain: if it could modulate $P2X_3$ and/or TRPV1 receptors, what might be its effects on the physiologically inhibitory $GABA_A$ receptors?

In this thesis, all these issues will be critically discussed on the basis of the original experimental data obtained during the PhD course, and linked to the current knowledge of the molecular and functional properties of P2X, TRPV1 and $GABA_A$ receptors.

MATERIALS, METHODS AND RESULTS

See enclosed papers.

DISCUSSION

The present thesis has provided new elements for the understanding of ATP-mediated transmission, and of the functional properties of ionotropic receptors for ATP (and for other transmitters) on sensory neurons. In particular, it has described PC12 cells as an *in vitro* model for the study of ATP release-mediated events, and cultures of trigeminal ganglion (TG) neurons in culture as a system in which the modulatory effect of putative algogenic substances (NGF, 5-HT, CGRP) can be effectively assessed.

The findings reported in this thesis have been already discussed in the individual papers; in addition, a more comprehensive discussion and interpretation of the results obtained from the experimental work will be provided below, in relation to their possible physiological implications and the most recent findings in the field.

1. Fast, quantal-like, $P2X_2$ receptor-mediated currents induced by vesicular ATP release in undifferentiated PC12 cells

A fundamental issue in the study of synaptic transmission is the understanding of the mechanisms involved in transmitter release and its effect on the activity of its post-synaptic receptors. In this framework, the study of the elementary release events and of the spontaneous (miniature) or evoked post-synaptic currents assumes particular interest, because it provides information about both the responsiveness of the post-synaptic element and the amount of transmitter released by the pre-synaptic one.

Unitary events occurring at synapses could be effectively mimicked by a system in which the transmitter of interest (ATP) is released at one side of the cell-cell contact and a fast, ionotropic receptor for it (P2X) is expressed at the other side. PC12 cells store ATP together with catecholamines in their releasable dense core vesicles (DCVs; Njus et al., 1986) and express $P2X_2$ receptors (Brake et al., 1994), therefore providing a potentially useful model to investigate the properties of purinergic fast signalling involving $P2X_2$ receptor.

PC12 cells and chromaffin cells are classical models for the study of exocytosis (Tischler, 2002; Martin, 2003) and PC12 cells are able to differentiate toward a neuronal phenotype in response to NGF treatment (Tischler, 2002). Nevertheless, since NGF also exerts many other actions in terms of cell survival, development and neuronal receptor plasticity (Sofroniew et

al., 2001; Pezet and McMahon, 2006), it seems likely that NGF-untreated, undifferentiated PC12 cells are a more reliable model for the study of the system in its basal conditions.

Purinergic nature of STICs

The work reported in the first part of this thesis illustrates that clustered PC12 cells show small transient inward currents (STICs) following application of either ATP or KCl.

$P2X_2$ receptors activated by ATP released from neighbour cells seem to be the origin of STICs, because these events are abolished by superfusion with a Ca^{2+}-free extracellular solution and by the P2X receptor antagonist PPADS.

The conclusion that STICs are due to ATP release from DCV also finds several points in its favour and, in this respect, the delayed appearance of STICs relative to the triggering input is an interesting issue. PC12 cells grown in the absence of NGF show a relatively long latency of catecholamine (and ATP) secretion from DCVs (seconds; Ninomiya et al., 1997). This fact is in full agreement with the reported 3-4 seconds latency of STICs, providing an additional, circumstantial evidence that STICs are due to ATP released from DCV. In addition, the single peak observed in the distribution of STICs amplitude and decay time suggests a mainly monoquantal ATP release. Consistently with the DCV origin of quantal ATP release, Grandolfo and Nistri (2005) have reported that the number of both total and perimembrane DCVs of PC12 cells falls after a pulse of ATP or KCl, thus indicating DCVs depletion following stimuli which are known to induce the appearance of STICs.

STICs, maturation and clusterization of PC12 cells

One necessary condition for the appearance of STICs is cell clusterization. Indeed, in only a few cases (5%) STICs have been detected in isolated cells and the scheme described (fig. 9 in the original paper) shows that the cells closely surrounding the patched one are likely to be the ATP source. The perfusion system employed in the experiments (multibarrel rapid solution changer; see Methods in the enclosed paper) is actually a suitable device for obtaining STICs after application of the triggering substances (ATP or KCl), because each glass capillary can be positioned quite close to a cell cluster and, owing to an internal capillary diameter of about 0.58 mm, these substances are simultaneously applied to the whole cell cluster. Consistently with a mechanism dependent on cell clusterization, vesicular ATP release and $P2X_2$ receptor activation, the appearance of STICs is correlated with time in culture (that is, with the number

and size of cell clusters), its Ca^{2+} response to a depolarizing KCl pulse (which is likely to trigger DCVs exocytosis) and the ability to respond to ATP. All these parameters gradually grow with time in culture. KCl responses show a strong decrease after the sixth day in culture, while ATP responsiveness continues to grow. Culture ageing, accompanied by a possibly differential temporal decay pattern for the activity of voltage-activated Ca^{2+} channels (contributing to the Ca^{2+} response to KCl pulses) and of ATP-activated $P2X_2$ receptors might account for this phenomenon.

The ability of PC12 cells to respond to ATP and its relation to cell maturation in culture is an interesting point, as STICs occurrence is very low in the first days after plating, when the current response to 1 mM ATP is also low. There are at least five different mechanisms which might account for the low probability of observing STICs in the above mentioned conditions (some of which have been already discussed in the enclosed paper). First, there could be no exocytosis of DCVs, for instance because of a small readily releasable DCVs pool: this is probably not the case, because sustained evoked exocytotic activity has been reported from acutely plated PC12 cells (Ninomiya et al., 1997). Second, DCVs might be loaded with a smaller amount of ATP, for example because of a weaker H^+ gradient across the vesicle membrane or a lower activity of the vesicular ADP/ATP translocase (both mechanisms being suitable systems for vesicular ATP loading; Pankratov et al., 2006). Because the H^+ electrochemical gradient usually drives the carrier-mediated neurotransmitter uptake into vesicles, it would be interesting to monitor the amount of catecholamines loaded in DCVs of PC12 cells at different time points in culture, in order to investigate a possible, parallel time-dependent catecholamine loading. The third possibility is that, in the first days in culture, ATP could be effectively released, but the density of $P2X_2$ receptors is so low that the resulting STICs have very low amplitude, which could be masked by current baseline noise (this would be in agreement with the lower responsiveness to exogenously applied ATP). Then, the low number of contacts between neighbouring cells could simply make the occurrence of DCV exocytosis in the narrow intercellular space less probable. Finally, the intracellular Ca^{2+} increase might be a limiting factor; in this respect, it would be interesting to characterize the possible developmentally regulated expression and function of Ca^{2+} channels in PC12 cells, which might account for a reduced DCVs exocytosis. The finding that isolated cells, even when patched without Ca^{2+}-buffers in the pipette solution, do not show STICs (with only few exceptions) well fits with a possible developmentally regulated activity of both ATP-releasing

machinery and $P2X_2$ receptors. This phenomenon, thus, depends on intercellular contacts, in a way similar to the already reported adhesion-dependent maturation and plasticity of synapses (Gerrow and El-Husseini, 2006).

PC12 cells as a model system to study ATP-mediated events

Miniature and evoked unitary currents induced by release of endogenous ATP have been described in acute slices of habenula and spinal cord (Edwards et al., 1992; Bardoni et al., 1997) and, more recently, in hippocampal slices and from dissociated stellate neurons (Pankratov et al., 2006; Tompkins and Parsons, 2006). These preparations can thus serve as reliable systems for the study of unitary events mediated by endogenous ATP release. STICs detected in PC12 cells could provide an additional (and maybe simpler) model system in which these events and their underlying mechanisms can be investigated.

PC12 cells possess a relatively small DCVs readily releasable pool (Martin, 2003) with respect to chromaffin cells and are then less efficient in terms of DCVs exocytosis. Nevertheless, if the focus is on P2X receptor-mediated events, native chromaffin cells are not a suitable model, because no ATP-mediated currents have been found in these cells. Evoked, quantal-like events have been detected only after chromaffin cells transfection with the $P2X_1$ subunit-encoding gene, but they display unusual kinetics (Hollins and Ikeda, 1997). PC12 cells seem then to be more useful, because of their endogenous $P2X_2$ expression and, not last, practical convenience in cell preparation with respect to the classical bovine (or rodent) chromaffin cells.

In order to consider PC12 cells as a good model to study events analogous to those occurring at synapses, attention should be paid to the choice of the trigger for STICs induction. The use of KCl to induce STICs should avoid interference with receptor occupancy , desensitization and deactivation. These complications might be expected to arise with the use of ATP, even if preliminary data (4 cells) have indicated that STICs evoked by ATP or KCl have similar characteristics in terms of amplitude, decay and time-course. It seems feasible that a careful choice of the stimulus intensity (and, thus, of the amount of Ca^{2+} entering into the cells) might help to calibrate STIC occurrence at the desired frequency, thus mimicking high- or low-probability release sites. This protocol might be useful, for instance, for testing drugs acting at the level of ATP release, its diffusion into the intercellular space and its binding to (and activation of) receptors on the post-synaptic-like membrane.

An additional, possible use of PC12 cells is as detectors of neurotransmitter release. Indeed, the methods usually employed to study ATP release-mediated events have a low temporal and spatial resolution. "Real time" ATP level measurements by luciferase assays (reported, for instance, for macrophageal ATP release; Beigi and Dubyak, 2000) and by ATP-sensitive microelectrodes (Dale et al., 2005; Llaudet et al., 2005) are not fast enough (seconds) and are still unable to signal ATP release events at the level of the single cell-to-cell contact. Furthermore, these methods might combine ATP release from many sites/cells, making them unsuitable to reliably monitor ATP release at the single cell level. In future, it will be interesting to investigate "sniffer" patches of PC12 cell membrane to detect (*via* $P2X_2$ receptor activation) release of endogenous ATP.

2. Trigeminal ganglion neurons in culture as model to study $P2X_3$ and TRPV1 receptors

The characterization of receptors involved in nociceptive stimuli transmission by sensory neurons ($P2X_3$ and TRPV1) has been investigated in another *in vitro* system, namely TG neurons in culture. In order to analyze this issue from the functional point of view, the characterization of receptors endogenously expressed by these neurons appears a necessary step. The ideal system to this aim would be direct recording from sensory neurons *in vivo* or from the intact ganglion, but only few studies of these preparations have been reported (Stebbing et al., 1998; Boada and Woodbury, 2006). In particular, for DRG, Stebbing et al. (1998) have not detected any response to topical ATP application in intact DRG neurons, in contrast with a number of studies reporting large responses of DRG (and TG) neurons in culture to ATP or its analogues (for representative examples, see Cook et al., 1997, Spehr et al., 2004 for TG; Cook et al., 1998; Burgard et al., 1999, Sokolova et al., 2001 for DRG). This discrepancy might be accounted for by the presence of low, $P2X_3$-desensitizing amounts of ATP in the intact ganglia as a consequence of experimental manipulation, which could mask the response to exogenously applied ATP. In addition, taking into account the fast rise time of $P2X_3$ receptor-mediated responses and their rapid, strong desensitization, the relatively slow method of agonist application and/or an extensive enzymatic breakdown of extracellular ATP could have led to the absence of response. As suggested by North (2002) and confirmed in our study, culturing neurons might have led to an increased $P2X_3$ receptor expression and/or trafficking to the neuronal membrane, thus facilitating observation of ATP-mediated responses.

Even if the technical difficulties of recordings from intact ganglia or from acutely dissociated TG (or DRG) neurons could be overcome, these cells are unsuitable models to continuously monitor changes in receptor expression or function during long time frames (days). For this reason, the development and characterization of TG cultures (described in the work reported in this thesis) has been carried out.

Both mouse and rat TG neurons have shown a good viability in culture, even if the latter appear to be more stable after a few days *in vitro*. The finding that the number of neurons expressing "pain receptors" ($P2X_3$ and TRPV1) is increased in culture in comparison to the ganglion is important, because it indicates, as expected, that culture model is a useful, but not perfect mirror of the *in vivo* condition, thus suggesting limits as well as advantages of the culture as a model system. In this respect, an interesting point is that, in contrast to $P2X_3$ and TRPV1, $P2X_2$ receptor expressing neurons are equally abundant in culture and in ganglia: previous reports have indicated a global increase in the expression of $P2X_3$ receptors in TG neurons after axotomy (Eriksson et al., 1998), thus suggesting that $P2X_3$ receptor expression can be modulated by axotomy (occurring during cell culture preparation), while that of $P2X_2$ receptor might not. Whether this means lack of involvement of $P2X_2$ receptors in the plastic changes of nociceptors following neuronal damage remains to be elucidated.

Nociceptors represent the principal interest of the present research, therefore functional experiments (calcium imaging and patch clamping) have been performed on neurons of small to medium somatic diameter, which were mainly purinergic nociceptive sensory neurons (see Introduction) as validated in the present project. Rat and mouse TG neurons in culture appear morphologically similar. In addition, when comparing neurons of the two species at the first day after cell plating, they are comparable in terms of cell size, as deduced by the similar values of cell capacitance (slow component; A. Fabbro, unpublished) and this might be an indication of a conserved phenotype of TG neurons among these two species.

P2X receptors

In addition to the expression of $P2X_3$ receptors by TG, the functionality of these receptors has been analyzed, not only because of the obvious interest in their responses, but also because the anti-$P2X_3$ antibody employed in the immunocytochemical study recognizes an intracellular epitope of $P2X_3$ receptor, thus labelling also intracellular receptors. This issue has been

addressed by using the ATP analogue α,β-meATP, in order to activate homomeric or heteromeric $P2X_3$ subunit-containing receptors (North, 2002).

α,β-meATP is usually referred to as a selective agonist for $P2X_3$ subunit-containing receptors, but it can also activate the $P2X_1$ ones (North, 2002). Since the mRNA coding for $P2X_1$ is also present in TG neurons (Xiang et al., 1998), it has been necessary to clarify if the latter are present and functional in TG cultures: the almost complete suppression of α,β-meATP-induced currents by A-317491, at a concentration selective for the $P2X_3$ receptor, but not for $P2X_1$ (1 μM; Jarvis et al., 2002), excluded a major role of $P2X_1$ receptors in the α,β-meATP-evoked responses in TG neurons. A full dose-response curve has been then constructed for this agonist, showing an almost perfect overlapping of the responses obtained from rat and mouse neurons.

Immunocytochemical experiments have shown a lower level of colocalization between $P2X_3$ and $P2X_2$ receptors in mouse with respect to rat neurons, implying that mouse neurons express either fewer homomeric $P2X_2$ receptors, or fewer heteromeric $P2X_{2/3}$ receptors. The analysis of the currents elicited by α,β-meATP or ATP application can help to understand this phenomenon from the functional point of view. $P2X_3$ receptors are known to desensitize almost completely during a 2 s-long α,β-meATP or ATP application. Heteromeric $P2X_{2/3}$ show minimal desensitization like homomeric $P2X_2$ receptors (the latter do not respond to α,β-meATP; Ralevic and Burnstock, 1998; North, 2002). These properties make the residual current detected at the end of a 2 s-long α,β-meATP application ($I_{residual}$) an indication of the expression of heteromeric $P2X_{2/3}$ receptors. Because $I_{residual}$ for α,β-meATP is larger in rat than in mouse neurons, it seems likely that the first express a larger amount of $P2X_{2/3}$ heteromers than the latter. These findings are in good agreement with immunocytochemistry data obtained both in culture and in the ganglia. The physiological meaning of the species-specific differential expression of $P2X_{2/3}$ receptors and, in general, their role in the transmission of sensory inputs by TG neurons remain unclear. The expression of $P2X_{2/3}$ receptors has been reported for both TG and DRG neurons and their slow or absent desensitization has led to the suggestion of a possible role in pain transmission under chronic or repetitive stimulation (North, 2002). Nevertheless, no direct evidence has been provided in this respect and, in contrast, their expression and function have been found to be unaffected in a neuropathic pain model (Kim and Chung, 1992). Nonetheless, even if the present study has shown that the

fraction of TG neurons expressing heteromeric $P2X_{2/3}$ receptors is not negligible, these receptors seem to play a limited role in the responses to α,β-meATP.

A fundamental point in the characterization of TG cultures as a model system is that, if the modulatory effects by long-term exposure to certain mediators are to be investigated, the functional expression of receptors should be stable in culture. In our project, this seems indeed to be the case, because both the fraction of neurons responding to α,β-meATP and the currents obtained in response to it were well conserved for three days after plating, thus making the cultures suitable for investigating long term plastic changes in receptor activity.

TRPV1 receptors

TG neurons in culture have also been characterized for their TRPV1 receptor function. To this aim, a standard test dose of 1 μM capsaicin has been used. This capsaicin concentration has been chosen because it readily evokes sub-maximal inward currents in sensory neurons (Koplas et al., 1997). Even if the construction of a full capsaicin dose-response curve is difficult because of strong tachyphylaxis (Koplas et al., 1997), a higher (10 μM) capsaicin dose evoked a larger current, thus indicating a non-saturated response to 1 μM capsaicin (A. Fabbro, unpublished). Functional and immunocytochemical studies showed that functional TRPV1 receptors were present in both rat and mouse TG neurons, but the first were more prone to express functional TRPV1 receptors than the latter.

Like $P2X_3$ receptors, TRPV1 receptor-mediated currents were quite stable during the first days in culture, with a trend for a decrease only at the third day after plating: as no protein loss has been detected at this time point, the response decrease might be accounted for by post-translational modifications or internalization of the receptor.

In summary, the first part of the work performed on TG neurons has shown that both rat and mouse cultures can be reliable models to study $P2X_3$ and TRPV1 receptors function. Neurons show good viability and express receptors stable in terms of expression and function. While rat TG neurons seem a convenient model to study TRPV1 receptors, in view of their wider receptor expression with respect to the mouse ones, both rat and mouse TG neurons are suitable systems to study the activity of $P2X_3$ receptors. Additionally, mouse TG neurons in culture rarely show functional heteromeric $P2X_{2/3}$ receptors, and might therefore serve as a reliable model for the study of native homomeric $P2X_3$ receptors.

3. Effects of chronic NGF or 5-HT exposure of cultured TG neurons on $P2X_3$ and TRPV1 receptor function

The characterization of the experimental system in its basal conditions has represented the background for the subsequent functional studies in which slow, delayed changes in "pain receptor" function induced by mediators involved in certain pathological states (e.g. inflammation and migraine; NGF, 5-HT) has been confirmed, thus showing that the plastic changes in $P2X_3$ and TRPV1 receptor expression found in the early culture period with respect to the ganglion did not prevent upregulation by putative algogens.

Nerve Growth Factor

Although the basal amount of endogenous NGF in TG cultures is relatively low, NGF levels strongly increase in certain chronic pain states (e.g. headache, inflammatory pain; see Introduction), and peak 24 hours after the induction of experimental inflammation (Donnerer et al., 1992). In addition, the effective NGF concentration at the level of peripheral terminals of sensory neurons is probably much higher than the one measured in the bulk tissue or in the serum. Therefore, the effect of a long term exposure of cultured TG neurons to an excessive amount of NGF has thus been investigated in the present thesis. The finding that the responsiveness to a test pulse of α,β-meATP was increased by such a treatment indicates that the function of $P2X_3$ receptors was significantly enhanced.

NGF levels in the cerebrospinal fluid are increased in some forms of headache, and NGF is known to induce the neosynthesis and release of CGRP by sensory neurons (Lindsay and Harmar, 1989; Supowit et al., 2001). Taking into account that CGRP strongly potentiates $P2X_3$ receptor-mediated responses, at least in the time frame of 1 hour (present thesis), the possibility that NGF exerts its action on $P2X_3$ receptors *via* CGRP should not be neglected: however, this seems not to be the case, because inhibition of CGRP receptor activity does not prevent the potentiation of $P2X_3$ receptor-mediated responses by chronic NGF treatment (A. Fabbro, unpublished). The action of NGF *in vitro* seems, thus, to be independent from CGRP.

The potentiation of $P2X_3$ receptor function by NGF reported in this thesis accords with an *in vivo* study reporting enhanced $P2X_3$ receptor expression in sensory neurons after chronic intrathecal NGF administration (Ramer et al., 2001) and indicates that, during inflammation,

the enhanced responsiveness of $P2X_3$ receptors by sensory neurons might account, in part, for the hyperalgesia typical of this pathological condition.

The small, but significant increase in the number of sensory neurons expressing functional $P2X_3$ receptors (i.e., responding to the application of α,β-meATP) might be an additional, indirect indication of a role of $P2X_3$ receptors in inflammatory states. The existence of a subset of nociceptors innervating skin, joints and viscera which do not respond to noxious stimuli in normal conditions, but can become responsive to a variety of chemical mediators as a consequence of inflammation or tissue injury have indeed been described (Millan, 1999): accordingly, the reported increase in the number of $P2X_3$ receptor-expressing neurons might take place during inflammation (that is, when NGF levels are significantly elevated; Pezet and McMahon, 2006), thus possibly contributing to inflammation-induced primary hyperalgesia.

The specificity of action of NGF has been then investigated, by analyzing its action on the activity of TRPV1 receptors. The lack of effect of a *chronic* (24 hours) NGF treatment on the amplitude of current mediated by these receptors is in contrast with the strong, potentiating action of NGF when it is *acutely* applied to TG neurons (and, analogously, to DRG ones; Shu and Mendell, 2001; Bonnington and McNaughton, 2003) and with its boosting effect on the activity of $P2X_3$ receptors. This behavior is unlikely to reflect a different sensitivity of TRPV1 receptors to NGF action with respect to $P2X_3$ receptors, because NGF acutely applied to TG neurons in control conditions can readily potentiate capsaicin-induced currents. A possible explanation might be the presence of negative-feedback mechanisms, for instance receptor dephosphorylation or internalization (Zhang et al., 2005), bringing the system back to its basal level of activity after an initial, transient potentiation by NGF. This hypothesis would, nevertheless, require further investigations outside the aims of the present work.

5-HT

TG neurons express several classes of receptor for 5-HT, including the ionotropic $5\text{-}HT_3$ and a number of metabotropic receptors (1B, 1D, 1F, 2, 7; Lazarov, 2002; Hu et al., 2004), which can be activated by 5-HT released mainly by platelets, mast cells and perivascular serotoninergic nerves (Millan, 1999). 5-HT is a substance typically viewed as a major player in migraine pathogenesis (Goadsby, 2000; Goadsby et al, 2002; Silberstein, 2004). Indeed, during migraine pain, serum 5-HT levels reach concentrations in the order of μM which last for

several hours after the beginning of the attack (Ferrari et al., 1989; Ribeiro et al, 1990). Thus, the possibility that the *chronic* exposure of TG neurons to a relatively large amount of 5-HT might modulate the activity of the main "pain receptors" on TG neurons has been preliminary tested. In contrast to NGF, 5-HT treatment shows no effect on the current mediated by $P2X_3$ receptors, but it strongly potentiates the TRPV1-mediated one, thus demonstrating (in analogy with the action of NGF on $P2X_3$ receptors) that TRPV1 receptors of TG neurons in culture could be pharmacologically potentiated . This finding is in accordance with the *acute* potentiating effect mediated by metabotropic 5-HT receptors on the activity of TRPV1 receptors expressed by visceral DRG afferents (Sugiura et al., 2004). Nakazawa and Ohno (1997) have demonstrated that *acute* 5-HT application has no effect on the current mediated by $P2X_3$ receptors in transfected oocytes, thus making a modulatory action of 5-HT on $P2X_3$ receptors unlikely in both the short and the long term.

The present findings are clearly only a preliminary description of the effect of a chronic NGF or 5-HT application on $P2X_3$ and TRPV1 receptors. Further investigations will be necessary to disclose the molecular mechanisms accounting for this phenomenon. However, the differential, selective effects of chronic NGF and 5-HT treatments on $P2X_3$ and TRPV1 receptors, respectively, suggest the intriguing hypothesis of a discrete regulation of these pain receptors by certain substances, whose balance might direct the nociceptive response toward a purinergic or a vanilloid phenotype.

4. Modulation of $P2X_3$ receptor activity by desensitization: new molecular determinants

The activity of $P2X_3$ receptors expressed by sensory neurons is largely influenced by their strong desensitisation and the slow, Ca^{2+} dependent recovery from it, a phenomenon which can account for a form of short-term receptor memory (Cook et al 1998; Giniatullin et al 2003; see Introduction). Therefore, the desensitization process raises particular interest as a potential site responsible for receptor dysfunction in pain states, and its molecular determinants have been analyzed in $P2X_3$ receptors expressed in HEK cells. Indeed, recent advances concerning the identity and function of ATP receptors and their participation in pain processes have been obtained by molecular cloning of both ionotropic and metabotropic ATP receptors (North, 2002; North, 2004; Ruan and Burnstock, 2003).

One of the most interesting points of the reported work is the demonstration of a spatial segregation of the molecular sites involved in the onset of receptor desensitization, recovery from it, sensitivity to the potentiating action of Ca^{2+}, and agonist potency. The segregation between desensitization onset and recovery, and the involvement of the extracellular domain of $P2X_3$ receptors in the desensitization process have also been described by Zemkova et al. (2004), who have shown that $P2X_2$ receptors display unaltered desensitization onset, but slower recovery, when expressed as chimeras including a portion of the extracellular domain of $P2X_3$ receptors, thus confirming the role of specific residues located in this domain in shaping receptor desensitization.

One of the most interesting mutants is D266A. The behavior of $P2X_3$ receptors mutated at this position is very similar to that of $P2X_2$ receptors, as far as the slow desensitization onset and the fast recovery from it are concerned. However, while $P2X_2$ receptors are readily inactivated by increased external Ca^{2+} levels (Ding and Sachs, 2000), Ca^{2+} has no effect on D266A-mutated $P2X_3$ receptors, further indicating that receptor desensitization and Ca^{2+} sensitivity can be structurally distinct phenomena. Another interesting feature of D266A mutant (and of D220A one) is its lower agonist potency and efficacy when compared to the wild type $P2X_3$ receptor. These two mutations might have negatively affected channel gating or agonist binding. In this respect, binding studies or single channel recordings could provide further insights into the mechanisms underlying the behavior characteristic of these mutated receptors.

An additional point suitable for further investigation is the complex structural and functional relationship between multiple, specific residues in modulating channel function. For instance, because some mutations (E161, E187, E270) only partially block the Ca^{2+} modulatory effect, it would be interesting to investigate the activity of double (or multiple) mutated receptors, in order to better clarify their contribution to the receptor functional features. Analogously, an attractive issue would be to test the results of two mutations with individually opposite effects: for instance, the E111A (or the D266A) and the E289A (or the D220A) mutations have contrasting consequences for receptor recovery from desensitization.

As already mentioned in the Introduction, $P2X_3$ receptor desensitization and its slow recovery could be a protective mechanism for uncontrolled neuronal excitation during sustained receptor stimulation by its endogenous agonist. Furthermore, it also offers a potential target for pharmacological treatments aimed at inhibiting the transmission of nociceptive inputs. As far

as nociceptive TG neurons are concerned, changes in desensitization properties of $P2X_3$ receptors might contribute to migraine pain, particularly when there are fluctuations in the extracellular levels of ATP, Ca^{2+} and Mg^{2+}. Likewise, relatively large Ca^{2+} amounts can spread from the outer to the inner epidermis in case of skin wounds (together with ATP released from damaged cells), thus increasing both the activity and the persistence of the response of $P2X_3$ receptors expressed on sensory neurons, enhancing the transmission of painful stimuli (see Introduction).

5. $P2X_3$ receptors as novel targets for CGRP action: implications for migraine

The principal aim of the development and characterization of TG cultures has been the mimicking of the conditions which trigeminal ganglion neurons are likely to encounter during pathological states, like inflammation or a migraine attack. To explore this issue, the effect of the putative migraine mediator CGRP on pain receptors of TG neurons has been systematically analyzed with this experimental model. Despite the demonstration of an increase in plasma CGRP levels and of the efficacy of CGRP receptor antagonists for migraine treatment (see Introduction), only few data exist on the precise cellular mechanisms involved in the action of this neuropeptide. In particular, acutely applied CGRP has been reported to potentiate TTX-insensitive Na^+ currents in DRG neurons (Natura et al., 2005); in contrast, even though CGRP can exert pronounced vasodilatory effects, CGRP-mediated vasodilation seems to be insufficient to excite nociceptors, at least in a 30 minute time frame (Levy et al., 2005), thus suggesting other potential targets for CGRP activity.

In full agreement with the putative pro-algesic action of CGRP, CGRP binding sites are mainly expressed in small-to-medium sized TG neurons, expected to be nociceptors, with a strong colocalization with the pain transducer receptor $P2X_3$. A trend for a potentiation of $P2X_3$ receptor-mediated responses by CGRP is present already at relatively low peptide concentrations, and reaches a plateau at about 0.5-1 μM. While these concentrations are higher than those reported in plasma, these measurements have been carried with jugular or cubital venous blood, in which the CGRP concentration is probably strongly diluted with respect to its putative, meningeal site of release (Goadsby et al., 1990; Sarchielli et al., 2000). Indeed, large CGRP levels *in vivo* near the TG neuronal terminals are likely because high amounts of CGRP ready for release are expressed by TG neurons, in particular by meningeal afferents (O'Connor and van der Kooy, 1988). It is suggested that the build-up of large local concentrations of the

neuropeptide occur, *via* autocrine stimulation, in conditions of high neuronal activity and/or neurogenic inflammation (Millan, 1999; Durham and Russo, 2002). In this respect, it would be interesting to explore the effects of CGRP on $P2X_3$ receptors of TG meningeal afferents, in order to assess the specificity of action of CGRP on them with respect to TG afferents innervating other cranial sites.

Mechanisms of CGRP action on pain-transducing receptors

The potentiating action of CGRP on $P2X_3$-mediated responses has been shown to depend on the activity of both protein kinase A (PKA) and protein kinase C (PKC), but their precise sites of action are not known. The PKA and PKC dependence might be indirect, *via* phosphorylation of unknown intracellular molecules by the two kinases, or direct by phosphorylation of the P2X N-terminal site by PKC (Vial et al., 2004). While no PKA consensus sites have, however, been described on the $P2X_3$ molecule, both kinases are involved in the trafficking of other receptors such as NMDA ones to the neuronal membrane (Lan et al., 2001; Scott et al., 2003). Furthermore, the potentiation of TTX-resistant Na^+ currents by CGRP reported for a subset of DRG neurons is also PKA- and PKC-dependent (Natura et al., 2005), thus providing further suggestions in favour of the recruitment of both kinases by CGRP receptor activation for trafficking of $P2X_3$ receptors.

TRPV1-mediated responses have not been enhanced by CGRP. Indeed, its action seems to be selective for $P2X_3$ receptors, probably because of the rare colocalization between TRPV1 and CGRP receptors. However, even if the functional colocalization of $P2X_3$ and TRPV1 receptors is limited (~20%), the $P2X_3$ receptor upregulation by CGRP is not restricted to TRPV1 expressing neurons, because CGRP can potentiate $P2X_3$-mediated responses to the same extent in both capsaicin responsive and non responsive TG populations (A. Fabbro, unpublished).

The high level of colocalization between $P2X_3$ receptors and CGRP binding sites (i.e., CGRP receptors) is circumstantial evidence for an action of CGRP specifically on CGRP receptor-expressing TG neurons, and the possibility that the action of CGRP could be indirect, *via* the release of a second messenger from neurons as well as from non-neuronal cells in culture, has no evidence to support it. Indeed, treatment of TG neurons with a CGRP-conditioned medium has not revealed any potentiation of $P2X_3$ receptor-mediated responses, thus providing no indication of the involvement of intermediate substances (A. Fabbro, unpublished).

CGRP in migraine pathogenesis

The CGRP concentration in plasma correlates with the timing and severity of headache during a migraine attack (Juhasz et al., 2003, Juhasz et al., 2005). A number of additional clinical data point to a fundamental role of CGRP in the pathogenesis of migraine. First, triptans (agonists of the serotonergic 5-$HT_{1B/D}$ receptors) are widely used to prevent or relieve migraine attacks. They exert a double action in the transmission of pain at the cranial level, namely by inhibiting synaptic transmission between primary afferents and second order neurons (Goadsby et al., 2002) and by antagonizing the elevation of CGRP levels in migraine sufferers (in parallel with a relief of pain), *via* block of synthesis and release of CGRP (Durham and Russo, 2002). Recent pharmacological trials show that a CGRP receptor antagonist (BIBN4096BS) is a potent and well tolerated antimigraine drug (Olesen et al., 2004; Edvinsson, 2005).

The upregulation of $P2X_3$ receptors of TG neurons reported in this thesis might then represent one of the possible sites of action of CGRP favouring pain transmission specifically at the level of the trigeminal ganglion (because this neuropeptide has no effect on DRG neurons, which poorly express its receptor). Although activation of the trigeminovascular system seems to be secondary to an intrinsic, abnormal brain activity in migraine pathogenesis (like cortical spreading depression; Bolay et al., 2002; Pietrobon and Striessnig, 2003), it represents the effective "pain-sensing" element: the release of vasoactive neuropeptides by meningeal nociceptors, including CGRP, might contribute to a long lasting sensitization of $P2X_3$ receptors expressed on TG neurons, an effect which is likely to persist well after the time during which local CGRP levels are increased. The response of $P2X_3$ receptors to ATP would then be enhanced: in this respect, it is worth noting that ATP could also stimulate CGRP release *via* P2Y receptors (Zimmermann et al., 2002), thus further contributing to propagate a noxious response. The findings reported in the present thesis are therefore in favour of a potentially important role of ATP as a pain transmitter for trigeminal sensory neurons during migrainous attacks, as originally proposed by Burnstock (1996).

A highly speculative, but intriguing possibility is that the functional consequences of an upregulation of $P2X_3$ receptors could take place at the peripheral terminals of (meningeal) nociceptors, but might also favour synaptic transmission between primary TG afferents and second order neurons in the brainstem. In fact, central terminals of sensory neurons express $P2X_3$ receptors (which can be activated by ATP locally released by primary afferents

themselves or by spinal cord -and, perhaps, also brainstem- neurons; Jo and Schlichter, 1999; Burnstock, 2001) and might also release CGRP (Storer et al., 2004; Fischer et al., 2005). Thus, it is not unlikely that, following long-lasting, sustained activity of CGRP-containing nociceptors, CGRP could reach a sufficiently high and peristent local concentration at the level of central terminals of primary afferents. This process might enhance neuronal depolarization and calcium entry in the presence of ATP, leading to the central sensitization observed in chronic pain states.

6. Specificity of NGF action: responses to GABA and ATP of DRG neurons

Our work has demonstrated that a long-term NGF exposure of TG neurons in culture boosts $P2X_3$ receptors activity, while leaving that of TRPV1 ones unaffected. The last part of the work described in the present thesis therefore deals with the specificity of action of NGF, by analyzing its effect on inhibitory receptors expressed on sensory neurons, namely the $GABA_A$ ones.

The lack of effect of NGF treatment on the amplitude of GABA-induced currents of DRG neurons in the first days in culture accords with previous findings (Aguayo and White, 1992; Bevan and Winter, 1995; Oyelese et al., 1997). However, previous reports have not taken into account changes in current kinetics, and in particular its fading during GABA application (desensitization), which could be potentially important for the phenomenon of primary afferent depolarization, providing an indication of the persistence of GABA action (see Introduction). NGF has been found to improve desensitization (and deactivation) of GABA-, but not ATP-, induced currents. The latter finding is in agreement with the unaltered desensitization of $P2X_3$ receptor-mediated currents with chronically-applied NGF to TG neurons (A. Fabbro and R. Giniatullin, unpublished). Remarkably, no significant increase in ATP current amplitude by NGF in DRG neurons has been found, in accordance with data by Bevan and Winter (1995) after a 1-2 week long NGF treatment of DRG neurons. Nevertheless, this finding contrasts with the potentiating action (1 day NGF exposure) of $P2X_3$ receptors of TG neurons (present thesis). It would therefore be interesting to explore if the distribution of the high-affinity receptor for NGF (TrkA) among $P2X_3$ receptor-expressing neurons is different between DRG and TG neurons. DRG and TG neurons differ in several biochemical markers as, for instance, $P2X_3$ receptors are expressed in small, medium and large size neurons in TG, while they are restricted to small size neurons in DRG; the colocalization between $P2X_3$ receptors and the

neuropeptide CGRP is significantly higher in TG with respect to DRG neurons (Ambalavanar et al., 2005); furthermore, they also differ in the extent of CGRP receptor expression and, therefore, in the sensitivity to its modulatory action (present thesis).

7. Conclusions

The work reported in the present thesis has clarified some aspects of the functional features of the main "pain transducing" receptors expressed by primary sensory neurons, in the context of a characterization of the *in vitro* model system suitable to study them. In particular, cultures of PC12 cells have been shown to be a good model to study quantal-like events (mediated by $P2X_2$ receptors) induced by release of endogenous ATP, suggesting a useful method to study ATP release-mediated events. The search for a suitable *in vitro* model to study P2X receptor activity has then moved to trigeminal ganglion neurons in culture. The focus on $P2X_3$ "pain receptors" has led to the finding of their selective potentiation by long-lasting exposures to NGF or CGRP, while the function of TRPV1 receptors has been shown to be specifically enhanced by a long-term 5-HT treatment, putting forward the possibility of a selective, differential regulation of distinct purinergic and vanilloid pain-signalling pathways.

These findings, together with the identification of molecular targets for the modulation of $P2X_3$ receptor function, add further evidence in favour of the fundamental role of $P2X_3$ (and TRPV1) receptors in pain transmission. These receptors, and the substances which positively modulate them, might be suitable targets for new pharmacological treatments aiming at inhibiting nociceptive inputs from primary nociceptors to higher levels of the central nervous system.

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Comparison of P2X and TRPV1 receptors in ganglia or primary culture of trigeminal neurons and their modulation by NGF or serotonin

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Abstract

Background: Cultured sensory neurons are a common experimental model to elucidate the molecular mechanisms of pain transduction typically involving activation of ATP-sensitive P2X or capsaicin-sensitive TRPV1 receptors. This applies also to trigeminal ganglion neurons that convey pain inputs from head tissues. Little is, however, known about the plasticity of these receptors on trigeminal neurons in culture, grown without adding the neurotrophin NGF which *per se* is a powerful algogen. The characteristics of such receptors after short-term culture were compared with those of ganglia. Furthermore, their modulation by chronically-applied serotonin or NGF was investigated.

Results: Rat or mouse neurons in culture mainly belonged to small and medium diameter neurons as observed in sections of trigeminal ganglia. Real time RT-PCR, Western blot analysis and immunocytochemistry showed upregulation of $P2X_3$ and TRPV1 receptors after 1–4 days in culture (together with their more frequent co-localization), while $P2X_2$ ones were unchanged. TRPV1 immunoreactivity was, however, lower in mouse ganglia and cultures. Intracellular Ca^{2+} imaging and whole-cell patch clamping showed functional P2X and TRPV1 receptors. Neurons exhibited a range of responses to the P2X agonist α, β-methylene-adenosine-5'-triphosphate indicating the presence of homomeric $P2X_3$ receptors (selectively antagonized by A-317491) and heteromeric $P2X_{2/3}$ receptors. The latter were observed in 16 % mouse neurons only. Despite upregulation of receptors in culture, neurons retained the potential for further enhancement of $P2X_3$ receptors by 24 h NGF treatment. At this time point TRPV1 receptors had lost the facilitation observed after acute NGF application. Conversely, chronically-applied serotonin selectively upregulated TRPV1 receptors rather than $P2X_3$ receptors.

Conclusion: Comparing ganglia and cultures offered the advantage of understanding early adaptive changes of nociception-transducing receptors of trigeminal neurons. Culturing did not prevent differential receptor upregulation by algogenic substances like NGF or serotonin, indicating that chronic application led to distinct plastic changes in the molecular mechanisms mediating pain on trigeminal nociceptors.

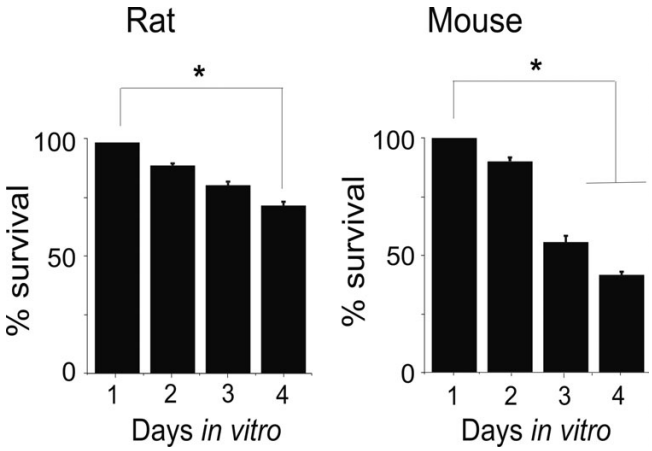

Figure 1
Survival of neurons in TG ganglia or in culture. Survival of neurons (calculated as neuron density expressed as number of β-tubulinIII positive cells per unit area) at 1–4 days in culture. Data are normalized with respect to those at 1 day (n = 250). *: P < 0.05.

Background

Trigeminal ganglion (TG) neurons convey sensory inputs including painful stimuli from head tissues like skin and mucosal surfaces, tooth pulp and meninges. The characterization of nociception-transducing receptors on TG neurons thus becomes important to understand certain forms of acute and chronic pain.

Important pain transducers of noxious stimuli are small and medium size neurons (nociceptors) that can express ATP-activated $P2X_3$ subunit-containing receptors and/or capsaicin (and heat) sensitive TRPV1 receptors [1,2]. Activation of TRPV1 receptors is associated with a slow inward current [1] while ionotropic ATP receptors generate fast currents mediated by $P2X_3$ receptors, and slow ones mediated by $P2X_2$ subunit-containing receptors [3,4]. Overexpression of heteromeric $P2X_{2/3}$ receptors is suggested to be associated with chronic pain states [2,5].

To understand the molecular basis of chronic pain, it would be helpful to use TG neurons in culture as models to study slow changes in the structure and function of P2X or TRPV1 receptors after exposure to mediators such as serotonin or NGF to mimic certain forms of chronic headache [6].

TG nociceptive neurons are modulated by serotonin (5-HT) in a complex fashion. In fact, 5-HT can excite them through $5\text{-}HT_3$ receptors [7] as well as depress their pain signaling via multiple subtypes of the $5\text{-}HT_1$ receptor group [8], an action which had led to the clinical use of $5\text{-}HT_1$ receptor agonists to treat migraine. Furthermore, acute application of 5-HT can strongly potentiate responses mediated by TRPV1 receptors, indicating rapid nociceptive sensitization [9]. Nevertheless, headache is usually a sustained form of pain and its molecular mechanisms including the modulatory action of 5-HT on pain signaling by TG neurons should be studied with long-term experimental models.

NGF may be an additional contributor to headache because of its increased levels in the cerebrospinal fluid of patients during headache attacks [10]. Application of NGF sensitizes spike firing and TRPV1 receptor activity of dorsal root ganglion (DRG) neurons [1,43] and facilitates release of algogenic substances like CGRP from TG neurons [11].

To the best of our knowledge, there is no information on the evolution of TG pain receptors (ATP P2X or TRPV1 ones) during culture since previous studies have simply investigated nociceptors *after* they had been plated for culture [4,6,12]. Thus, the current study characterized the expression, distribution and function of ATP P2X and TRPV1 receptors in cultured trigeminal neurons in comparison with ganglia. We chose to study rat and mouse neurons because the former had been used in other studies of pain and the latter can provide fundamental new data concerning genetic models of chronic pain. While information concerning P2X receptors in TG is less abundant than those for DRG, it is clear that extrapolating data from DRG to TG is inadvisable in view of the very different distribution, expression and modulation of $P2X_3$ receptors between these ganglia [13].

Using TG preparations, we addressed the following questions: 1. How do P2X and TRPV1 receptors of rat or mouse TG neurons grown in culture compare with those of ganglia? 2. Are these markers stable in culture and are they functional? 3. Are these pain receptors similar in rat and mouse TGs? 4. Can 5-HT or NGF modulate the function of P2X and TRPV1 receptors on cultured TG neurons? We report significant differences in the expression and pharmacological modulation of P2X and TRPV1 receptors of nociceptive neurons cultured from rat or mouse TGs.

Results

Rat and mouse neurons are stable in culture without adding NGF

Scanning electron microscopy was used to investigate if the surface ultrastructure of rat or mouse TG neurons had changed after 24 h in culture. After enzymatic dissection of the ganglion at one d in culture, rat or mouse TG neurons retained their spherical shape with somewhat irregular contour probably resulting from the detachment of satellite cells. The surface of TG neurons in culture was characterized by the presence of small microvilli of different length (200–500 nm) emerging from cell body as well

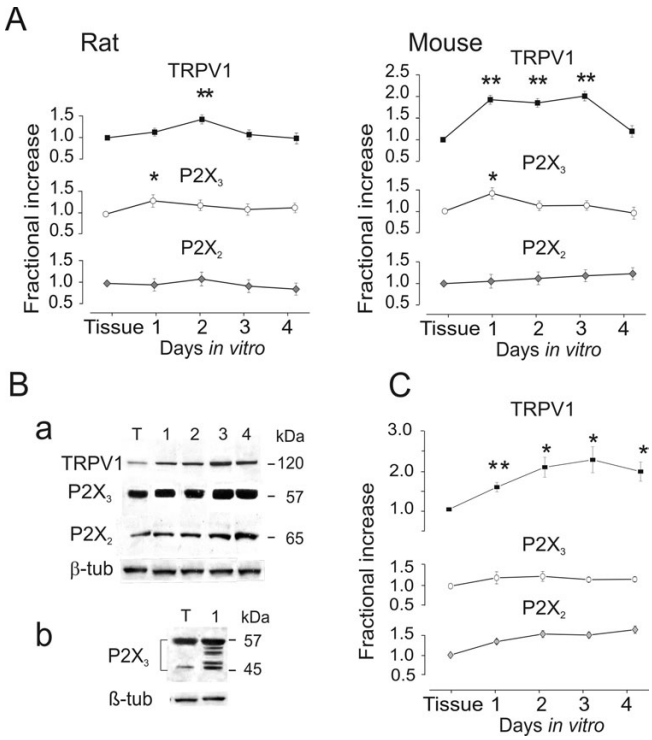

Figure 2

Real time RT-PCR and western immunoblot of ganglia and cultures. A, real time RT-PCR of ganglia and cultures from rat and mouse. Ordinate: relative increment with respect to the ganglion products. For each receptor, amplification values were normalized with β-tubulinIII mRNA levels and compared with the ganglion mRNA levels. GAPDH amplification control was the same in all reactions. B, western immunoblots of equal amounts of neuronal protein lysates (β-tubulinIII) derived from ganglia or culture. Immunodetection of $P2X_3$, $P2X_2$ and TRPV1 mature proteins revealed proper migration (a) in accordance with Vulchanova *et al.*[53] for the predominant $P2X_3$ form (57 kDa), with Newbolt *et al.* [54] for the $P2X_2$ mature receptor (65 kDa) and with Kedei *et al.* [55] for the TRPV1 mature receptor (120 kDa). Panel B b shows $P2X_3$ native (45 kDa) and intermediate polypeptides (up to 50 kDa) detected one d in culture (1) but not in the ganglion (T) as reported by Nicke *et al.* [56] C, relative optical density values of mature receptors at 1–4 days in culture (normalized with respect to β-tubulinIII) and compared to ganglion values. For all experiments shown in A-C n = 3 animals for ganglion or day in culture datapoint (each point is mean ± SEM). *: $P < 0.05$; **: $P < 0.01$.

as by filamentous structures (2–10 μm long, with a cross-section of 120 nm) that remained in contact with the neuron surface for their entire length. These morphological features of TG neurons are typical of rat sensory neurons [14], confirming that culturing conditions without exogenous NGF did not change neuronal phenotype. Rat β-tubulinIII positive cells demonstrated good viability *in vitro* (<30 % loss at 4 d; Fig. 1, left, in which data expressed as neuronal density per unit area are normalized with respect to the number of neurons found at 24 h in culture). Mouse neurons were more labile as their number was more consistently reduced after the second d in culture (Fig. 1, right). Nevertheless, at each one of the four days in culture, only 3–10 % of total mouse neurons was labeled with antibodies against early markers of apoptosis as JNK or activated caspase3 (not shown). Apoptotic nuclei were also rarely detected.

mRNA and protein expression

We next assessed if rat and mouse TG neurons in culture demonstrated changes in the levels of mRNA transcripts of P2X and TRPV1 receptors with respect to the ganglion. To quantify changes in neuron specific mRNA neosynthesis for P2X and TRPV1 receptors in culture with respect to the ganglion, we performed real time RT-PCR experiments as a sensitive method to detect changes in gene expression [15]. Fig 2A shows that, when comparing samples containing an equal amount of the housekeeping gene GAPDH mRNA, there was significant upregulation of $P2X_3$ and TRPV1 transcripts for rat and mouse neurons *in vitro* with respect to the ganglion after normalization for the neuronal-specific β-tubulinIII marker. In particular, $P2X_3$ mRNA showed a peak at 24 h (1.29 fold, $P < 0.05$, and 1.39 fold, $P < 0.01$, for rat and mouse, respectively). Rat TRPV1 mRNA analysis showed a peak at 48 h (1.4 fold, $P < 0.01$), while in the mouse TRPV1 mRNA increased more substantially (1.9 fold, $P < 0.001$) already at the first d and remained elevated for the following two days. $P2X_2$ mRNA signals did not show any significant change.

Because $P2X_2$ and TRPV1 receptors are also expressed in non-neuronal cells, while the $P2X_3$ receptor is not [16,17], it was important to assess the contribution of $P2X_2$, $P2X_3$ and TRPV1 mRNAs in 3 week-old cultures, containing no neurons (β-tubulinIII mRNA negative). Real time amplification experiments showed that equivalent amount of samples (measured as GAPDH mRNA amplification levels) gave undetectable amplification of $P2X_2$, $P2X_3$ and TRPV1 mRNAs, indicating that the contribution of non-neuronal mRNAs to PCR experiments was minimal.

Western immunoblotting experiments were performed to investigate protein expression levels for $P2X_3$, $P2X_2$ and TRPV1 receptors in rat and mouse ganglia as well in culture (Fig. 2B). To study the time course of the protein expression profiles in culture and in the ganglion, we investigated differences in the mature form of rat $P2X_3$, $P2X_2$ and TRPV1 receptors (bands at 57, 65, 120 kDa) under equal protein loading conditions assessed with β-tubulinIII staining (Fig. 2B, a and Fig. 2C). While in the

case of the TRPV1 receptor there was a significant increment already at 24 h in culture (1.5 fold, $P < 0.05$), this was not paralleled by $P2X_2$ and $P2X_3$ expression that showed no substantial change in culture with respect to ganglion (Fig. 2C). Note that the 120 kDa TRPV1 protein was more prominent in culture than in the ganglion (Fig. 2B a). Moreover, in the case of the $P2X_3$ receptor, intermediate bands (45–55 kDa) were observed in culture but not in the ganglia (Fig. 2B b), indicating that, despite a constant amount of the mature 57 kDa protein, there was a significant increment of $P2X_3$ protein expression occurring in culture (2.0 fold, $P < 0.05$). Analysis of mouse 1–3 d cultures revealed similar protein expression behaviour (not shown). The different time profile of data related to TRPV1 expression obtained with real time RT-PCR and western blotting might have been due to a rapid increase in protein translation rate dependent on already present mRNA as reported for the same protein in DRG neurons *in vitro* [18]. Immunocytochemical analysis was then carried out to further investigate changes in receptor protein expression in culture.

Immunoreactivity of TG neurons

The distribution among rat and mouse neurons of $P2X_3$, $P2X_2$ and TRPV1 receptors in freshly excised ganglia and TG cultures was studied as shown by the example of Fig. 3A in which rat ganglion immunoreactivity for $P2X_3$ receptors is compared with 1 d cultured neurons. Neurons labeled by the $P2X_3$ antibody were clearly detected, as much as neurons immunoreactive for TRPV1 receptors (right panel). Fig. 3B quantifies the percentage of TG neurons immunoreactive for $P2X_3$, $P2X_2$ and TRPV1 receptors under different experimental conditions. $P2X_2$ receptors in both rat and mouse TGs were equally represented in ganglion and in culture (approximately 40 %). The number of rat and mouse $P2X_3$ or TRPV1 immunoreactive neurons grew by about 20% with respect to the ganglion (see Fig. 3). Fig. 3C shows that the considerable difference between mouse and rat neurons expressing TRPV1 receptors in culture was not simply due to the *in vitro* condition: in fact, TRPV1 expressing neurons were clearly more abundant in rat ganglia than in mouse ones as quantified by comparing histograms in Fig. 3B.

Table 1 presents pooled data for the distribution of these receptors among rat and mouse neurons of different somatic diameter in the ganglion or in culture. $P2X_3$, $P2X_2$ and TRPV1 receptors were expressed by all three subpopulations of TG neurons (small, medium and large size cells) in the ganglion. It is noteworthy that the relative numbers of neurons expressing these receptors showed only minimal variations in culture (Table 1). Despite an increment in the $P2X_3$ immunopositive cells in culture, $P2X_3$ was predominantly confined to small size neurons for both rat and mouse (60%). In culture, $P2X_2$ was expressed predominantly in medium (50%) while TRPV1 in small size neurons (65–70%). These values remained virtually unchanged during the time *in vitro*.

Receptor co-expression

We next studied the co-existence of two markers on same neurons. Pooled data are given in Fig. 4. Notwithstanding a large number of neurons co-expressing two markers, it was apparent that the number of $P2X_3$ positive cells also positive for $P2X_2$ receptors was higher in rat than in mouse cultures (53% vs 35%, respectively), a phenomenon observed also on ganglion tissue (63% vs 40%, respectively). Likewise, $P2X_3$ positive cells had a higher probability of expressing TRPV1 receptors in rat than in mouse culture (75% vs 54%), a result confirmed with rat and mouse ganglia (61 and 43%, respectively). Although the global number of $P2X_2$ receptor expressing neurons was always comparatively low (see Fig. 3 and Table 1), once in culture, the percentage of $P2X_2$-positive neurons expressing also $P2X_3$ receptors largely rose. A strong rise in the number of TRPV1-positive neurons that could also co-express $P2X_3$ receptors occurred in rat TG culture and, less intensely, in mouse TG culture.

Calcium imaging of TG neurons

To characterize the functional properties of a large number of intact (non-patched) neurons, we first performed intracellular Ca^{2+}-imaging of 24 h cultured TG neurons activated by agonists of $P2X_3$ and TRPV1 receptors as this method is a potent screening tool for P2X receptor assay [19]. As a pharmacological tool to differentiate between $P2X_2$ and $P2X_3$ receptors and to avoid activation of metabotropic P2Y receptors that are natively expressed by TG neurons [20], we used α, β-methyleneadenosine 5'-triphosphate (α, β-meATP), a selective agonist for $P2X_3$ (and $P2X_1$) subunit-containing receptors [16], and capsaicin to activate TRPV1 receptors [21].

Neurons were identified by their responsiveness to pulses of KCl (15 or 50 mM; 1 s). Fig. 5A shows examples of Ca^{2+} transients in mouse TG neurons following 2 s application of 10 μM α, β-meATP or 1 μM capsaicin. Note that capsaicin-induced transients were often long lasting (see example in Fig. 5A b). KCl-sensitive cells could be classified into three groups: 1. responsive to α, β-meATP only ('purinergic phenotype'; Fig. 5A a); 2. responsive to capsaicin only ('vanilloid phenotype'; Fig. 5A b); 3. responsive to both agonists ('mixed phenotype'; Fig. 5A c). Figure 5B summarizes results obtained from 346 mouse and 41 rat TG neurons. In agreement with our immunocytochemistry results, most (68%) mouse neurons responded to α, β-meATP (regardless of their sensitivity to capsaicin), and 33% responded to capsaicin (regardless of their sensitivity to α, β-meATP). Twenty percent of mouse neurons responded to both drugs. Interestingly, the fraction of rat

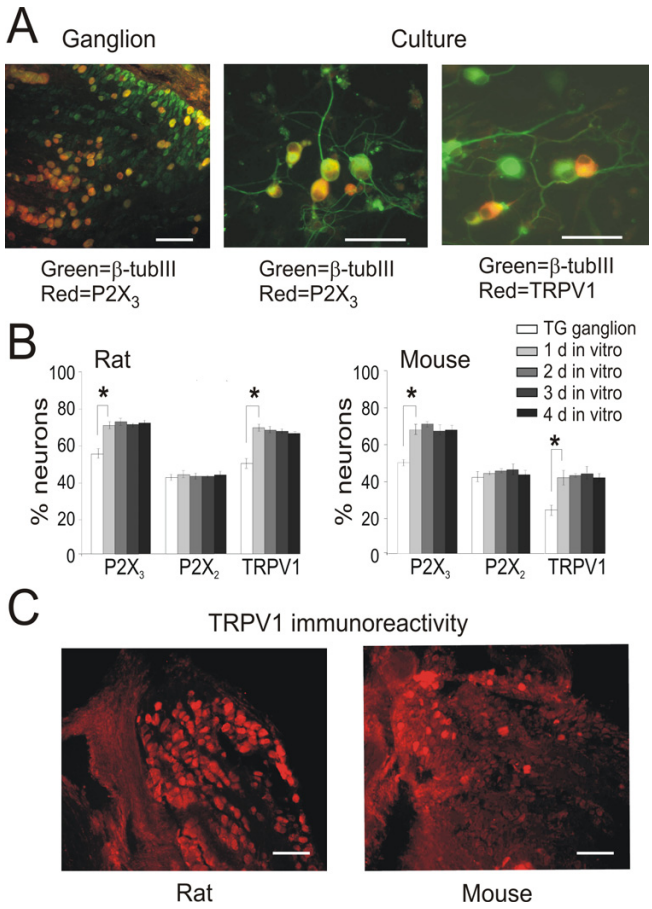

Figure 3

P2X and TRPV1 immunoreactivity in ganglia and cultures. A, example of $P2X_3$ receptor expression by β-tubulinIII labeled neurons in a fixed ganglion (left; calibration bar = 50 μm). After one day in culture, medium-size neurons that are also labeled by β-tubulinIII are immunoreactive for $P2X_3$ (middle) or TRPV1 (right; calibration bar = 50 μm) receptors. B, percentage of neurons (β-tubulinIII positive) immunoreactive for $P2X_3$, $P2X_2$ and TRPV1 in the ganglion and at different d in culture, for rat (left) and mouse (right; n = 3 animals in all cases). The number of $P2X_3$ and TRPV1 immunoreactive neurons increase significantly after dissection and remain constant in culture, while $P2X_2$ receptor labeled neurons remain unchanged. *: $P < 0.05$ for indicated pair of histograms. Values at later d in culture are also significantly different from ganglia. C, comparison of TRPV1 receptor immunoreactivity of rat (left) or mouse (right) ganglia. Note larger number of immunoreactive neurons in the rat tissue. Calibration bars = 50 μm.

cells activated by α, β-meATP was almost the same (70%) as in the mouse, while the percentage of cells responding to capsaicin was higher (55%). The fraction of rat cells sensitive to both agonists was 44% (Fig. 5B). To sum up, imaging data from intact neurons validated the molecular biology and immunocytochemical profile of mouse and rat TG neurons.

P2X or TRPV1 receptor-mediated currents generated by TG neurons in culture

Since in various tissues there are differences in P2X receptor function between rat and mouse cells [22,23], patch clamp recording was used to further characterize receptor-mediated currents of rat or mouse TG neurons (small and medium size).

Fig. 6A shows typical examples of the currents induced by agonists of $P2X_3$-containing or TRPV1 receptors. Application (2 s) of α, β-meATP (10 μM) to either rat or mouse neurons (top row) elicited a fast-developing inward current, which peaked and then strongly desensitized during agonist application, a behavior typical of currents mediated mainly by $P2X_3$ receptors [16]. Plots of peak amplitude responses versus increasing concentrations of α, β-meATP are depicted in Figure 6B. For both species the plots attained analogous maximum response, and the EC_{50} values for α, β-meATP were almost the same in both species (5 ± 1 μM for rat neurons, n = 9, and 6 ± 1 μM for mouse neurons, n = 12). This observation is consistent with the realization that $P2X_3$ receptors of rat and mouse share 98.7% identity of their primary sequence (Blast analysis: [NP_663501] and [NP_112337] for mouse and rat receptors, respectively).

Since one characteristic feature of $P2X_3$-containing receptors is their fast-developing desensitization [16], the current decay during application of α, β-meATP (10 μM, 2 s) was also analyzed. The rapid phase of current decay could be fitted monoexponentially with a time constant (τ_{fast}) which was the same for rat and mouse cells (44 ± 3 ms and 48 ± 3 ms for rat and mouse; n = 24 and 39, respectively), indicating that the onset of fast desensitization was the same for both species.

While α, β-meATP activates homomeric $P2X_3$ receptors and has no effect on homomeric $P2X_2$ receptors [16], this agonist can also activate heteromeric $P2X_{2/3}$ receptors with characteristically-slow current decay during agonist application [24,25]. Thus, co-activation of $P2X_3$ and $P2X_{2/3}$ receptors would produce mixed responses with a fast peak and a residual current [24,25]. One index for the presence of heteromeric $P2X_{2/3}$ receptors on TG neurons was considered the presence of a residual current ($I_{residual}$ in inset to Fig. 6C) at the end of a 2 s-long α, β-meATP application (10 μM). $I_{residual}$ was significantly ($P < 0.01$) different between rat and mouse neurons at 24 h in culture (-73 ± 22 pA, n = 27, and -15 ± 3 pA, n = 41 for rat and mouse cells, respectively). It was interesting to examine how many neurons would express responses indicative of heteromeric receptors. This issue is shown in Fig. 6C with

Table 1: P2X or TRPV1 of small, medium and large TG neurons in ganglia or in culture

	$P2X_3$					
	Rat			**Mouse**		
	Small	**Medium**	**Large**	**Small**	**Medium**	**Large**
Tissue	44 ± 4%	33 ± 3%	23 ± 4%	49 ± 3%	36 ± 4%	15 ± 4%
24 h	55 ± 3%	28 ± 3%	17 ± 2%	60 ± 4%	31 ± 3%	9 ± 2%
48 h	54 ± 2%	33 ± 3%	13 ± 3%	56 ± 4%	29 ± 3%	15 ± 2%
72 h	58 ± 5%	30 ± 4%	12 ± 2%	62 ± 5%	30 ± 4%	8 ± 2%
96 h	56 ± 4%	31 ± 4%	13 ± 3%	60 ± 4%	33 ± 3%	7 ± 2%
	$P2X_2$					
	Rat			**Mouse**		
	Small	**Medium**	**Large**	**Small**	**Medium**	**Large**
Tissue	30 ± 2%	55 ± 3%	15 ± 4%	32 ± 3%	52 ± 2%	16 ± 4%
24 h	34 ± 4%	56 ± 3%	10 ± 4%	36 ± 2%	51 ± 3%	13 ± 3%
48 h	36 ± 4%	57 ± 4%	7 ± 3%	29 ± 3%	55 ± 3%	16 ± 4%
72 h	33 ± 3%	57 ± 4%	10 ± 3%	22 ± 3%	65 ± 2%	13 ± 3%
96 h	34 ± 3%	60 ± 2%	6 ± 3%	21 ± 3%	69 ± 6%	10 ± 3%
	TRPV1					
	Rat			**Mouse**		
	Small	**Medium**	**Large**	**Small**	**Medium**	**Large**
Tissue	60 ± 3%	31 ± 4%	9 ± 3%	59 ± 2%	32 ± 3%	9 ± 2%
24 h	65 ± 4%	29 ± 4%	6 ± 2%	66 ± 2%	31 ± 3%	3 ± 1%
48 h	64 ± 2%	29 ± 3%	7 ± 2%	70 ± 3%	28 ± 3%	2 ± 1%
72 h	66 ± 4%	29 ± 2%	5 ± 2%	68 ± 5%	30 ± 4%	2 ± 1%
96 h	68 ± 4%	27 ± 2%	5 ± 2%	72 ± 4%	26 ± 3%	2 ± 1%

Data are % distribution of $P2X_3$, $P2X_2$ or TRPV1 immunopositive neurons according to their somatic size (small: dia <15 μm; medium: 15 μm–25 μm; large: dia > 25 μm). In each box 100% = the total number of cells immunoreactive for the given receptor.

the distribution of $I_{residual}$ (as % of the initial peak, I_{peak}) for rat (filled bars) and mouse (grey bars) neurons. We assumed that currents comprising $I_{residual}$ at least 5 % of I_{peak} were suggestive of heteromeric $P2X_{2/3}$ receptors (vertical dashed line in Fig. 6C) because, under the present conditions, α, β-meATP was a selective agonist for $P2X_3$ subunit-containing receptors. Using this criterion [26], 54 % of rat neurons generated mixed currents, while only 16 % of mouse neurons did so. These data suggested that α, β-meATP-mediated responses comprised a limited contribution by heteromeric $P2X_{2/3}$ receptors. This notion was further supported by experiments with the selective $P2X_3$ receptor antagonist A-317491 (1 μM; [27]) that almost completely abolished (5 ± 1 %; n = 13) the currents induced by α, β-meATP, confirming that $P2X_3$ receptors were the target for the action of α, β-meATP.

Since approximately 40 % TG neurons were immunopositive for $P2X_2$ subunits (Fig. 3B), we further explored the functional role of this subunit by comparing currents evoked by ATP with those induced by α, β-meATP (as the latter is ineffective on $P2X_2$ receptors). On the same mouse TG neurons (24 h in culture), 10 μM ATP elicited currents with peak amplitude significantly larger (131 ± 9 %; $P < 0.05$) that the ones produced by α, β-meATP (n = 27). Interestingly, taking the criterion of 5 % size of $I_{residual}$, while 3/27 neurons generated $I_{residual}$ in response to α, β-meATP, 10/27 produced a slow current to ATP application.

Fig. 7A summarizes the proportion of rat and mouse cells (24 h in culture) responding to a test concentration of α, β-meATP or capsaicin. In the case of rat neurons, the number of cells sensitive to α, β-meATP was about 80 %, while 60 % responded to capsaicin. The percent value of

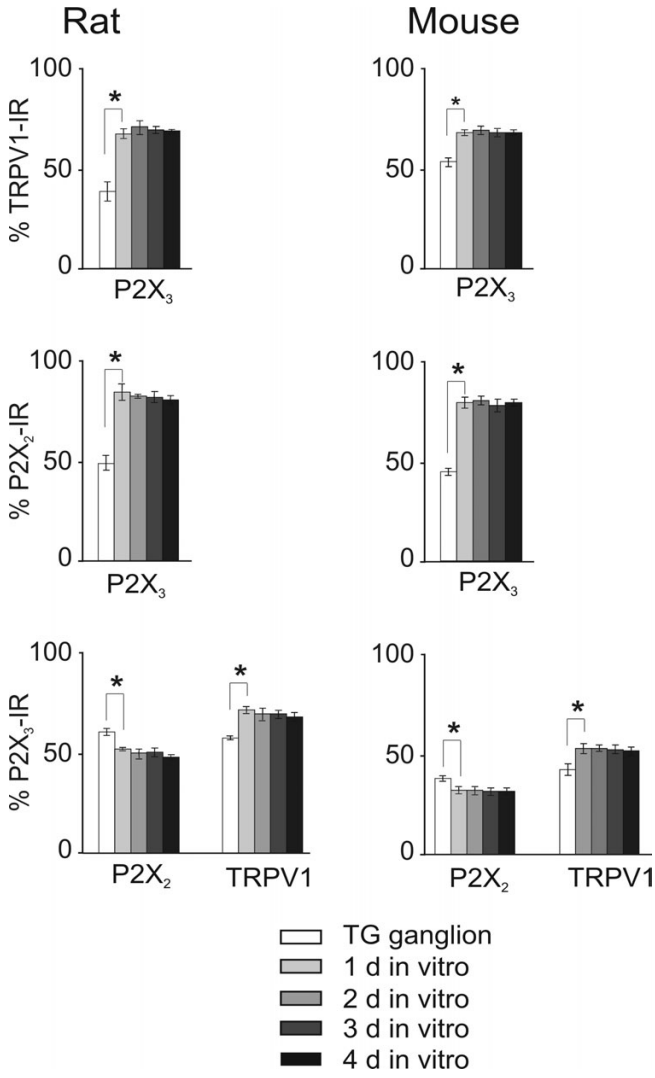

Figure 4

Receptor co-localization investigated with double immunofluorescence. Percent of neurons showing double immunoreactivity for pairs of $P2X_3$, $P2X_2$ and TRPV1 as indicated (rat data are on the left while mouse ones are on the right). The "reference" receptor type is taken as 100%. Data from ganglia (about 1,000 neurons) or at different d in culture (about 500 cells per culture) are shown with differently shaded histograms. n = 3 animals for each bar. *: $P < 0.05$. Values at later d in culture are also significantly different from ganglia. Further details are in the legend to Fig. 3.

cells responding to both agonists was about 50 %. In the case of mouse TG cells, approximately 80 % of neurons were activated by α, β-meATP, while about 20 % responded to capsaicin and only a small minority of neurons could respond to capsaicin as well as to α, β-meATP.

The next issue was the stability of functional receptors during culturing conditions. This was examined with rat TG neurons as shown in Fig. 7B a (filled bars) where the fraction of cells responding to α, β-meATP was large after 24 h in culture and only slightly increased later. The peak amplitude of their response, however, remained essentially unchanged (Fig. 7B b, filled bars). The number of cells responsive to capsaicin was, however, smaller and remained at a relatively steady level in culture (Fig. 7B a, grey bars), as the apparent decrease in response amplitude was not statistically significant (Fig. 7B b, grey bars).

Chronically-applied NGF differentially modulated $P2X_3$ and TRPV1 receptors

Although exogenous NGF is often used to grow sensory neurons in culture [4,12,28], we decided as a routine to avoid adding NGF to the medium because it is a potent algogenic substance and might thus alter the phenotype and function of pain receptors [1]. An ELISA bioassay was performed to measure the amount of NGF in the bulk medium of TG neurons after 24 h in culture. In rat and mouse culture medium NGF concentrations were 9.1 ± 1.3 and 12.5 ± 3.1 pg/ml (absolute concentrations were 6.4 ± 1.5 and 13.4 ± 1.7 pg, respectively): these values are not distant from the binding dissociation constant (0.6 nM) for the high affinity NGF receptor [29], especially when considering the large dilution in the bulk solution. Endogenous NGF was also detected in homogenates of ganglia or cells after 24 h in culture. Indeed, NGF was more concentrated in cultured cells (rat cultures = 19.5 ± 0.9 pg/μg of DNA content, n = 3 cultures; mouse cultures = 23.3 ± 2.2 pg/μg; n = 4) than in the whole ganglion (rat ganglia = 11.4 ± 1.1, n = 3 rats; mouse ganglia = 12.7 ± 0.1, n = 4).

We next investigated what effects a dose of 50 ng/ml of NGF [12] applied for 24 h may produce on the function of $P2X_3$ and TRPV1 receptors of mouse TG neurons. As a screening assay we first assessed changes in the percent of neurons responsive to α, β-meATP (10 μM) in terms of Ca^{2+} transients: Fig. 8A a shows that, after NGF application, their number became significantly larger. Conversely, the fraction of neurons responsive to capsaicin (1 μM) remained unchanged (Fig. 8A b). These data were expanded with patch clamp investigations as indicated by the examples of Fig. 8B a that depicts larger membrane currents evoked by α, β-meATP (10 μM) and unchanged ones induced by capsaicin (1 μM) after NGF application. The histograms in Fig. 8B b summarize these data, indicating that chronically-applied NGF could selectively upregulate $P2X_3$ receptor function with no significant change in the amplitude of capsaicin-induced currents. The lack of change in TRPV1 receptor mediated responses was not attributable to inability of the present cultures to show upregulation of capsaicin-induced responses by NGF. In fact, acutely applied NGF (50 ng/ml for 5 min) could

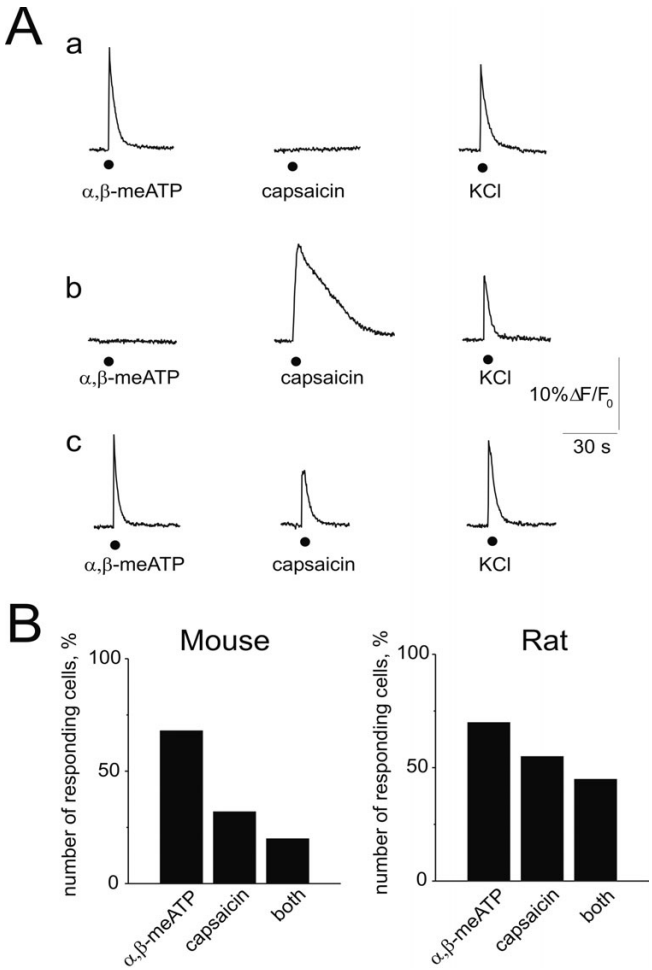

Figure 5

Calcium imaging indicates functional expression of P2X and TRPV1 receptors. A, examples of Ca^{2+} transients in mouse TG neurons activated by 2 s application of 10 μM α, β-meATP or 1 μM capsaicin. a, example of cell responding to α, β-meATP only ('purinergic phenotype'); b, responding to capsaicin only ('vanilloid phenotype'); c, responding to both agonists ('mixed phenotype'). All cells respond to pulse application of KCl. B, number of neurons sensitive to α, β-meATP (regardless of their response to capsaicin), to capsaicin (regardless of their response to α, β-meATP) or to both agonists, for mouse (n = 346; left) and rat (n = 41; right) TG neurons. Neurons (kept in culture for 24 h) were identified by their responsiveness to 15 mM KCl (1 s), while satellite cells and fibroblasts did not respond to this agent. Note that the percent of cells responding to capsaicin was higher in the rat (55%) than in the mouse (33%).

upregulate responses (344 ± 158 %; n = 5; $P < 0.05$) induced by small concentrations of capsaicin (0.1 μM).

Chronically-applied 5-HT selectively upregulated TRPV1 receptors

The differential upregulation of $P2X_3$ and TRPV1 receptors in culture by NGF begged the question whether other mediators believed to be involved in headache might elicit similar long-lasting changes. Since short application of 5-HT has no action on $P2X_3$ receptors [30], we investigated the effect of 5-HT (10 μM; [31]) applied for 24 h to rat TG neurons. Figure 8C a shows typical examples of α, β-meATP (10 μM)- or capsaicin (10 μM)- induced currents obtained in control or after treatment with 5-HT. Note that, while the α, β-meATP response was essentially the same, the response to capsaicin grew much larger. This phenomenon was statistically significant as indicated in Fig. 8C.

Discussion

By combining morphology, molecular biology, immunocytochemistry, Ca^{2+} imaging and electrophysiology, the current study provides the novel characterization of the expression, time-profile and function of P2X and TRPV1 receptors on rat or mouse TG neurons in culture. Comparing TG ganglia and cultures had not been done before and allowed us to discern distinctive changes in key pain transducing molecules in a species-dependent fashion and to monitor their early adaptive changes *in vitro*, thus demonstrating limits as well as usefulness of the model. Furthermore, the differential upregulation of $P2X_3$ and TRPV1 receptors observed after chronically applying either NGF or 5-HT suggested diversity in adapting pain signaling systems to algogenic substances.

Expression of P2X and TRPV1 receptors in ganglia and culture

Our culture conditions ensured that small and medium size neurons, normally associated with pain transducing function [21], were the largest population, in analogy with observations on sections of trigeminal ganglia. One important question was whether cultured TG neurons expressed P2X and TRPV1 receptors like those found in tissue sections of the TG. To the best of our knowledge, this issue has not been investigated before by directly comparing these markers in ganglion sections and in TG cultures.

Real time RT-PCR and Western blot analysis indicated that there was a significant increase in TRPV1 and $P2X_3$ receptors in culture with little change for $P2X_2$ receptors. In addition, the emergence of immature forms of $P2X_3$ receptors may suggest neosynthesis and trafficking of these proteins to reflect ongoing plasticity. Although both RT-PCR and Western blot yielded unidirectional results, we observed a mismatch in the timecourse of RT-PCR and Western blot signals for TRPV1 receptors. A discrepancy between transcription and translation is not an uncom-

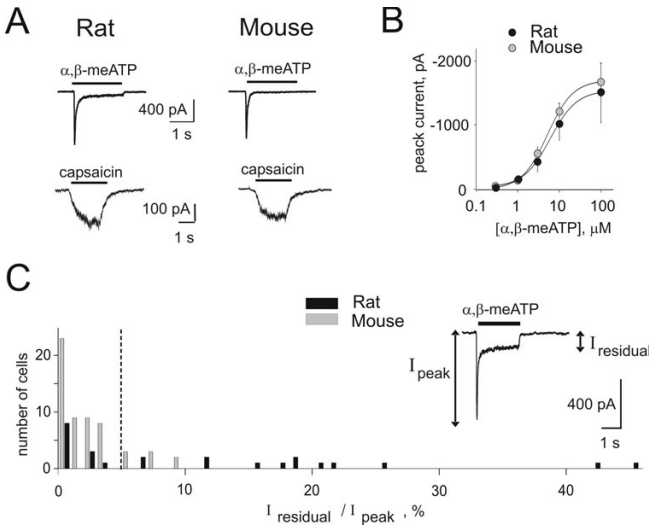

Figure 6

Characteristics of pain receptors of cultured TG neurons. A, examples of currents recorded from rat and mouse TG neurons in culture (24 h) during application of α, β-meATP (10 μM, 2 s; upper row) and capsaicin (10 μM, 2 s for rat; 1 μM, 2 s for mouse; bottom row). B, log dose-response curves for rat (filled symbols; n = 9) and mouse (grey symbols; n = 12) neurons cultured for 24 h. Both potency and efficacy of α, β-meATP were similar for rat and mouse. C, distribution of values of the residual current ($I_{residual}$) present at the end of an application of α, β-meATP (10 μM, 2 s) in rat and mouse neurons cultured for 24 h, expressed as a fraction of the peak current (I_{peak}; n = 24 and 58 for rat and mouse, respectively). Inset shows an example of the mixed type of current recorded from a subset of rat TG neurons. Dashed line indicates the 5% arbitrary threshold above which currents were considered to be mixed.

mon phenomenon, as amply discussed in a recent review, because of the complex relationship between mRNA and protein as transcription and translation are governed by independent mechanisms and separate time constants [32].

While mouse ganglion P2X immunoreactivity data were comparable to those by Ruan *et al.*[33], immunostaining of cultured neurons showed that, in rat and mouse cultures, the number of cells containing $P2X_3$ and TRPV1 subunits was significantly increased already at 24 h in culture and remained stable for the following 3 d. Increased immunoreactivity was, however, absent in the case of $P2X_2$ receptors.

Co-expression of P2X and TRPV1 receptors was present in a large number of rat TG neurons, suggesting potential bimodal signaling to pain stimulants [2,21]. Interestingly, the small number of neurons immunoreactive for the $P2X_2$ receptors acquired significant $P2X_3$ receptor immunoreactivity in culture. This change in expression profile might provide the substrate for heteromeric assembly of $P2X_{2/3}$ receptors, typically signaling chronic pain [2,5]. Overall, data from real time RT-PCR, Western blotting and immunocytochemistry suggest upregulation of $P2X_3$ and TRPV1 receptors in culture with respect to the ganglion.

Intracellular calcium imaging

Measuring responses only from excitable cells which generated Ca^{2+} transients to a depolarizing pulse of KCl, we found the proportion of neurons responsive to α, β-meATP and the TRPV1 agonist capsaicin similar to the one obtained with immunocytochemistry data. Ca^{2+} transients induced by α, β-meATP comprised Ca^{2+} influx partly via open $P2X_3$ receptors and mainly via depolarization activated Ca^{2+} channels [34]. The complex nature of this Ca^{2+} signal thus accounts for the slow decline of the α, β-meATP-evoked transients in comparison with the faster decay of membrane currents generated by the same agent (Fig. 6A). Our present imaging data thus show that functional receptors were expressed at membrane level on the majority of TG neurons.

Membrane currents induced by α, β-meATP or capsaicin

Fast inward currents evoked by α, β-meATP were mediated by $P2X_3$ receptors because of their block by the selective $P2X_3$ blocker A-317491, and accord with knockout mouse model data indicating that sensory neurons almost exclusively express $P2X_3$ and α, β-meATP-insensitive $P2X_2$ receptors [35]. On a significant number of rat TG neurons, α, β-meATP produced mixed-type currents suggestive of co-expression of $P2X_3$ and $P2X_{2/3}$ receptors (the latter responsible for the residual current component). By using ATP it was possible to assess the functional role of $P2X_2$ receptors. The amplitude of current responses evoked by ATP was somewhat larger and more frequently followed by a slow residual current, suggesting that $P2X_2$ receptors demonstrated with molecular biology or immunocytochemistry could be functional, although their contribution to purinergic signaling was quite limited, in comparison to $P2X_3$ receptors, in terms of response amplitude and percent of neurons expressing them.

Capsaicin-mediated responses were more rarely observed on mouse neurons than rat ones in accordance with the low expression level of TRPV1 receptors. Low TRPV1 immunoreactivity is reported also for other murine ganglia [36] while high expression is typical of the rat TG [37]. It is likely that, in the mouse, thermal nociception (which is normally mediated by TRPV1 receptors in the rat) is transduced, at least in part, by other nociceptive sensor proteins, in addition to TRPV1 receptors [36,38].

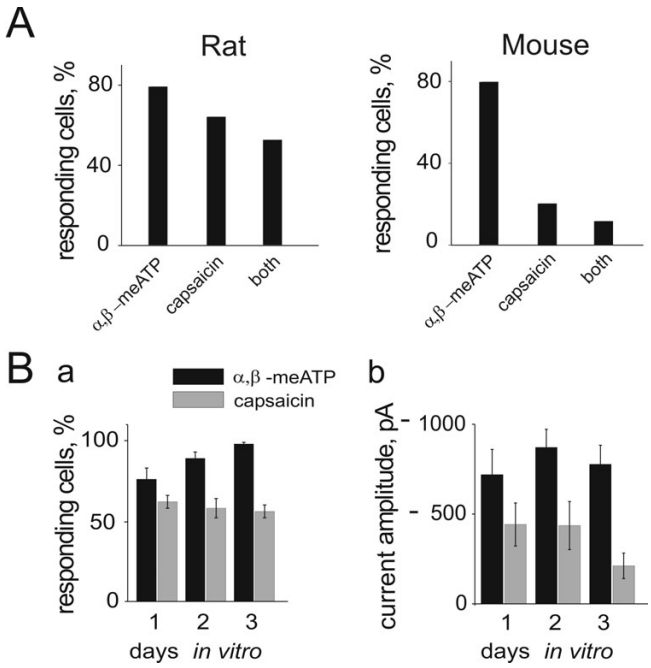

Figure 7

Functional characterization of rat and mouse TG neurons. A, fraction of cells responding to α, β-meATP (independently from their response to capsaicin), to capsaicin (independently from their response to α, β-meATP) or to both agonists for rat (left panel; n = 33) and mouse (right panel; n = 50). B, persistence of the responsiveness of rat TG neurons in culture to α, β-meATP and capsaicin. a, Proportion of cells responding to α, β-meATP (black bars; n = 7, 7, 10 cell cultures for the 1st, 2nd and 3rd day, respectively) and capsaicin (grey bars; n = 7, 8 and 8 cell cultures for the 1st, 2nd and 3rd day). b, peak amplitude of currents elicited by α, β-meATP (black bars; n = 22, 11 and 31 cells for the 1st, 2nd and 3rd day, respectively) and capsaicin (grey bars; n = 16, 13 and 10 cells for the 1st, 2nd and 3rd day). Only cells responding to the agonist were included in the current analysis.

NGF-induced modulation of $P2X_3$ receptor function

Cultures of sensory neurons are often grown in the presence of high concentrations of NGF, known to be a strong algogen operating via multiple metabolic pathways [1,39]. Our study demonstrated that TG neurons could be grown in culture without exogenous NGF probably because the standard medium contained a small concentration of NGF synthesized by cultured cells as shown by the presence of NGF in homogenates of ganglia and cell cultures. Since the high-affinity binding of NGF to neuronal membranes has a 0.6 nM dissociation constant [29], it is probable that, in standard culture conditions, there was adequate production of NGF to preserve neuronal viability. It is likely that other endogenously-produced neurotrophins (like for example NT-3, BDNF and GDNF; [40-42] could also play a role in shaping the activity of these receptors.

Chronically-applied NGF upregulated the function of $P2X_3$ receptors without changing TRPV1 receptor activity. This observation suggests a model system to test how an excess of NGF expected to occur during certain chronic pain states can bias the pain transducing properties of nociceptors towards purinergic rather than vanilloid function. Such effects by chronic NGF are distinct from the consequence of acute, brief application of this substance that enhanced TRPV1 receptor function as previously reported for DRG neurons [1,43].

5-HT-evoked modulation of TRPV1 receptors

While TG neurons express several types of 5-HT receptors [44] that can mediate acute nociception via rapid sensitization of TRPV1 receptors [9], much less is known about long-term consequences of 24 h exposure to this monoamine. This is an interesting issue because plasma levels (in the micromolar range) of 5-HT remain elevated for many hours during headache [45]. Chronic application of 5-HT to TG neurons in culture was followed by a large upregulation of TRPV1 receptor function without affecting $P2X_3$ receptors. Because 5-HT induces a large, long-lasting increase in intracellular Ca^{2+} [31], it is likely that Ca^{2+} dependent changes in second messenger systems triggered such a plasticity of TRPV1 receptor function.

Conclusion

Primary cultures of TG neurons could be maintained for a few days without the need of exogenous NGF. This approach provided a useful preparation to explore how chronically-applied algogens, implicated in headache pathophysiology, could generate heightened pain sensitivity. When NGF or 5-HT was tested on such a system, there was differential modulation of purinergic and vanilloid receptors, indicating distinct types of plasticity of nociceptors depending on the type of algogenic substance.

Methods

Cell culture preparation of TG neurons

Primary cultures of TG ganglion sensory neurons were obtained from C57-Black/6Jico mice or Wistar rats (P10–14; an age reported to show a quasi-adult phenotype for P2X receptors [33]). Animals were anesthetized by diethyl ether and decapitated (in accordance with the Italian Animal Welfare Act and approved by the Local Authority Veterinary Service). TG were rapidly excised and enzymatically dissociated in F12 medium (Invitrogen Corp, S.Giuliano Milanese, Italy) containing 0.25 mg/ml trypsin, 1 mg/ml collagenase and 0.2 mg/ml DNAse (Sigma) at 37°C. Cells were plated on poly-L-lysine-coated petri dishes in F12 medium with 10% fetal calf

serum. For molecular biology or Ca^{2+} imaging experiments, 2×10^6 cells were plated. For patch clamp experiments, mouse and rat cells were diluted twice. Three week-old TG cultures, that lacked neurons (i.e., negative for the neuron specific marker β-tubulinIII), served as negative control for molecular biology experiments.

Real time RT-PCR

For PCR experiments, total RNA was extracted from TG ganglia or from culture using Trizol reagent (Invitrogen). After DNAse treatment (Ambion, Austin, TX, USA), cDNA synthesis and amplification were obtained using SuperScript III Two-step qRT-PCR kit (Invitrogen). Thirty ng cDNA were amplified with specific oligonucleotides and fluorogenic probes (TaqMan gene expression assays, Applied Biosystems, Applera, Norwalk, CT, USA) in ABI PRISM 7000 Sequence Detection System (Applied Biosystems) in the presence of ROX (Invitrogen) as internal reference dye. mRNA samples from fresh ganglia or cultures at different times were calibrated to obtain similar amplification of the GAPDH housekeeping mRNA. In preliminary experiments, analogous amplification of the samples was obtained also with 18S RNA probes. Nevertheless, to normalize the real time PCR results only with respect to neuronal mRNA, amplification of the neuronal specific β-tubulinIII housekeeping gene was chosen.

Specific TaqMan assays for mouse and rat target mRNA encoding $P2X_3$, $P2X_2$ and TRPV1, neuronal specific β-tubulinIII, GAPDH mRNA and 18S RNA (respective Applied Biosystems catalogue numbers: Mm00523699_m1, Mm00462952_m1, Mm01246282_m1, Rn01460299_m1, Mm00727586_s1, 4352339E FG, 4308329; www.appliedbiosystems.com) were chosen. All assays were validated for linearity of amplification efficiency and quantitative standard curves were obtained using serial dilutions of ganglia rat or mouse TG cDNA. To ensure absence of amplification artifacts, end point PCR products were initially assessed on ethidium bromide-stained agarose gels that gave a single band of the expected size for each assay. Negative controls containing no template cDNA were run in each condition and gave no results. The reactions were quantified when the PCR product of interest was first detected (cycle threshold). Calculations for relative mRNA transcript levels were performed using the comparative C_T method ($\Delta\Delta C_T$) between cycle thresholds of different reactions [46]. In particular, the parameter C_T (threshold cycle) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold. The calculation is based on the difference (ΔC_T) between the C_T values of the target receptor and the neuron-specific housekeeping gene (β-tubulinIII) at each time-point in culture, and then normalized with respect to the ΔC_T value of the ganglion.

Western immunoblot

Rat or mouse TG ganglia or cultures were homogenized in ice-cold lysis buffer containing 10 mM TrisHCl (pH 7.5), 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 8 M urea and protease inhibitors (Roche, Basel, Switzerland). The procedure was essentially the same as described by Fabbretti *et al.*[47]. The following polyclonal antibodies were used: $P2X_3$ (1:2000 Neuromics, Bloomington, MN, USA), $P2X_2$ or TRPV1 (1:400 Alomone, Jerusalem, Israel), β-tubulinIII (1:400, Chemicon, Temecula, CA, USA). To ensure correct equal loading reflecting the neuronal cell content in different lysates, protein extracts were quantified with bicinchonic acid (Sigma) and calibrated for the neuronal specific β-tubulinIII. The amount of loaded proteins was in the 20–50 μg/ml range.

Immunocytochemistry

TG ganglion tissue was used with a free-floating immunostaining procedure. TG culture cells were fixed in 4% paraformaldehyde for 20 min at room temperature. The following rabbit polyclonal antibodies were used: $P2X_3$ (1:500 from Chemicon), $P2X_2$ and TRPV1 (1:200 from Alomone), and anti-cleaved caspase3 (1:100, Cell Signaling Technology, Beverly, MA, USA). The mouse monoclonal antibodies against the neuron specific β-tubulinIII (1:100, from Chemicon), GFAP (1:200, Sigma) and JNK (1:100, Santa Cruz) were used. Immunofluorescence reactions were visualized using the secondary antibodies AlexaFluor 488 or AlexaFluor 594 (1:500 dilution; Molecular Probes, Invitrogen). The $P2X_3$ antibody used for double immunofluorescence experiments was obtained by immunizing a guinea-pig with the peptide CVEKQSTDSGAYSIGH. The specificity of the guinea-pig anti-$P2X_3$ antibody was evaluated by western immunoblotting and immunofluorescence experiments (dilution 1:500 and 1:200, respectively) of HEK-293 cells transfected with pCDNA-$P2X_3$ [47]. Tissue or cells stained with the secondary antibody only showed no immunostaining. To minimize tissue autofluorescence, TG ganglia were treated with Sudan Black. All images were captured under the same brightness and contrast settings. Control experiments using pre-immune guinea-pig serum gave no signal. In each experiment the number of positive neurons for a given antibody was normalized by dividing the number of positive cells by the number of β-tubulinIII-stained cells (equal to 100 %). For double immunofluorescence experiments the number of neurons stained with a certain antibody was referred as a percent of the total number of cells stained with the other antibody. An average of 500 cells in culture or 1,000 cells in the tissue were counted for each condition. Each data is the mean of at least 3 independent experiments. Results were analyzed with the ImagePro Express software (Media Cybernetics, L.P., Silver Spring, MD, USA).

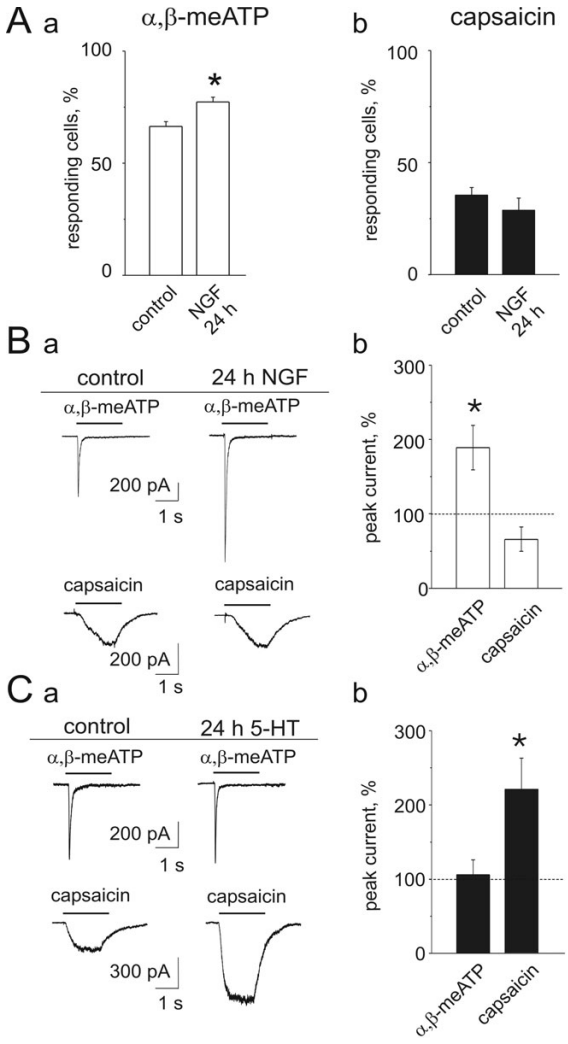

Figure 8

Modulation of receptor function by chronically applied NGF or 5-HT. A, Ca^{2+} imaging of single neurons shows percent increase in mouse TG cells responsive to α, β-meATP (a) in control or after application of 50 ng/ml NGF (24 h; n = 6 culture dishes). In 6 sister cultures there was no significant change as far as responses to capsaicin (b) were concerned. *: P < 0.05. B, a, patch clamp current records show increased amplitude of mouse responses to α, β-meATP after 24 h NGF treatment, while responses to capsaicin remained equiamplitude. B, b, histograms summarizing the significant (*: P < 0.05) rise in α, β-meATP evoked current amplitude (n = 26) without significant change in capsaicin responses (n = 14). Data are expressed as % of control amplitude in sister cultures. C, 5-HT (10 μM; 24 h) upregulates the amplitude of rat capsaicin current without affecting responses to α, β-meATP (a). C,b shows significant rise in the peak current induced by capsaicin (n = 31) with no change in the α, β-meATP-evoked current (n = 31). Data are expressed as % of control amplitude in sister cultures. *: P < 0.05.

Scanning electron microscopy

For scanning electron microscopy, 24 h rat or mouse TG cultures were fixed in 2.5% glutaraldehyde (Sigma; in 0.1 buffered phosphate; pH 7.3) for 30 min at 4°C and post-fixed with 1% OsO_4 (Sigma), dehydrated in ethanol and dried by the critical-point method [44]. Ganglia or cultures were sputter-coated with gold (Electron microscopy sciences, Hatfield, PA, USA) as described [48]. Specimens were observed under a Stereoscan 430i microscope (Leica, Houston, TX, USA). Three hundred cells obtained from 4 different rats and 6 mice were observed. Freshly dissected TGs from two rats were slit open after de-sheathing without enzymatic treatment. Ganglia were fixed for 3 h at 4°C and treated as above.

ELISA

The level of NGF present in the supernatant or in the cell lysates of TG culture was assessed with using the Emax NGF immunoassay system (Promega, Madison, WI, USA). Rat and mouse TG culture medium was collected after 24 h from plating and concentrated 4-fold for analysis (n = 4). Furthermore, mouse or rat ganglia or cell cultures were homogenated in 200 μl of a buffer containing 137 mM NaCl, 20 mM TrisHCl (pH 8), 1% NP40, 10% glycerol and protease inhibitors (Roche). Samples were diluted (1:6) in the same buffer and processed for ELISA assay. Results were corrected for blank and normalized. In the case of ganglia or cells, quantification was normalized with respect to the genomic DNA content (purified using GenElute mammalian genomic DNA kit, Sigma). The recovery of exogenously added NGF (100 ng/ml) was 63 ± 11 % (n = 3); data were not corrected for recovery. No NGF was detected in the fetal calf serum before adding it to the cultures.

Patch-clamp recording

Cells were continuously superfused (2 ml/min) with physiological solution containing (in mM): 152 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES (pH adjusted to 7.4 with NaOH, osmolarity adjusted to 320 mOsm with glucose). Single cells were patch clamped in the whole-cell configuration using pipettes with a resistance of 3–4 MΩ when filled (in mM) with 140 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 2 Mg_2ATP_3, 2 GTP, 10 HEPES and 10 EGTA (pH adjusted to 7.2 with KOH; osmolarity 285 mOsm). Currents were recorded from medium-sized (15–25 μm) nociceptive TG neurons [1]. TG cells were voltage clamped at membrane potential ranging from -70 to -60 mV. Series resistance was compensated by 70 %. α, β-meATP concentration-response curves were obtained by applying the same dose range to each tested cell; results were fitted with a sigmoidal curve (Origin 6.0, Microcal, Northampton, MA, USA) in order to express agonist potency in terms of EC_{50} values (concentration producing 50 % of the maximum response). Each concentration of

α, β-meATP was applied for 2 s every 7 min to obtain full response recovery from desensitization. Capsaicin induces responses with strong tachyphylaxis during repetitive applications [49] and it can kill primary afferent nociceptors [50]: to circumvent these problems, a single dose of capsaicin was applied to each TG neuron. On mouse neurons, 1 μM capsaicin was used as standard test dose to yield reproducible inward currents, because even a small increment in concentration (10 μM) produced very slowly reversible inward currents as previously reported for sensory neurons [51]. On rat neurons, reproducible responses were evoked with a test (1–10 μM) concentration of capsaicin. This concentration is in excess of the EC_{50} value for TG neurons [52]. In order to minimize any possible difference in responses between TG neuron preparations, sister dishes were used on each occasion to compare control neurons and neurons treated with 5-HT or NGF (acute or chronic treatment).

Calcium imaging

Cells were incubated for 40 min at 20–22°C in physiological solution containing Fluo3 (AM ester cell-permeable compound; 5 μM; Molecular Probes), followed by a 30 min washout period. Fluorescence emission was detected with a fast CCD camera (Coolsnap HQ; Roper Scientific, Duluth, GA, USA). Images were acquired with 150 ms exposure time and single cell responses were analysed with the Metafluor software (Metafluor Imaging Series 6.0, Universal Imaging Corporation, Downingtown, PA, USA). Intracellular Ca^{2+} transients were expressed as percent amplitude increase ($\Delta F/F_0$, where F_0 is the baseline fluorescence level and ΔF is the increment over baseline). Each event was also visually inspected to exclude artifactual components.

Drug delivery in functional experiments

α, β-meATP, NGF and capsaicin (all from Sigma) were diluted with physiological solution to final concentration and applied by a rapid superfusion system (Rapid Solution Changer RSC-200, BioLogic Science Instruments, Grenoble, France). The time for solution exchange was about 30 ms [34]). Chronic application of NGF (50 ng/ml) was done by applying this substance to TG cultures for 24 h; cells were patch clamped immediately after washing out this dose of NGF. Twenty-four hour long treatment with 5-HT (10 μM) was carried out in the continuous presence of the monoaminoxidase inhibitor pargyline (100 μM; Sigma) to prevent enzymatic breakdown of this monoamine. Parallel controls were treated with the same concentration of pargyline alone.

Data analysis

Data are presented as the means ± standard error of the mean (n = number of cells, unless otherwise indicated). The statistical significance was assessed with Mann-Whitney rank-sum test and the Wilcoxon test for non parametric data, and with Student's *t*-test for parametric data (KyPlot, version 2.0, Qualest Co., www.qualest.co.jp). For RT-PCR and Western blot, the films were scanned and band density was measured using CorelDraw Photopaint software (Corel, Berkshire, UK), normalized with β-tubulinIII control band and compared to the tissue value. For real time PCR, the relative mRNA expression of $P2X_3$, $P2X_2$ and TRPV1 in the different samples was normalized to the neuronal β-tubulinIII mRNA content in each condition and correlated with the one of the TG tissue. These experiments were performed in duplicate and repeated thrice for mouse and rat samples. Differences between groups were compared using ANOVA. A P value of < 0.05 was accepted as indicative of a statistically significant difference.

Abbreviations

α, β-meATP: α, β-methyleneadenosine 5'-triphosphate. DRG: dorsal root ganglion. EC_{50}: concentration producing 50 % of the maximum response. NGF: nerve growth factor. P2X: membrane receptors gated by ATP. TG: trigeminal ganglion. TRPV1: membrane channel gated by capsaicin. 5-HT: serotonin.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MS, MD and EF performed the molecular biology and immunocytochemical studies. MZ performed the electron microscopy. AF and RG performed electrophysiology and imaging. AN oversaw the research. The manuscript was jointly prepared by all authors.

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Identification of Negative Residues in the $P2X_3$ ATP Receptor Ectodomain as Structural Determinants for Desensitization and the Ca^{2+}-sensing Modulatory Sites*

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On nociceptive neurons, one important mechanism to generate pain signals is the activation of $P2X_3$ receptors, which are membrane proteins gated by extracellular ATP. In the presence of the agonist, $P2X_3$ receptors rapidly desensitize and then recover slowly. One unique property of $P2X_3$ receptors is the recovery acceleration by extracellular Ca^{2+} that can play the role of the gain-setter of receptor function only when $P2X_3$ receptors are desensitized. To study negatively charged sites potentially responsible for this action of Ca^{2+}, we mutated 15 non-conserved aspartate or glutamate residues in the $P2X_3$ receptor ectodomain with alanine and expressed such mutated receptors in human embryonic kidney cells studied with patch clamping. Unlike most mutants, D266A ($P2X_3$ receptor numbering) desensitized very slowly, indicating that this residue is important for generating desensitization. Recovery appeared structurally distinct from desensitization because E111A and D266A had a much faster recovery and D220A and D289A had a much slower one despite their standard desensitization. Furthermore, E161A, E187A, or E270A mutants showed lessened sensitivity to the action of extracellular Ca^{2+}, suggesting that these determinants were important for the effect of this cation on desensitization recovery. This study is the first report identifying several negative residues in the $P2X_3$ receptor ectodomain differentially contributing to the general process of receptor desensitization. At least one residue was important to enable the development of rapid desensitization, whereas others controlled recovery from it or the facilitating action of Ca^{2+}. Thus, these findings outline diverse potential molecular targets to modulate $P2X_3$ receptor function in relation to its functional state.

$P2X_3$ receptors of nociceptive sensory neurons transduce the action of extracellular ATP into painful signals especially during chronic pain states (1). Similar to other ligand-gated ionotropic receptors, $P2X_3$ receptors undergo rapid, full desensitization in the continuous presence of their agonist (2). However, a distinctive property of $P2X_3$ receptors is the prompt re-attainment of function in high extracellular Ca^{2+} solution that operates by facilitating recovery from desensitization (3, 4). Thus, Ca^{2+} can exert a profound, rapid action on the ability of $P2X_3$ receptors to transmit sensory inputs to the central nervous system. However, the precise sites mediating the effect of Ca^{2+} remain unknown and carry considerable interest for any attempts to manipulate transduction of pain signals.

The large family of ionotropic ATP receptors ($P2X_{1-7}$) shares a similar topology that comprises two transmembrane domains joined by one large extracellular loop with 10 disulfide bonds and intracellular N- and C-terminal regions (2, 5, 6). The facilitating action of extracellular Ca^{2+} is exclusively produced on desensitized $P2X_3$ receptors, perhaps via extracellular sites (3, 4). To identify the receptor region involved in this action and the amino acid sites important for it, we focused on negatively charged residues of the ectodomain of $P2X_3$ receptor that are not conserved in other P2X receptors. In fact, other subtypes of the P2X receptor family have either very slow desensitization (typical of the $P2X_2$ receptor class) (7, 8) or fast desensitization (*e.g.* the $P2X_1$ receptor class) not modulated by high extracellular Ca^{2+} (9).

Because on $P2X_3$ receptors the effects of Ca^{2+} are closely related to desensitization, our approach also provided an opportunity to explore the role of extracellular negative residues in controlling desensitization development and recovery from it as these processes are not completely understood as far as $P2X_3$ receptors are concerned.

For other P2X receptors, the development of desensitization is believed to be determined by receptor transmembrane and intracellular segments (10–15). However, recent experiments have indicated that the ectodomain not only controls agonist binding (16) but also desensitization via its coupling to the C-terminal domain (17). Furthermore, the chimeras of $P2X_2$ receptors containing the N-half of the $P2X_3$ receptor ectodomain develop desensitization, indicating that this region influences the desensitized conformation state and the process of recovery of receptor function (18).

Because the latter study has narrowed the number of potential ectodomain residues important for desensitization (18), the present investigation based on single mutations of certain negatively charged amino acids allowed us to examine whether sites mediating recovery from desensitization may be distinct from those involved in the onset of desensitization.

MATERIALS AND METHODS

Mutagenesis of the $P2X_3$ Receptor—The pCDNA3-r$P2X_3$ plasmid was kindly provided by Prof. R. A. North (Sheffield University). The alignment of the amino acid sequences of $P2X_1$, $P2X_2$, and $P2X_3$ was deduced from NCBI accession numbers P47824 (r$P2X_1$), 2020424A (r$P2X_2$), and CAA62594 (r$P2X_3$) (2).

The non-conserved, negatively charged residues of the extracellular domain of the $P2X_3$ receptor were mutated to the neutral amino acid alanine (Fig. 1). Single point mutations were introduced using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Each mutated

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FIG. 1. **Amino acid sequence of the rat P2X$_3$ subtype of ATP receptor (rP2X$_3$; NCBI accession number: CAA62594).** The 15 negatively charged amino acids that were mutated into alanine are highlighted in *gray*. *Underlined* sequences indicate putative hydrophobic membrane-spanning regions.

plasmid P2X$_3$ DNA was obtained with a single PCR reaction using specifically designed mirror-image oligonucleotides containing the mutation of interest. "Sense" and "antisense" oligonucleotides (Roche Applied Science) used for the mutagenesis are listed in Table I. For all of the mutants, the introduction of the correct mutation and the absence of spontaneous mutations were confirmed by automated DNA sequencing.

Cell Culture and Transfection—HEK[1] 293T cells, supplied by the in-house SISSA cell bank, were maintained in culture in Dulbecco's modified Eagle's medium-Glutamax medium supplemented with 10% fetal calf serum and penicillin/streptomycin. For each transient transfection experiment, 5×10^{-5} cells were plated and transfected 24 h later with the calcium/phosphate method using 1 μg of high quality purified P2X$_3$ plasmid DNA (Sigma), either WT- or point-mutated. Transfected cells were used for further experiments 48 or 72 h later. Correct cell expression was confirmed with immunofluorescence and Western immunoblot assays as described previously (19).

Western Immunoblotting—Western immunoblots of transfected or untransfected HEK 293T cells were performed as recently reported (19). These cells were lysed using a buffer containing 100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 20% glycerol, and a mixture of protease inhibitors (Sigma) and separated on 10% polyacrylamide gel. After blocking with Tris-buffered saline containing milk, Tween 20, and preimmune serum, proteins were incubated overnight at 4 °C with an anti-P2X$_3$ antibody (1:2000, Neuromics, Milan, Italy). Immunocomplexes were incubated for 1 h with a peroxidase-conjugated secondary antibody (1:4000, Sigma) and detected with a chemiluminescence ECL kit (Amersham Biosciences). Negative controls were obtained by mock transfection of HEK 293T cells with the pEGFP-N1 plasmid (Clontech). Controls for efficient loading of different lysate materials were carried out by using an anti-β-actin antibody (mouse monoclonal, 1:2000, Sigma).

Electrophysiological Recording—Details of the recording protocols can be found in Sokolova *et al.* (19). HEK 293T cells were continuously superfused with control solution containing (in mM) the following: 152 NaCl; 5 KCl; 1 $MgCl_2$; 2 $CaCl_2$; 10 glucose; and 10 HEPES with pH adjusted to 7.4 with NaOH. Patch pipettes had a resistance of 3–4 megaohms when filled (in mM) with the following: 130 CsCl; 20 HEPES; 1 $MgCl_2$, 3 Mg_2ATP_3; and 5 EGTA with pH adjusted to 7.2 with CsOH. Cells were voltage-clamped at −60 mV. Currents were filtered at 1 kHz and acquired with pCLAMP 8.0 software (Axon Instruments, Foster City, CA).

Drugs and Their Application—α,β-Methyleneadenosine 5′-triphosphate (α,β-meATP; lithium salt) was used as a selective P2X$_3$ agonist to avoid activation of P2Y receptors natively expressed by HEK 293T cells (20). Unless otherwise stated, we used 100 μM α,β-meATP as a routine test concentration to evoke responses of maximal amplitude. All of the drugs were applied via a rapid superfusion system (Rapid Solution Changer RSC-200, BioLogic Science Instruments, Grenoble, France) placed 100 μm near the cell. Time for the solution exchange at the cell membrane level was ~30 ms. The α,β-meATP applications were 2-s long. For construction of dose-response plots, it was necessary to minimize receptor desensitization, which developed rapidly especially at high doses of agonist and prevented response reproducibility also in view of slow recovery. Thus, the agonist concentrations of ≥100 μM were applied sequentially every 6 min, whereas for lower concentrations, the applications were spaced at 2–5-min intervals.

All of the chemicals including enzymes for cell culture were from Sigma. Culture mediums were obtained from Invitrogen, and the antibody against P2X$_3$ was from Neuromics, whereas the secondary anti-rabbit antibody was from Sigma. G418 was from Invitrogen.

Data Analysis—All of the data are presented as the mean ± S.E. (n = number of cells) with statistical significance assessed with Student's t test (for parametric data) or Mann-Whitney rank sum test (for non-parametric data). The best fits of the data obtained with a sigmoid function (Origin software, version 6.0) were compared with respective control fits using SigmaStat (Jandel Scientific, version 2.0). A value of $p < 0.05$ was accepted as indicative of statistically significant difference. The fitting function for recovery from desensitization as a function of time was as reported earlier (19).

TABLE I
Oligonucleotides used for the mutagenesis reaction

Codons containing the mutation are underlined. In this table and in Tables II and III, amino acid numbers refer to the P2X receptor numbering.

D53A	5′-TAC CAA GTG AGG GCC ACC GCC ATT GAG-3′
E57A	5′-GAC ACC GCC ATT GCG TCC TCA GTA G-3′
E100A	5′-ATG ATC GTT ACT GCA AAT CAA ATG CAA GG-3′
E111A	5′-TGT CCA GAG AAT GCA GAG AAG TAC C-3′
E125A	5′-CAG TGT GGG CCT GCA CGC TTC CCA G-3′
E161A	5′-GTG GAC ACC GTG GCG ATG CCT ATC ATG-3′
E187A	5′-CTC TTC AAC TTT GCG AAG GGA AAC C-3′
D197A	5′-CCT AAC CTC ACC GCC AAG GAC ATA AAG AGG-3′
D199A	5′-CTC ACC GAC AAG GCC ATA AAG AGG TG-3′
E208A	5′-CGC TTC CAC CCT GCA AAG GCC CCA TTT TGC-3′
D220A	5′-TTG AGG GTA GGG GCT GTG GTT AAG TTT G-3′
D266A	5′-TTC ACT CGG CTG GCT GGA GTT TCT G-3′
E270A	5′-GGA GTT TCT GCG AAA AGC AGT GTT TCC-3′
E289A	5′-TAC TAT AAG ATG GCG AAC GGC AGC G-3′
E293A	5′-AAC GGC AGC GCG TAC CGC ACA CTC C-3′

RESULTS

Desensitization of Wild Type P2X$_3$ Receptors and Its Sensitivity to High Extracellular Ca^{2+}—The basic properties of activation and desensitization of native P2X$_3$ receptors expressed by HEK 293T cells are shown in Fig. 2*A*. The inward current induced by 100 μM α,β-meATP peaked and then fully decayed

[1] The abbreviations used are: HEK, human embryonic kidney; WT, wild type; α,β-meATP, α,β-methyleneadenosine 5′-triphosphate.

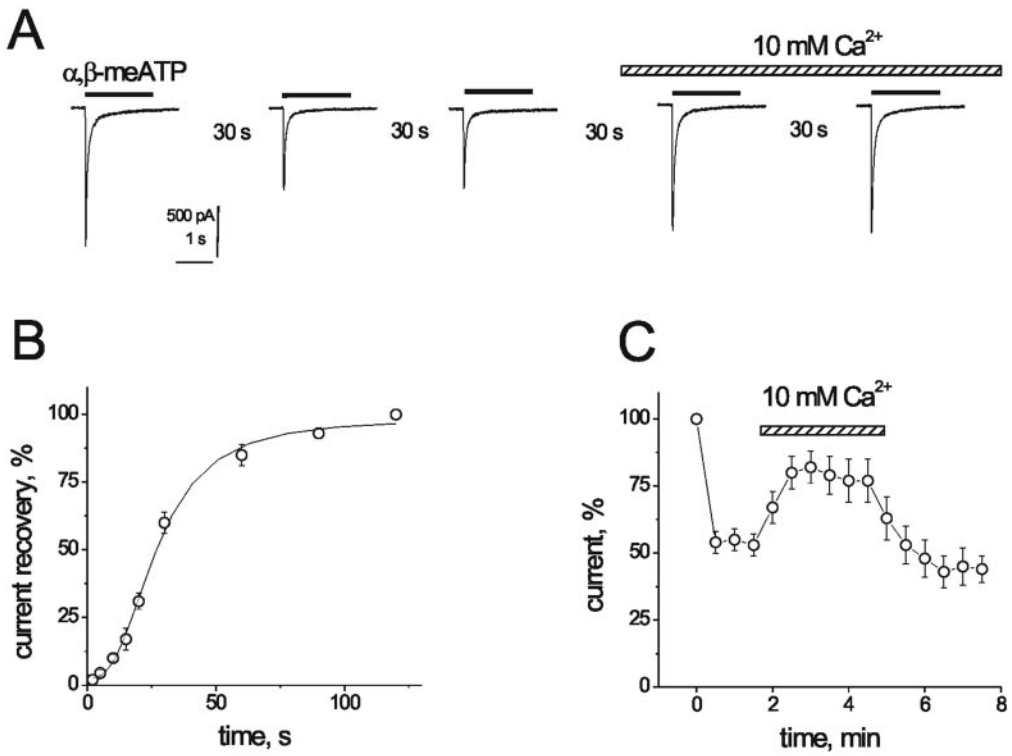

FIG. 2. **Extracellular Ca^{2+} transiently up-regulates WT $P2X_3$ receptors.** *A*, inward currents induced by 100 μM α,β-meATP decayed fully to base line during a 2-s application of this agonist and possessed smaller amplitude when the agonist application was spaced at 30-s intervals. High extracellular Ca^{2+} (10 mM) strongly potentiated current peaks almost to control level. *B*, to measure recovery from desensitization, the α,β-meATP current amplitude (as percentage of non-desensitized current peak) was plotted *versus* time of testing subsequent agonist application. Full recovery was attained after ~100 s (time for 50% recovery was 24 s). Data are from 6–11 cells. *C*, time course of the enhancing action of high extracellular Ca^{2+} on α,β-meATP currents tested at 30-s intervals. The action of Ca^{2+} was rapidly lost on a washout with control solution (n = 11).

TABLE II

Current amplitude and desensitization onset of single point mutants of P2X$_3$ receptors

τ_1 and τ_2 values refer to the first and second time constants of the biexponential decay of current to base line during a 2-s application of α,β -meATP (100 μM).

Mutant	Amplitude	τ_1	τ_2
	pA	*ms*	*ms*
Wild type P2X	1222 ± 178 (n = 43)	123 ± 9 (n = 21)	1346 ± 190 (n = 21)
D53A	660 ± 117 (n = 11)[a]	133 ± 18 (n = 11)	1395 ± 300 (n = 11)
E57A	681 ± 138 (n = 22)[a]	146 ± 10 (n = 15)	1016 ± 125 (n = 15)
E100A	935 ± 212 (n = 11)	139 ± 13 (n = 9)	1343 ± 231 (n = 10)
E111A	1014 ± 294 (n = 13)	133 ± 9 (n = 11)	1425 ± 417 (n = 13)
E124A	1092 ± 377 (n = 10)	119 ± 12 (n = 9)	1395 ± 350 (n = 10)
E161A	1106 ± 257 (n = 13)	126 ± 17 (n = 11)	1197 ± 245 (n = 13)
E187A	1139 ± 150 (n = 16)	113 ± 11 (n = 14)	1416 ± 209 (n = 15)
E197A	1351 ± 373 (n = 9)	111 ± 16 (n = 10)	739 ± 170 (n = 10)[a]
D199A	1680 ± 318 (n = 9)	135 ± 10 (n = 11)	1480 ± 148 (n = 11)
E208A	1048 ± 245 (n = 10)	145 ± 21 (n = 9)	950 ± 84 (n = 10)
D220A	110 ± 15 (n = 34)[a]	100 ± 9 (n = 12)	828 ± 164 (n = 14)
D266A	636 ± 122 (n = 19)[a]		1066 ± 96 (n = 17)
E270A	1233 ± 211 (n = 20)	127 ± 15 (n = 14)	1416 ± 225 (n = 18)
E289A	1165 ± 126 (n = 15)	115 ± 16 (n = 15)	1039 ± 160 (n = 15)
E293A	1478 ± 87 (n = 16)	117 ± 13 (n = 12)	1031 ± 217 (n = 12)

[a] $p < 0.05$.

with biexponential time course to base line (average data of current amplitude and decay are in Table II), indicating full desensitization. Response recovery was complete after a 6-min washout and partial (60 ± 4%; n = 21) when agonist applications were spaced at 30-s intervals. In this case, the protocol of applying agonist pulses at varying intervals after the desensitizing response enabled quantification of recovery from desensitization, which showed the typical sigmoidal time course (Fig. 2*B*) previously observed with native P2X$_3$ receptors (19). Membrane currents evoked when P2X$_3$ receptors were desensitized could be readily enhanced by a high extracellular Ca^{2+} solution (50 ± 6%; n = 11, Fig. 2*A*) in a prompt and persistent fashion (Fig. 2*C*). Hence, the P2X$_3$ receptors expressed by HEK 293T cells appeared to display all of the main properties (including sensitivity to high extracellular Ca^{2+}) of native receptors of dorsal root ganglion nociceptors (3, 4).

Effect of Single Amino Acid Mutations on the Amplitude of P2X$_3$ Receptor Currents and Their Desensitization—We examined the extracellular loop for negatively charged sites potentially involved in mediating the action of Ca^{2+} and Mg^{2+} (3, 4) and identified 15 non-conserved amino acids (Fig. 1), which were subjected to mutation. Table II compares the functional properties of the WT and mutated P2X$_3$ receptors following the application of 100 μM α,β-meATP. Although the majority of

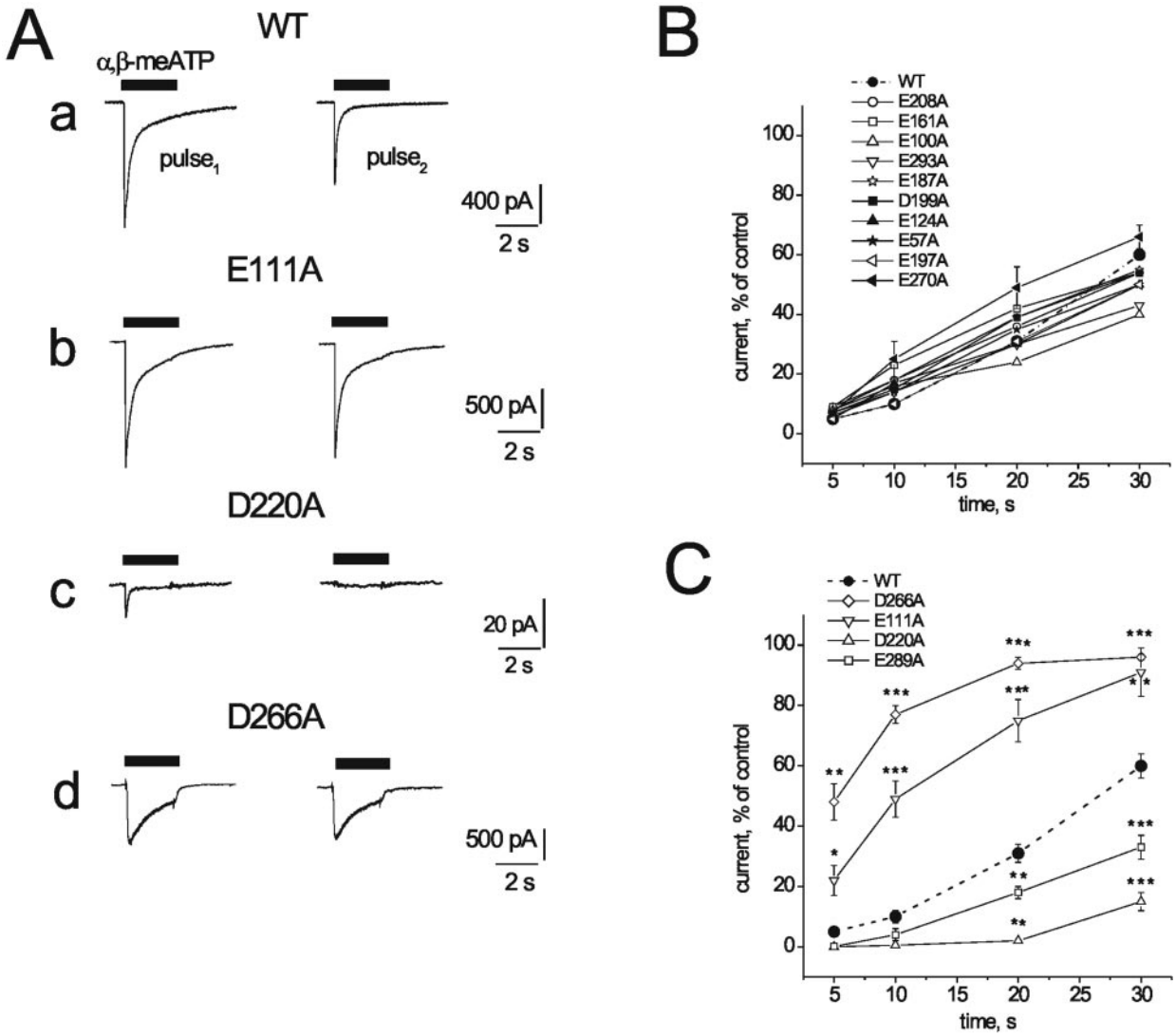

FIG. 3. **Differential recovery from desensitization of mutated P2X$_3$ receptors.** *A*, *a–d*, examples of currents recorded from the activation of WT, E111A, D220A, or D266A P2X$_3$ receptors, respectively. All of the responses were generated by 100 μM α,β-meATP at 30-s intervals (pulse$_1$ and pulse$_2$). Note different current amplitude and kinetics for D220A and D266A (dissimilar current calibrations are indicated alongside records). *B*, plots of recovery from desensitization (for details see Fig. 2*B*) for a range of mutated P2X$_3$ receptors. This data group comprised mutants with recovery not significantly different from that of WT (n = 4–8). *C*, plots of recovery from desensitization to include mutants with a significantly faster (D266A, E111A) or slower (D220A and E289A) recovery time course than that of WT (n = 4–12; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

mutants generated current amplitudes very similar to the WT (Table II), the responses recorded from mutants D53A, E57A, D220A, and D266A had a significantly smaller amplitude. Examples of control currents (pulse$_1$) recorded from mutants E111A, D220A, and D266A are given in Fig. 3*A*, *b–d*. Although the E111A response was closely similar to that of WT, D220A generated responses of very limited size (note larger current calibration) but with a shape analogous to WT. Conversely, the D266A response was considerably slower because it produced currents with monoexponential decay, suggesting that, in this case, the onset of receptor desensitization was largely impaired. All of the other mutants had $\tau_{1,2}$ values close to those of WT (see Table II), indicating that, in those cases, receptor desensitization developed unabatedly.

Mutated Receptors Display Different Recovery from Desensitization—Mutants could be classified into three groups as far as recovery from desensitization was concerned. Fig. 3*A* shows examples of current recovery (compare pulse$_2$ records taken 30 s after the corresponding pulse$_1$ traces) after α,β-meATP-induced desensitization. The first group (Fig. 3*B*) comprised 10 mutants (of 15) with recovery time not significantly different from the WT recovery. The second group (Fig. 3*C*) showed significantly faster recovery and included E111A (with onset of desensitization similar to the WT P2X$_3$; see also Fig. 3*Ab*) and D266A (with very slow onset of desensitization; Fig. 3*Ad*). The third group comprised mutants with much slower recovery (Fig. 3*C*), namely D220A (Fig. 3*Ac*) and E289A, both of them with onset characteristics close to WT.

In summary, by single point mutations of certain non-conserved, negatively charged amino acids in the extracellular loop of P2X$_3$ receptor, we observed that Glu-111, Asp-220, Asp-266, and Glu-289 differentially contributed to the process of recovery from desensitization.

Effect of Single Point Mutations on the Ability by Extracellular Ca^{2+} to Facilitate Recovery from Desensitization—Using the same protocol shown in Fig. 2*A*, we investigated how single point mutations of the extracellular loop of P2X$_3$ receptors might affect the effectiveness of high extracellular Ca^{2+} to facilitate responses to α,β-meATP. On WT receptors activated by repeated applications of α,β-meATP to produce stable, low amplitude currents, the application of 10 mM Ca^{2+} potentiated (~150%) the agonist-induced current with respect to the one in

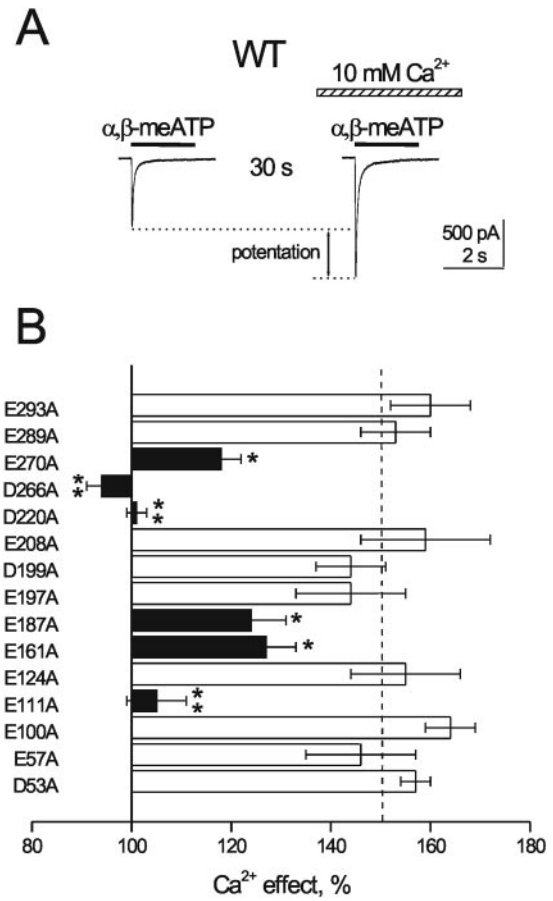

FIG. 4. **Differential effectiveness of high extracellular Ca^{2+} on currents from mutated P2X$_3$ receptors.** All of the responses were evoked by 100 μM α,β-meATP applied every 30 s. *A*, examples of WT P2X$_3$ receptor currents to show how the potentiating action of Ca^{2+} was calculated. *B*, histograms indicating the extent of Ca^{2+} potentiation for 15 mutants. *Dotted line* shows the standard potentiation by Ca^{2+} on WT receptors. *Open bars* indicate data not significantly different from WT. *Filled bars* show effects significantly smaller than the effects on WT (n = 6–13; *, $p < 0.05$; **, $p < 0.01$).

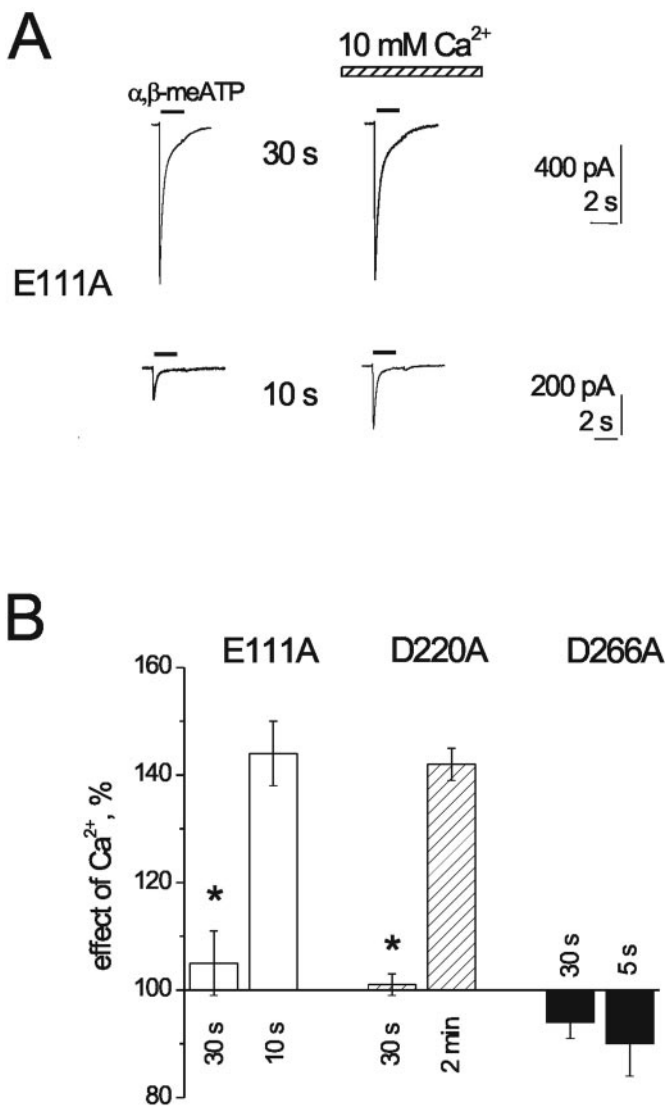

FIG. 5. **Changes in agonist application timing can restore the effect of high Ca^{2+}.** *A*, on E111A P2X$_3$ receptors, Ca^{2+} was ineffective when α,β-meATP was applied at a 30-s interval (*top*); however, on the same cell, Ca^{2+} potentiation could be observed when the agonist was applied every 10 s with consequently smaller amplitude of P2X$_3$ receptor-mediated current. *B*, histograms summarizing data for mutants tested with the same concentration of α,β-meATP at different intervals. In the case of E111A, the enhancing action of Ca^{2+} was similar to the WT for the 10-s interval. For D220A, the facilitation by Ca^{2+} could be restored when the agonist was applied every 2 min. However, for D266A, neither a 30- nor 5-s interval manifested any enhancing action by Ca^{2+} (n = 3–5; *, $p < 0.05$).

standard saline solution as exemplified in Fig. 4*A*.

Most mutants displayed the same degree of Ca^{2+} modulation as the WT P2X$_3$ (see *open bars* in Fig. 4*B*). However, six mutants did not (Fig. 4*B*, *filled bars*). In particular, E161A, E187A, and E270A generated responses significantly less sensitive to high Ca^{2+} (despite the fact that their recovery from desensitization was similar to that of WT). Even more strikingly, D266A, D220A, and E111A completely lost sensitivity to high Ca^{2+}. In the latter case, we wondered whether the lack of sensitivity to high Ca^{2+} was related to differential recovery from desensitization (see Fig. 3*C*). To this end, we applied α,β-meATP at different intervals because, for each mutant, the response amplitude became ~50% of the control (obtained at a 6-min interval) as in the case of WT receptors.

On E111A, 10 mM Ca^{2+} produced clear potentiation when responses were evoked by applying α,β-meATP every 10 s instead of every 30 s (Fig. 5*A*). The same approach was used for D220A receptors tested at 120-s intervals and D266A receptors tested at 5-s intervals. These results summarized in the histograms of Fig. 5*B* indicate that currents generated by E111A and D220A could then display strong Ca^{2+}-dependent potentiation, whereas those produced by D266A remained Ca^{2+}-insensitive.

These data show that Glu-161, Glu-187, and Glu-270 contributed to the facilitating action by Ca^{2+}. For E111A, D220A, and D266A, their low sensitivity to Ca^{2+} seemed to be secondary to primary changes in receptor desensitization properties.

Potency and Efficacy of the Agonists on Mutants with Changed Ca^{2+} Sensitivity—To explore this issue, we first constructed an α,β-meATP dose-response relation for WT P2X$_3$ receptors, which provided EC_{50} (1.4 ± 0.4 μM; n = 6) and n_H (1.2) values very similar to those of native receptors of rat dorsal root ganglion neurons (19). Fig. 6*A* shows that E111A, E161A, E187A, and E270A produced maximal current amplitudes not significantly different from WT. Although the EC_{50} values of these four mutants were relatively close to those of WT, they all remained significantly larger but with similar n_H values (Table III). On the other hand, Fig. 6*B* shows that D266A and D220A had lower potency and efficacy than WT (see Tables II and III). The reduced ability of D266A and D220A to respond to α,β-meATP could not be overcome even by applying a 10-fold larger agonist concentration (1 mM), suggesting that such mutations probably impaired channel gating rather than

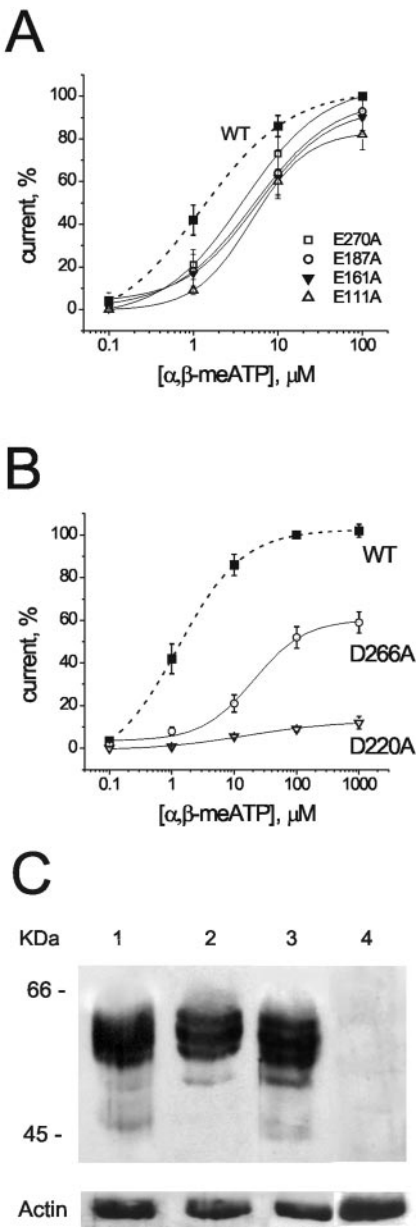

FIG. 6. **Dose-response plots for α,β-meATP applied to WT or mutated P2X$_3$ receptors.** *A*, WT plot is shown with a *dotted line*. Mutated receptors (E111A, E161A, E187A, and E270A) generated responses with maximal amplitude similar to WT, although their plots are slightly displaced to the *right* (corresponding EC_{50} values are in Table III). *B*, D220A and D266A P2X$_3$ receptors generated responses of considerably smaller amplitude than WT, and their plots are compressed rightwards (n = 5–11). In *A* and *B*, all of the responses were normalized with respect to the one generated by 100 μM α,β-meATP on WT receptors. *C*, Western immunoblot analysis of HEK 293T-transfected cells. WT (*lane 1*), D220A (*lane 2*), and D266A (*lane 3*) P2X$_3$ proteins demonstrated the same expression pattern, namely bands from 55 to 60 kDa in accordance with previous reports (25, 26). Mock-transfected cells (*lane 4*) gave a negative result. Equal amounts (20 μg of the total lysate) of protein were loaded into each gel lane. Actin (stained with anti-actin antibody) was used to validate correct loading.

agonist binding (see comparable data with P2X$_2$ receptors) (21). Finally, we tested E293A because this mutant had standard desensitization properties and sensitivity to Ca^{2+} (Table II and Fig. 4*B*). Also in this case, the EC_{50} value was slightly but significantly larger than that for the WT (Table III).

Fig. 6*C* shows an example of Western blot analysis of WT, D220A, and D266A P2X$_3$ receptor expression. It is apparent that, in each case, there was the comparable pattern of P2X$_3$ receptor protein, indicating that the large disparity in agonist sensitivity could not be simply due to insufficient receptor expression. Similar levels of P2X$_3$ receptor expression were also detected for all of the other mutants (data not shown).

TABLE III
Calculated EC_{50} and n_H values for WT and mutated P2X$_3$ receptors

Mutant	EC_{50}	n_H
	μM	
WT	1.4 ± 0.5	1.1 ± 0.2
E111A	5.9 ± 1.2[a]	1.7 ± 0.5
E161A	5.4 ± 1.3[a]	1.7 ± 0.4
E187A	5.3 ± 1.2[a]	1.0 ± 0.3
D220A	7.2 ± 1.2[a]	1.2 ± 0.3
D266A	28 ± 2.2[a]	1.5 ± 0.4
E270A	5.3 ± 1.5[a]	1.3 ± 0.1
E293A	4.5 ± 1.7[a]	1.1 ± 0.1

[a] $p < 0.05$.

DISCUSSION

The principal finding of this study is the identification of non-conserved, negatively charged residues in the extracellular loop of P2X$_3$ receptors involved in sensing Ca^{2+}. Furthermore, we observed distinct ectodomain residues responsible for desensitization development or for recovery from it. Thus, the present data might suggest new targets for novel analgesic strategies based on discrete regulation of P2X$_3$ receptor states.

Single Point Mutations of the P2X$_3$ Receptor Strongly Affect Its Ca^{2+} Sensitivity—The strong and rapid desensitization of P2X$_3$ receptors followed by rather slow recovery (2, 3) is an important process to regulate the activity of such receptors. Because extracellular Ca^{2+} can powerfully facilitate recovery of receptor function whenever the receptor is desensitized (3), understanding the mechanisms underlying this phenomenon is a major issue, especially because extracellular Ca^{2+} concentrations can rapidly fluctuate during intense neuronal activity (22) and thus modulate P2X$_3$ receptor function. Although previous data suggested the action of Ca^{2+} to be on the extracellular loop of P2X$_3$ receptors (3, 4), supportive evidence was only indirect. By focusing on non-conserved negatively charged residues that should be potential sites for Ca^{2+} binding (6), the present data indicated Glu-161, Glu-187, and Glu-270 to be important for the action of Ca^{2+}. Because the effect of Ca^{2+} on these mutated receptors was significantly reduced but not abolished, it is probable that such residues acted in concert to fully express the action of Ca^{2+}. Furthermore, because E161A, E187A, and E270A mutants possessed the onset of desensitization and recovery similar to the WT P2X$_3$, this observation suggests that the molecular sites involved in the action of extracellular Ca^{2+} were structurally and operationally distinct from those controlling desensitization.

Altered Desensitization Properties Can Mask the Action of Ca^{2+}—Another cause for impaired Ca^{2+} action was found when testing E111A, D220A, and D266A mutants with standard protocols of desensitization. However, this result was simply the result of the mutant altered properties of desensitization onset and recovery. For instance, E111A displayed very rapid recovery from desensitization, thus depriving Ca^{2+} of its receptor state target when the normal experimental protocol was used. Applying the agonist at a faster rate to make this process proportionally similar to the WT promptly restored the facilitatory action of Ca^{2+}. Conversely, on D220A, spacing agonist applications at much longer intervals than in the standard protocol reinstated the facilitatory action by extracellular Ca^{2+}. Hence, such experiments suggested that neither Glu-111 nor Asp-220 was crucial for the effect of Ca^{2+}, which, to be fully expressed, required an intermediate level of desensitization to be manifested.

A special case was observed with the D266A mutant (with a rather slow onset of desensitization and recovery), because extracellular Ca^{2+} could not improve the recovery of D266A, even after changing the extent of the desensitization state.

In conclusion, the finding that slow onset or very fast recovery was accompanied by reduced sensitivity to Ca^{2+} was consistent with data showing that non-desensitized P2X$_3$ receptors are almost insensitive to Ca^{2+} (3, 4).

Structural Determinants of Desensitization Onset—Studies of chimeras of P2X$_2$/P2X$_3$ and P2X$_1$/P2X$_2$ receptors have indicated that the onset of desensitization is controlled by a concerted interaction between transmembrane and intracellular domains (10, 15–17, 23). Whereas recent investigations using chimeras demonstrated the role of the ectodomain N-region to stabilize the desensitized receptor conformation (18), this study showed that a single negative residue (Asp-266) was important to control the desensitizing properties of the P2X$_3$ receptor because its mutation conferred unusually slow onset and rapid recovery. Thus, it seems probable that the multiple regions of P2X$_3$ receptors are involved in shaping development of desensitization.

In accordance with recent data on P2X$_4$ receptors (24), this report indicated that a single mutation in the extracellular domain could control agonist potency, which was reduced in the tested mutants, a phenomenon not attributable to insufficient receptor protein expression. However, decreased receptor sensitivity was not necessarily intertwined with the faster development of receptor desensitization because it was possible to observe very slow desensitization together with reduced agonist potency (*e.g.* D266A), normal desensitization with reduced agonist potency (see Table III), and rather weak efficacy coupled with standard desensitization (D220A).

All together, these results suggested a complex interplay between the agonist binding sites (and activated channels) and the extracellular and intracellular domains controlling the onset of desensitization.

Structural Determinants of Recovery from Desensitization—Our previous work with distinct P2X$_3$ agonists (19) suggested the onset of desensitization and recovery to be governed by independent mechanisms since agonists with identical receptor activation and onset of desensitization generated different rates of recovery. Desensitization recovery in chimeric P2X$_2$/P2X$_3$ receptors is controlled by the extracellular domain (18, 23), indicating a special protein structure regulating this process. Consistent with this notion, we observed that mutations in the extracellular loop, which left the onset of desensitization unaltered with respect to WT, displayed either faster (E111A) or slower (E289A and D220A) recovery from desensitization.

Since our previous work has suggested that desensitization is a multi-step process involving several receptor conformational states (19), it seems plausible to hypothesize that the ability to generate (or exit from) discrete conformations is governed by distinct molecular determinants within the P2X$_3$ receptor protein.

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Development/Plasticity/Repair

Delayed Upregulation of ATP $P2X_3$ Receptors of Trigeminal Sensory Neurons by Calcitonin Gene-Related Peptide

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Recent evidence indicates a key role for the neuropeptide calcitonin gene-related peptide (CGRP) in migraine pain, as demonstrated by the strong analgesic action of CGRP receptor antagonists, although the mechanisms of this effect remain unclear. Most trigeminal nociceptive neurons releasing CGRP also express ATP-activated purinergic $P2X_3$ receptors to transduce pain. To understand whether the CGRP action involves $P2X_3$ receptor modulation, the model of trigeminal nociceptive neurons in culture was used to examine the long-term action of this peptide. Although 79% of CGRP-binding neurons expressed $P2X_3$ receptors, acute application of CGRP did not change $P2X_3$ receptor function. Nevertheless, after 1 h of CGRP treatment, strong enhancement of the amplitude of $P2X_3$ receptor currents was observed together with accelerated recovery from desensitization. Receptor upregulation persisted up to 10 h (despite CGRP washout), was accompanied by increased *$P2X_3$* gene transcription, and was fully prevented by the CGRP antagonist $CGRP_{8-37}$. Surface biotinylation showed CGRP augmented $P2X_3$ receptor expression, consistent with confocal microscopy data indicating enhanced $P2X_3$ immunoreactivity beneath the neuronal membrane. These results suggest that CGRP stimulated trafficking of $P2X_3$ receptors to the cell-surface membrane. Using pharmacological tools, we demonstrated that this effect of CGRP was dependent on protein kinase A and PKC activation and was prevented by the trafficking inhibitor brefeldin A. Capsaicin-sensitive TRPV1 vanilloid receptors were not upregulated. The present data demonstrate a new form of selective, slow upregulation of nociceptive $P2X_3$ receptors on trigeminal neurons by CGRP. This mechanism might contribute to pain sensitization and represents a model of neuronal plasticity in response to a migraine mediator.

Key words: **plasticity; pain; purinergic receptor; nociception; neuropeptide; receptor trafficking**

Introduction

Calcitonin gene-related peptide (CGRP), a potent vasodilator and pro-inflammatory agent, is contained in vesicles of trigeminal nerve endings (Arulmani et al., 2004). Accumulating evidence suggests that CGRP has a key role in migraine, one of the commonest neurological disorders (Pietrobon and Striessnig, 2003; Goadsby, 2005). Although the CGRP plasma concentration closely correlates with the time course and severity of migraine (Sarchielli et al., 2000; Juhasz et al., 2005), the crucial role of this peptide is supported by the efficacy of CGRP antagonists for the treatment of migraine pain (Olesen et al., 2004; Edvinsson, 2005; Rudolf et al., 2005). Because CGRP does not have acute effects on the excitability of meningeal nociceptors (Levy et al., 2005), it is, however, difficult to understand how CGRP could induce persistent headache mediated by trigeminal ganglion (TG) neurons. This issue might be addressed by studying a model system that allows investigating structural and functional properties of TG neurons over time.

Because CGRP operates via G-protein-coupled receptors, it seems feasible to look for its downstream effectors among the pain-transducing receptors of TG neurons. On nociceptive sensory neurons, extracellular ATP is one of the main algogenic transmitters (Chizh and Illes, 2001; North, 2003) acting on ionotropic receptors containing the purinergic $P2X_3$ subunit (Cockayne et al., 2000; Souslova et al., 2000). $P2X_3$ receptors show rapid desensitization (North, 2003; Sokolova et al., 2004) controlled by intracellular messengers (Koshimizu et al., 1999). Therefore, $P2X_3$ receptors and their desensitization properties appear to be one potential target for expressing the algogenic action of CGRP. In support of this hypothesis, other neuropeptides such as substance P and bradykinin facilitate $P2X_3$ receptor signaling by speeding up recovery from desensitization, although with a faster time course (Paukert et al., 2001). Nevertheless, sensory neurons also use other pain-transducing receptors, such as TRPV1 (vanilloid receptor), that are activated by a wide range of stimuli [acidity, pressure, chemical irritants such as capsaicin, heat, etc. (Julius and Basbaum, 2001; Wang and Woolf, 2005)]. Using, as a model, cultures of mouse TG neurons, we demonstrated that sustained application of CGRP selectively potentiated $P2X_3$ receptor function, while leaving TRPV1 receptors unaffected.

Materials and Methods

Cultured TG neurons. Primary cultures of TG or thoraco-lumbar dorsal root ganglion (DRG) neurons were obtained from postnatal day 10–12

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C57-Black/6Jico mice. Animals were anesthetized by diethyl ether and decapitated (in accordance with the Italian Animal Welfare Act and approved by the Local Authority Veterinary Service). Ganglia were isolated and dissociated for 10–20 min at 37°C in a solution containing 0.25 mg/ml trypsin, 1 mg/ml collagenase, and 0.2 mg/ml DNase (Sigma, St. Louis, MO) in F-12 medium (Invitrogen, San Diego, CA). Cells were used 24 h after plating.

Unless indicated otherwise, neurons were incubated with 1 μM CGRP for 1 h at 37°C. HPLC analysis demonstrated CGRP (dissolved in physiological solution) to be stable for at least 5 h at 37°C (Dr. O. Jahraus, personal communication). This observation is consistent with the reported stability (90%) of CGRP when incubated for 5 h at 37°C (Ichikawa et al., 2000). The following drugs were preapplied for 30 min and coapplied together with CGRP for 1 h at 37°C: the CGRP receptor antagonist $CGRP_{8-37}$ (2 μM), actinomycin D (5 μg ml^{-1}), the protein kinase A (PKA) inhibitor fragment 14-22 (3 μM), chelerythrine chloride (5 μM), brefeldin A (5 μg ml^{-1}), forskolin (1 μM), and phorbol 12-myristate 13-acetate (PMA; 325 nM) (all from Sigma).

Patch-clamp recording. After 1 d in culture, cells were superfused continuously (2 ml min^{-1}) with physiological solution containing (in mM) 152 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES (pH adjusted to 7.4 with NaOH). Single cells were patch clamped in the whole-cell configuration by means of an L/M-EPC 7B patch-clamp amplifier (List Medical, Darmstadt, Germany) using pipettes with a resistance of 3–4 MΩ when filled with (in mM) the following: 140 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 2 Mg_2ATP_3, 2 GTP, 10 HEPES, and 10 EGTA (pH adjusted to 7.2 with KOH). TG or DRG cells were voltage clamped at −60 mV (series resistance compensation, 70%). Currents were filtered at 1 kHz and acquired by means of a DigiData 1200 Interface and the pClamp 8.2 software (Molecular Devices, Sunnyvale, CA). To assess $P2X_3$ receptor function, the potent synthetic agonist α,β-methylene-adenosine-5′-triphosphate (α,β-meATP) and the natural agonist ATP were applied with a fast superfusion system (Rapid Solution Changer RSC-200; BioLogic Science Instruments, Claix, France); the time for solution exchange was ~30 ms. The rapid desensitization of the current during agonist application together with its block by the selective antagonist A-317491 (5-[[[(3-phenoxyphenyl)methyl][(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt) (1 μM; 95% inhibition; $n = 13$) (Jarvis, 2003) indicated its origin as a $P2X_3$ receptor-mediated response. In a few experiments, α,β-meATP (200 μM; 10 ms pulse) was applied via a puffer pipette close to the recorded cell. Responses were measured in terms of peak amplitude and fitted with a logistic equation (Origin 6.0; Microcal, Northampton, MA) to express agonist potency in terms of EC_{50} values (concentration producing 50% of the maximum response). For standard tests of cell responsiveness, agonist applications (10 μM, 2 s; fast superfusion) were spaced at 5 min intervals to obtain full response recovery from desensitization (see Results). Paired-pulse experiments with α,β-meATP applications over shorter intervals were used to measure recovery from desensitization (Sokolova et al., 2004). The peak of α,β-meATP currents generated by the second pulse was expressed as the percentage of the peak amplitude of the control response so as to express recovery from desensitization as the time needed to regain 50% of the control peak amplitude ($t_{1/2}$). To quantify the effect of a certain drug on the α,β-meATP-induced current amplitude for each drug-treated neuron, the peak response was expressed as a percentage of the mean peak current amplitude obtained from control neurons from sister dishes used in parallel. Capsaicin was applied at the standard test dose of 1 μM (2 s) to evoke reproducible inward currents. Recording of functional responses started just after the washout of the CGRP-containing medium and continued for 1–1.5 h (if not indicated otherwise).

Calcium imaging. Cells were incubated for 40 min at 20–22°C in physiological solution containing Fluo3-AM (5 μM; Invitrogen, Eugene, OR), followed by a 30 min washout period. Fluorescence emission was acquired with a CCD camera (Coolsnap HQ; Roper Scientific, Duluth, GA) at 150 ms intervals. Data were collected from cells that produced a rapid response to a pulse of KCl (50 mM, 1 s), thus indicating they were neurons. Images were analyzed with the Metafluor software (Metafluor Imaging Series 6.0; Universal Imaging, Downingtown, PA). Intracellular Ca^{2+} transients were expressed as fractional amplitude increase ($\Delta F/F_0$, where F_0 is the baseline fluorescence level and ΔF is the increment over baseline).

Real-time reverse transcription-PCR. Total mRNA was extracted from TG cultures using Trizol reagent (Invitrogen). After DNase treatment (Ambion, Austin, TX), 1 μg of total RNA was retro-transcribed using SuperScript III (Invitrogen) with a mixture of oligo-dT and random primers (Invitrogen). cDNAs (30 ng) were amplified with JumpStart *Taq* ReadyMix (Invitrogen), specific oligonucleotide primers, and TaqMan fluorogenic probes (Applied Biosystems, Foster City, CA) in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Prevalidated assays, specific for amplification of mouse $P2X_3$, β-tubulin III, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Applied Biosystems code numbers: Mm00523699_m1, Mm00727586_s1, and 4352339E FG m MGB) were used. The end-point PCR amplicons (analyzed on an agarose gel) had the expected length. Initial calibration of the samples gave similar amplification of the GAPDH housekeeping mRNA, and all assays were validated for linearity of amplification efficiency. Negative controls containing no template cDNA were run in each condition and gave no results. The relative mRNA expression of $P2X_3$ in the different samples was normalized to the neuronal β-tubulin III mRNA content. Absolute calculations for relative mRNA transcript levels were performed using the comparative method between cycle thresholds of different reactions (Livak and Schmittgen, 2001).

Membrane biotinylation and Western immunoblot. Total protein lysates of TG cultures were extracted 2 or 5 h after 1 h CGRP treatment. For the procedure of membrane protein biotinylation, intact TG neurons were incubated with 1 mg ml^{-1} Sulfo-NHS-Biotin (Pierce, Rockford, IL) for 30 min at 4°C. After quenching with 10 mM Tris-HCl, pH 7.5, cells were lysated for 30 min on ice in 40 μl of a buffer containing10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM EDTA, and 1% Triton X-100 plus protease inhibitors (Roche Products, Welwyn Garden City, UK). Pull-down of biotinylated proteins was obtained with ImmunoPure Immobilized Streptavidin beads (Pierce) for 2 h at 4°C. The beads were washed three times with radioimmunoprecipitation assay buffer and eluted with SDS-PAGE sample buffer (Invitrogen). Sample were separated on Nu-PAGE Novex 4–12% Bis-Tris gel (Invitrogen) and processed for Western immunoblot using antibodies against the $P2X_3$ receptor (dilution 1:2000; Neuromics, Edina, MN), the TRPV1 receptor (dilution 1:1000; Alomone Laboratories, Jerusalem, Israel) or β-tubulin III (dilution 1:200; Chemicon, Temecula, CA). As secondary antibodies, specific HRP-conjugated antibodies were used and signals were detected with the enhanced chemiluminescence light system ECL (Amersham Biosciences, Piscataway, NJ). Biotinylation experiments resulted free of intracellular protein contaminants. For control of correct gel loading in biotinylation assays, we checked the β-tubulin III expression in the intracellular fraction. To quantify Western blot signals, band density was measured using CorelDraw Photopaint software (Corel, Ottawa, Ontario, Canada) and normalized with respect to the control.

Fluorescent markers. For immunofluorescent staining, paraformaldehyde-fixed TG neurons were processed with antibodies against the $P2X_3$ or the TRPV1 receptor (dilution 1:200; Alomone Laboratories) and the neuron-specific β-tubulin III (dilution 1:100; Chemicon). Immunofluorescence reactions were visualized using secondary antibodies AlexaFluor 488 or AlexaFluor 594 (dilution 1:500; Invitrogen). Confocal microscopy was performed by using a Zeiss (Thornwood, NY) LSM 510 microscope equipped with an argon–helium double laser, and images were analyzed and quantified with its dedicated software. Cells stained with the secondary antibody only showed no immunostaining. To visualize neurons sensitive to CGRP with standard fluorescence microscopy, we used live TG or DRG neurons incubated with 0.5 μM CGRP directly conjugated with rhodamine (CGRP-RITC; Phoenix, Belmont, CA) for 1 h at 4°C (Cottrell et al., 2005). Cells were then fixed and processed for indirect immunofluorescence for $P2X_3$, TRPV1, or β-tubulin III proteins. An average of 500 cells were analyzed in each test, and data are the mean of three independent experiments.

Competition experiments (run in duplicate) with excess, unlabeled CGRP (10 μM; 15 min preincubation at 37°C) showed a minimal (<5% of control) fluorescence signal by subsequent application of CGRP-RITC

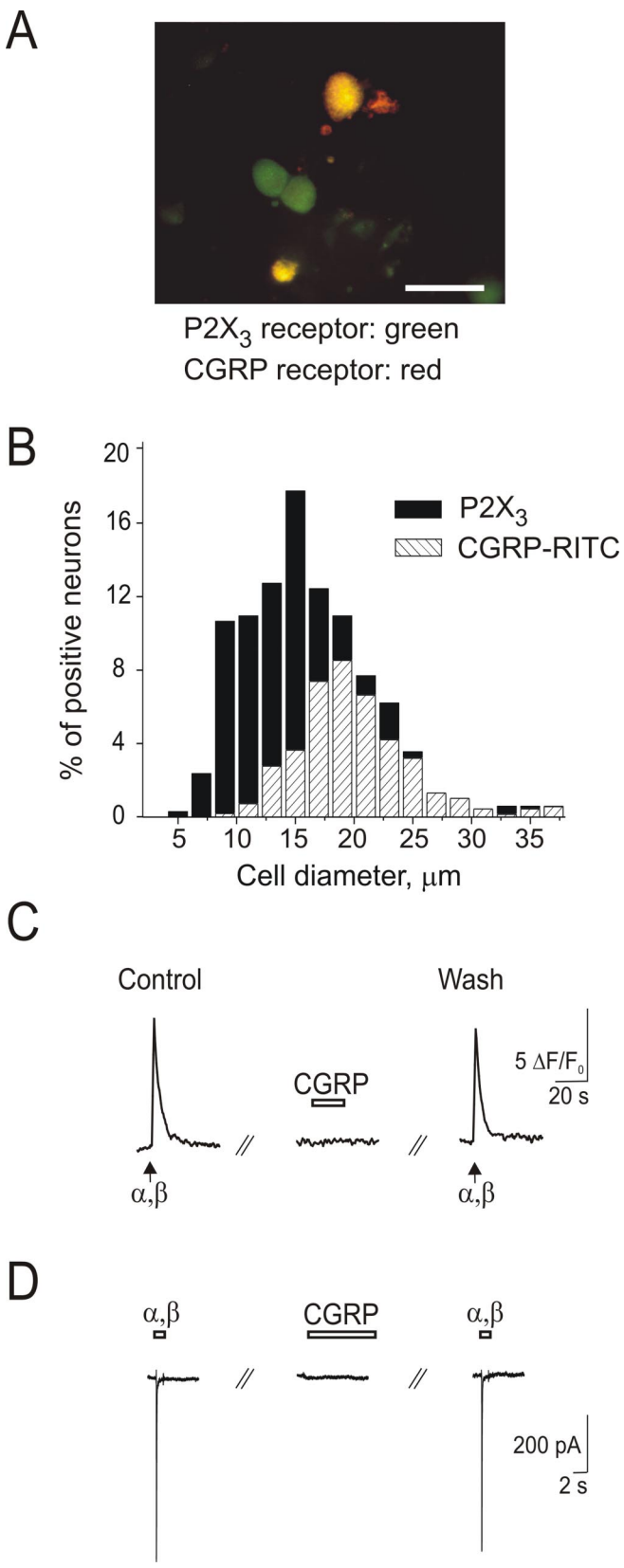

Figure 1. CGRP binds to $P2X_3$-immunopositive TG neurons without acute changes in membrane current, intracellular Ca^{2+} level, or $P2X_3$ receptor-mediated responses. ***A***, Microphotograph depicts TG neurons labeled with rhodamine-conjugated CGRP (0.5 μM; red) and $P2X_3$ receptor antibody (green). Scale bar, 50 μm. ***B***, Somatic size distribution of TG cells immunostained with $P2X_3$ receptor antibody (■) and labeled with CGRP-RITC (▨). Data are from ~1500 cells (3 independent experiments). ***C***, Example of 20 s application of CGRP (1 μM; open bar) that has no effect on fast responses induced by α,β-meATP pulses (10 μM, 2 s; arrows) recorded as Ca^{2+} transients from a single neuron loaded with Fluo-3 AM. ***D***, Example of lack of effect by CGRP (0.5 μM) applied for 6 min on the peak current induced by α,β-meATP (different cell from ***C***). α,β, α,β-meATP.

(0.5 μM). These results were analyzed with the ImagePro Express software (Media Cybernetics, Silver Spring, MD).

Data analysis. In each experiment, sister dishes of TG cultures were used to compare control neurons with neurons treated with CGRP or other drugs. Data are expressed as mean ± SEM. Statistical analysis was performed using the Student's *t* test, the Mann–Whitney rank sum test, or the ANOVA test (using the KyPlot software, version 2.0; Qualest). A p value of <0.05 was accepted as indicative of a statistically significant difference.

Results

Does CGRP induce rapid responses of trigeminal neurons?

Figure 1*A* shows an example of colocalization of CGRP binding and $P2X_3$ immunoreactivity on TG cells in culture. The histograms of Figure 1*B* demonstrate the cell diameter distribution according to these two markers with overlap at the ~20 μm value. On average, 79 ± 3% ($n = 3$ experiments) of CGRP-positive cells also expressed $P2X_3$ receptors. Double fluorescence experiments with the neuronal marker β-tubulin III and CGRP-RITC confirmed that CGRP was bound by neurons (supplemental Fig. S1*A*,*B*, available at www.jneurosci.org as supplemental material) mainly of 15–25 μm somatic diameter.

To study the acute effects of CGRP on TG neurons in culture, we first investigated the action of this neuropeptide on the level of intracellular Ca^{2+}. CGRP (0.5–1 μM) applied for 20–60 s did not induce Ca^{2+} signals in the majority of neurons (134 of 146 cells). In a subset of neurons (12 of 146), CGRP induced small Ca^{2+} transients (~5% amplitude of those induced by 50 mM KCl). Figure 1*C* shows that the acute application of CGRP (1 μM, 20 s) neither induced Ca^{2+} response nor changed Ca^{2+} transients evoked by the $P2X_3$ receptor agonist α,β-meATP (10 μM). Even longer (6 min) application of CGRP had no effect on α,β-meATP-induced Ca^{2+} responses (84 ± 8% of control; $n = 9$; $p = 0.12$).

Likewise, under patch-clamp conditions, α,β-meATP-induced membrane currents were not significantly changed (85 ± 4% of control; $n = 4$; $p = 0.11$) (Fig. 1*D*) by CGRP. Furthermore, there was no change in the rate of recovery of $P2X_3$ receptors from desensitization tested at 30 s interval with paired pulses of α,β-meATP (11.3 ± 0.5% recovery in the control condition vs 9.3 ± 0.9% recovery after 6 min exposure to 0.5 μM CGRP; $n = 4$). CGRP (0.5–1 μM) per se produced no detectable membrane currents ($n = 6$) (Fig. 1*D*).

Delayed upregulation of $P2X_3$ receptors by CGRP

The highest peak of CGRP in the effluent blood from the brain of migraine patients occurs 1 h from the start of the attack (Sarchielli et al., 2000). To find out whether TG neurons in culture might be suitable models to investigate the cellular action of CGRP, we explored whether this peptide could induce effects on $P2X_3$ receptors over a longer timescale than the one of the previous acute experiments. Thus, sister culture dishes were used in parallel for control data and for experiments testing the consequences of 1 h CGRP application that was washed out before studying neurons with Ca^{2+} imaging or patch clamping.

After 1 h exposure to 1 μM CGRP, the amplitude of Ca^{2+} transients induced by α,β-meATP (expressed as a percentage of KCl responses on the same cell) was significantly potentiated (from 27 ± 2%, $n = 86$, to 45 ± 2%, $n = 95$; $p < 0.0001$) (Fig.

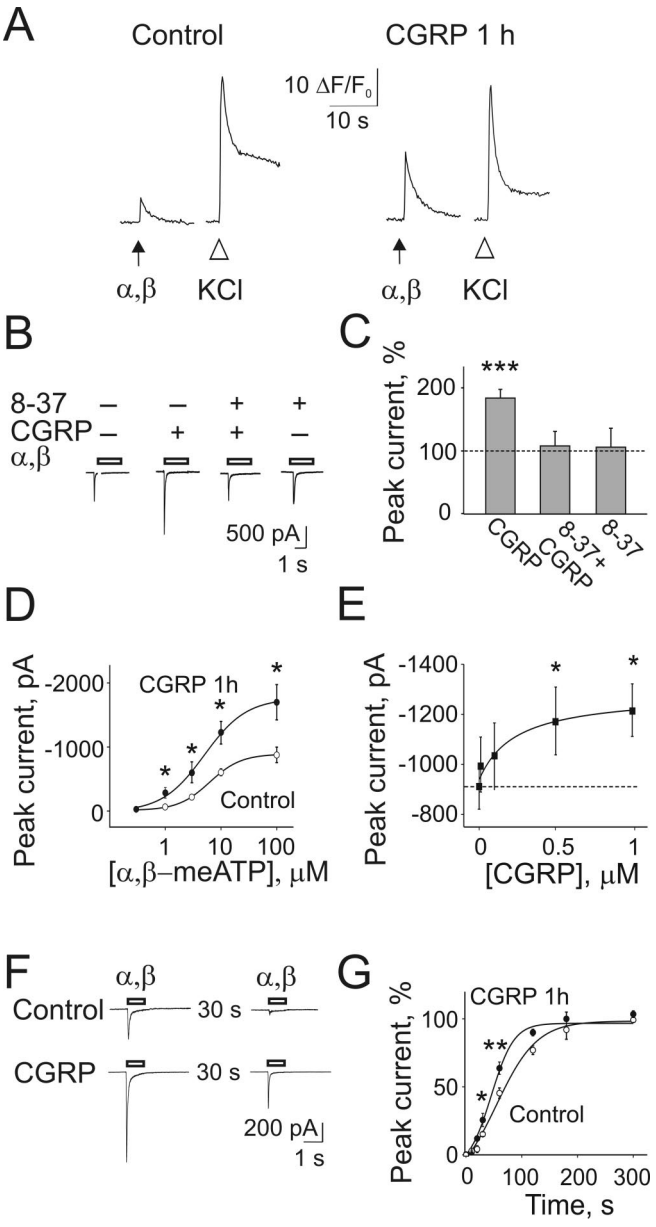

Figure 2. One-hour CGRP treatment upregulates $P2X_3$ receptor-mediated responses in TG neurons. Sister culture dishes were used in parallel for control data and for experiments testing the consequences of a 1 h application of CGRP, washed out before studying neurons with Ca^{2+} imaging or patch clamping. ***A***, Example of α,β-meATP (10 μM, 2 s; arrow) or KCl (50 mM, 1 s; open arrowheads) evoked Ca^{2+} transients in control condition. After 1 h CGRP (1 μM) treatment, the effect of α,β-meATP is comparatively larger, whereas the response to KCl is similar (different cell from control). ***B***, Examples of currents evoked by α,β-meATP (10 μM, 2 s; open bars) in control or after a 1 h treatment with CGRP, in the presence or absence of the CGRP receptor antagonist $CGRP_{8-37}$ (2 μM), which fully prevents the potentiation of current responses. ***C***, CGRP significantly increases α,β-meATP-induced peak current (expressed as percentage of control; $n = 78$), an effect antagonized by $CGRP_{8-37}$ ($n = 11$), which has no effect per se ($n = 15$). ***D***, Dose–response curves for α,β-meATP in the control condition (○; $n = 10$) and after 1 h CGRP treatment (●; $n = 12$). The EC_{50} value is not altered by CGRP, whereas the peak current amplitude is increased. ***E***, Concentration-dependent effect of CGRP on α,β-meATP-induced peak currents saturates near 1 μM. The dashed line indicates the mean value in the control condition ($n = 16$); $n = 10–15$ for CGRP treatments. ***F***, ***G***, CGRP accelerates $P2X_3$ receptor recovery from desensitization evoked by α,β-meATP (open bar) tested in this example at a 30 s interval between pulses. CGRP treatment significantly decreases the half-time of recovery (measured as percentage of the first response amplitude and tested over an extended time interval; ○, $n = 15$ control; ●, $n = 11$ CGRP). *$p < 0.05$; **$p < 0.01$; ***$p < 0.00001$. α,β, α,β-meATP; 8-37, $CGRP_{8-37}$.

2*A*), whereas the amplitude of KCl-evoked responses remained unchanged. Consistent with this observation, the peak amplitude of membrane currents induced by α,β-meATP after 1 h CGRP exposure was significantly increased (184 ± 13% of control; $n = 78$; $p < 0.00001$) (Fig. 2*B*,*C*). This effect was fully blocked by the CGRP receptor antagonist $CGRP_{8-37}$ (107 ± 20% of control; $n = 11$), which had no effect when applied alone (108 ± 24% of control; $n = 15$) (Fig. 2*B*,*C*). There was no change in cell input resistance after 1 h CGRP application (control: 874 ± 57 MΩ, $n = 66$; CGRP: 912 ± 60 MΩ, $n = 65$; $p = 0.64$), ruling out nonselective increases in cell responsiveness. CGRP application practically did not alter the number of cells immunoreactive for $P2X_3$ receptors (from 67 ± 1 to 72 ± 1%; $n = 3$ experiments).

Figure 2*D* shows that CGRP treatment enhanced α,β-meATP-induced currents without change in agonist potency (control: $EC_{50} = 5.6 \pm 0.7$, $n = 10$; CGRP: 5.4 ± 0.8 μM, $n = 12$). The effect of CGRP was apparently saturated at 1 μM (Fig. 2*E*). If ATP was used instead of α,β-meATP as a P2X receptor agonist, there was similar potentiation of peak membrane currents after 1 h CGRP treatment (supplemental Fig. S2*A*, available at www.jneurosci.org as supplemental material).

On DRG neurons grown in identical culture conditions, CGRP (1 μM; 1 h exposure) did not significantly increase the peak amplitude of $P2X_3$ receptor-mediated currents when compared with sister cultures (control: −521 ± 68 pA, $n = 33$; after CGRP: −611 ± 88 pA, $n = 31$). This difference between TG and DRG neurons prompted us to investigate the extent of colocalization of CGRP binding and $P2X_3$ receptors in DRG neurons. Unlike TG neurons, only 17% of $P2X_3$-immunoreactive DRG neurons (that represent the majority of neurons in this sensory ganglion) (Ruan et al., 2004) possessed CGRP binding, suggesting that topographic segregation of CGRP and $P2X_3$ receptors accounted for the lack of peptide-evoked facilitation of $P2X_3$ currents in DRG neurons.

Effects of CGRP on $P2X_3$ receptor desensitization

Fast desensitization followed by slow recovery is a key property of $P2X_3$ receptors (Cook et al., 1998; Sokolova et al., 2004). Using the patch-clamp technique and the paired-pulse protocol of agonist application, we tested whether desensitization of $P2X_3$ receptors had been changed by sustained CGRP application. CGRP treatment for 1 h did not change the time constant (τ_{fast}) of current decay (control: 47 ± 2 ms; $n = 52$; after CGRP: 48 ± 2 ms, $n = 49$), indicating no effect of CGRP on the onset of $P2X_3$ receptor desensitization (supplemental Fig. S2*B*, available at www.jneurosci.org as supplemental material). Nevertheless, 1 h CGRP treatment significantly accelerated recovery from desensitization (Fig. 2*F*,*G*) by decreasing the half-time of recovery ($t_{1/2}$) from 75 ± 4 s ($n = 15$) to 51 ± 4 s ($n = 11$; $p = 0.001$). These data indicate that $P2X_3$ receptors underwent two functional changes after CGRP treatment, namely enhanced responsiveness plus faster recovery from desensitization.

CGRP increased membrane expression of $P2X_3$ receptors

To test whether the delayed changes produced by CGRP were mediated by translocation of $P2X_3$ receptors to the membrane, we performed membrane biotinylation experiments. The amount of the $P2X_3$ receptors on surface membranes was significantly increased 2 and 5 h after CGRP treatment, as demonstrated by Western blots of the purified receptor protein (Fig. 3*A*). This time-dependent enhancement was not observed when the antagonist $CGRP_{8-37}$ was applied 30 min before CGRP and maintained throughout (Fig. 3*A*). CGRP also increased the ratio

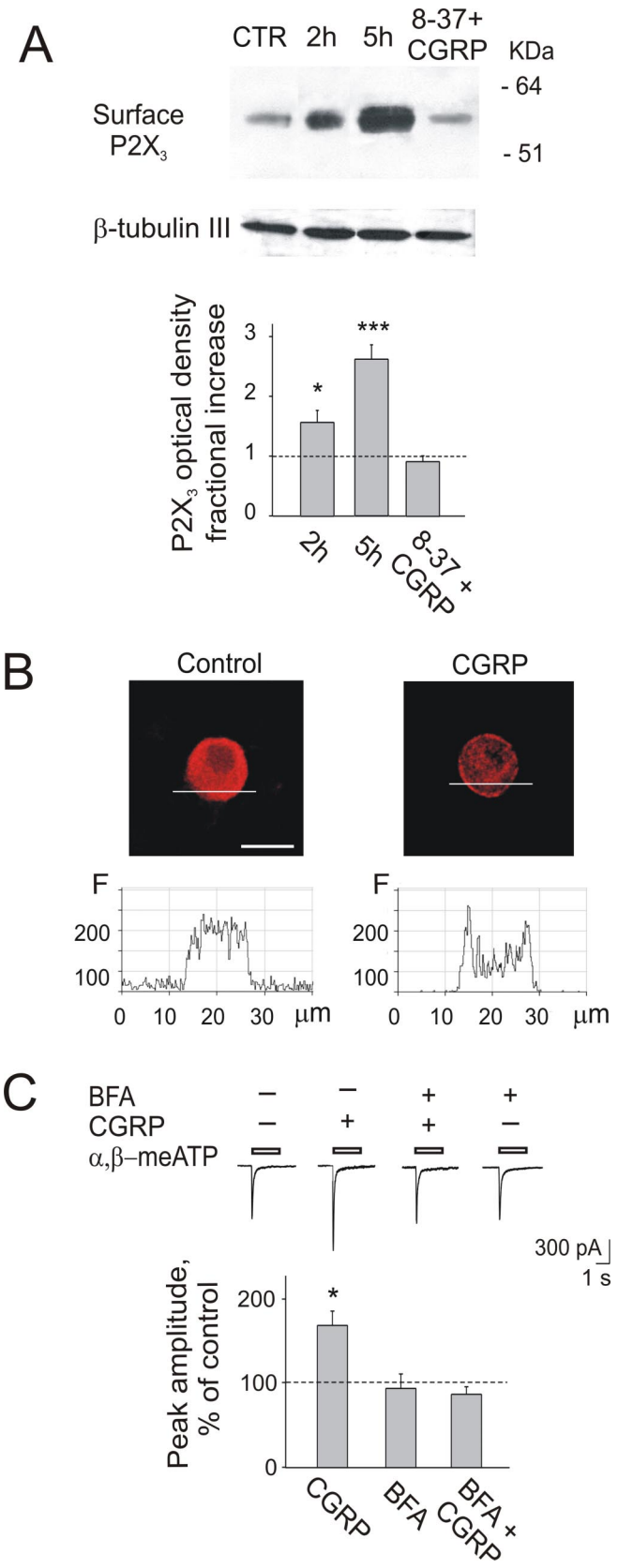

Figure 3. CGRP (1 μM, 1 h application) enhances expression of $P2X_3$ receptors at membrane level. ***A***, Example of Western immunoblots of the $P2X_3$ receptor after membrane biotinylation, showing a single band for the membrane form of the receptor (57 kDa). Note the increase in $P2X_3$ receptor expression after a 2 or 5 h washout of CGRP. When CGRP is coapplied with the receptor antagonist $CGRP_{8-37}$ (8-37; 2 μM), there is no change in $P2X_3$ receptor expression 2 h later. Control loading represents intracellular β-tubulin III. The plot shows protein expression increase as a fraction of control (histograms show $P2X_3$ receptor expression measured with optical density in arbitrary units); $n = 4$ experiments, $^*p = 0.034$ for 2 h; $n = 3$ experiments, $^{***}p = 0.0005$ for 5 h. ***B***, Example of confocal microscopy images of TG neurons treated (or untreated) with CGRP. $P2X_3$ receptor immunoreactivity shows perimembrane location 1 h after CGRP treatment, whereas it has homogenous distribution in control conditions. Plots beneath confocal images are the fluorescence profiles (ordinate, fluorescence intensity in arbitrary units) obtained by scanning along the lines shown in the photographs. Scale bar, 20 μm. ***C***, Top, The potentiating effect of CGRP on α,β-meATP-induced currents is prevented by brefeldin A (BFA; 5 μg/ml). Bottom, Quantification of blocking action of BFA on CGRP potentiation ($n = 7, 12$, or 11 for CGRP, BFA, or BFA + CGRP, respectively). $^*p < 0.05$.

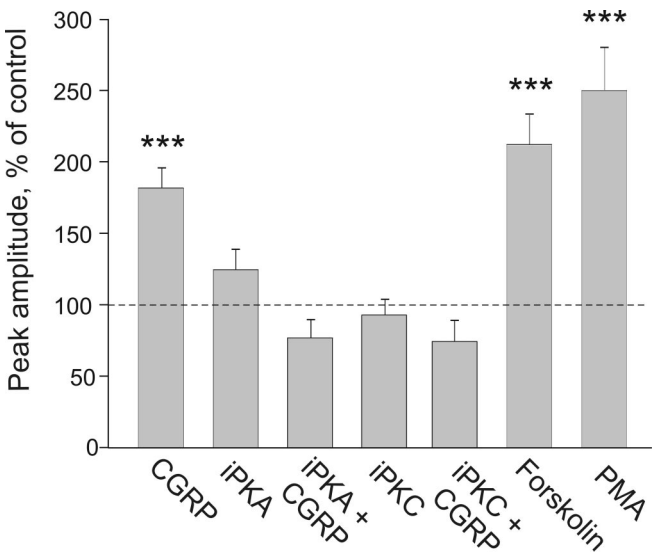

Figure 4. Potentiation of $P2X_3$-mediated currents by CGRP is PKA and PKC dependent. The plot summarizes the peak current amplitudes (expressed as percentage of the control value) of α,β-meATP-induced currents in TG neurons treated with CGRP and protein kinase inhibitors or activators. The PKA inhibitor 14-22 peptide (iPKA; 3 μM; $n = 14$) or the PKC inhibitor chelerythrine chloride (iPKC; 5 μM; $n = 8$) completely blocks the CGRP-mediated $P2X_3$ receptor potentiation ($n = 78$), whereas there is no effect when they are applied alone ($n = 36$ and $n = 11$, respectively). Conversely, forskolin (1 μM; $n = 37$) or PMA (325 nM; $n = 27$) potentiates $P2X_3$ receptor-mediated currents. $^{***}p < 0.001$.

between the surface and the total protein content in control and 2 h CGRP-treated samples (2.5 ± 0.6-fold increment; $n = 3$ experiments).

Confocal microscopy showed a ring-like structure enriched with $P2X_3$ receptor immunoreactivity in the area immediately beneath the surface membrane in 51 ± 5% of the $P2X_3$-positive neurons treated with CGRP ($n = 118$) (Fig. 3*B*). Conversely, only 3 ± 2% of neurons ($n = 56$) had a similar perimembrane signal in control ($p < 0.0001$) (Fig. 3*B*). To quantify these observations, we performed confocal line-profile analysis of neurons (Rathee et al., 2002) in control or after 1 h CGRP exposure by scanning along a line across the perimembrane region as indicated in Figure 3*B*. The plots corresponding to such scans are displayed below each cell image: note that, in control, $P2X_3$ immunoreactivity was evenly distributed throughout the sampled region, whereas after CGRP treatment, the signal was concentrated near the membrane, giving rise to two peaks in correspondence with the membrane area. These data suggest that CGRP treatment stimulated trafficking of $P2X_3$ receptors from cytoplasmic pools toward the membrane. In support for this notion, brefeldin A (5 $\mu g\,ml^{-1}$), a selective inhibitor of the trafficking processes (Chardin and

←

McCormick, 1999), prevented CGRP-induced potentiation of $P2X_3$ receptor-mediated currents (86 ± 9%; $n = 11$) (Fig. 3*C*).

$P2X_3$ potentiation is mediated by PKA and PKC

CGRP operates via G-protein-coupled receptors that, in many cell types, lead to activation of the cAMP/PKA cascade (Durham and Russo, 1999; Arulmani et al., 2004) and directly or indirectly to PKC activation (Drissi et al., 1998). Indeed, both PKA and PKC are involved in the enhancement of excitability of DRG neurons by CGRP (Natura et al., 2005). Thus, we first explored the role of PKA in the upregulation of $P2X_3$ receptors. As shown in Figure 4, the PKA inhibitor 14-22 peptide (3 μM) completely blocked the CGRP-mediated $P2X_3$ potentiation (76 ± 13% of control; $n = 14$; $p = 0.18$). Next, we tested the role of PKC in the action of CGRP on $P2X_3$ receptors. The PKC inhibitor chelerythrine (5 μM) fully prevented the enhancement of $P2X_3$ receptors by 1 h CGRP treatment (74 ± 15% of control; $n = 8$; $p = 0.14$) (Fig. 4). Both PKA and PKC inhibitors had no effect when applied alone (Fig. 4). Consistent with the involvement of PKA and PKC in the modulation of $P2X_3$ receptors, we observed that forskolin (1 μM) or PMA (325 nM), activators of PKA or PKC, respectively, strongly enhanced currents mediated by $P2X_3$ receptors (Fig. 4). In particular, α,β-meATP-induced currents were 211 ± 22% of control after 1 h forskolin treatment ($n = 37$; $p < 0.001$), whereas 1 h PMA treatment increased these currents to 249 ± 30% of control ($n = 27$; $p < 0.001$).

Persistence of CGRP effects

One point to be tested before considering the hypothesis that upregulated $P2X_3$ receptor function triggered by CGRP might contribute to sustained pain is the persistence of receptor potentiation long after removal of CGRP. For this purpose, current responses to α,β-meATP were monitored at different times after CGRP washout after 1 h exposure to 1 μM CGRP. Figure 5*A* shows that the potentiation of the $P2X_3$ receptor-mediated responses continued to grow after CGRP washout, peaking 5 h later and decreasing to control level after 24 h ($n = 81$ for control and $n = 14$–17 for CGRP data points). Likewise, improved recovery from $P2X_3$ receptor desensitization was also a long-lasting feature persisting for several hours after CGRP washout ($n = 6$–15) (Fig. 5*B*).

Upregulation of $P2X_3$ mRNA and protein synthesis could have been a mechanism responsible for such long-lasting facilitation of $P2X_3$ receptor-mediated responses. To explore this issue, real-time PCR and Western blotting experiments were performed. Figure 5*C* shows that 1 h incubation with CGRP (1 μM) significantly upregulated $P2X_3$ transcription (4.6 ± 1-fold increment in mRNA levels; $n = 4$ experiments; $p = 0.01$), an effect absent in cells incubated with CGRP plus the receptor antagonist $CGRP_{8-37}$ (1.1 ± 0.1-fold increment; $n = 3$) (Fig. 5*C*). $P2X_3$ mRNA neosynthesis was completely prevented by actinomycin D (1.1 ± 0.1-fold; $n = 3$ experiments) (Fig. 5*C*), thus demonstrating that CGRP activated gene transcription. Increased $P2X_3$ mRNA synthesis was coupled to translation into new $P2X_3$ protein as shown by Western blot experiments in which the differently glycosylated intracellular forms of the $P2X_3$ receptor polypeptides (50–57 kDa) (Vulchanova et al., 1997) were significantly increased 1 h after CGRP treatment (1.2 ± 0.05-fold increment; $n = 4$ experiments; $p = 0.01$) (Fig. 5*D*).

We also investigated whether CGRP altered the number of $P2X_3$-immunoreactive cells. $P2X_3$ immunofluorescence analysis, however, revealed that the number of immunoreactive cells was not increased after 1 h CGRP application (67 ± 1% control vs 72 ± 1% after CGRP; $n = 3$ experiments).

CGRP has no effect on TRPV1 receptor function

Because TG neurons also express capsaicin-sensitive TRPV1 receptors as transducers of nociception (Julius and Basbaum, 2001; Wang and Woolf, 2005), we explored whether CGRP treatment (1 μM) preincubated for 1 h could modify them. Currents evoked by 1 μM capsaicin (−101 ± 21 pA; $n = 40$) were not changed by CGRP (−107 ± 19 pA; $n = 37$) (see example in supplemental Fig. 1*SC*, available at www.jneurosci.org as supplemental material). The number of cells sensitive to capsaicin (40 ± 5% in control condition; $n = 17$ experiments) was also unchanged by CGRP (41 ± 4%; $n = 16$), a result validated with TRPV1 immunofluorescence analysis (36 ± 1% of positive cells in control and 37 ± 1% after CGRP; $n = 3$ experiments for each condition). Finally, Western immunoblotting of extracts from TG neurons showed no difference in expression of TRPV1 protein by TG neurons treated with 1 μM CGRP for 1 h ($n = 3$ experiments; data not shown). We next examined whether TG neurons labeled with fluorescent CGRP were also immunoreactive for the TRPV1 receptors: colocalization of such signals was observed in 12 ± 3% neurons only ($n = 3$ experiments) (supplemental Fig. S1 *D*, available at www.jneurosci.org as supplemental material).

Discussion

The principal finding of the present study is the demonstration that $P2X_3$ receptors of TG neurons were selectively upregulated by CGRP. This slow modulation was mediated by neosynthesis and increased trafficking of $P2X_3$ receptors to the plasma membrane. The present model thus outlines a mechanism for the persistent sensitization of a nociceptive system in chronic pain states such as migraine, associated with a high level of CGRP.

CGRP action on $P2X_3$ receptors

Short-lasting application of CGRP had no direct effect on the membrane current or resistance of trigeminal neurons in culture, except for a few isolated cells showing a subtle rise in intracellular Ca^{2+}. These observations accord with a recent *in vivo* study demonstrating lack of effect of acute administration of CGRP to meningeal nociceptors (Levy et al., 2005). Because we were interested in delayed effects of CGRP on an extended timescale that resembles the duration of pain in migraine, we took advantage of cultured TG neurons.

A role of ATP in migraine was first suspected in conjunction with the vascular theory of this disorder. Because the focus on migraine pathophysiology has shifted to neuronal dysfunction, we considered the possibility that ATP released during a pain attack (Burnstock, 2000) contributes to headache by activating ionotropic $P2X_3$ receptors in trigeminal sensory neurons (Cook and McCleskey, 1997; Ruan et al., 2004). In keeping with this hypothesis, we detected frequent coexpression of $P2X_3$ receptors with CGRP binding sites in mouse TG neurons in culture, thus providing the substrate for $P2X_3$ receptor modulation by CGRP at single-cell level.

Although CGRP enhanced the maximum response of $P2X_3$ receptors, it did not alter the agonist potency, suggesting an increase in the number of functional $P2X_3$ receptors rather than in agonist sensitivity. The faster recovery of $P2X_3$ receptors from desensitization would have significantly contributed to potentiation of $P2X_3$ receptor function, because such receptors possess an unusually long recovery that curtails their ability to generate pain signals (Cook et al., 1998). This phenomenon is different from the modulation of desensitization by other algogenic peptides such as substance P or bradykinin occurring over a much

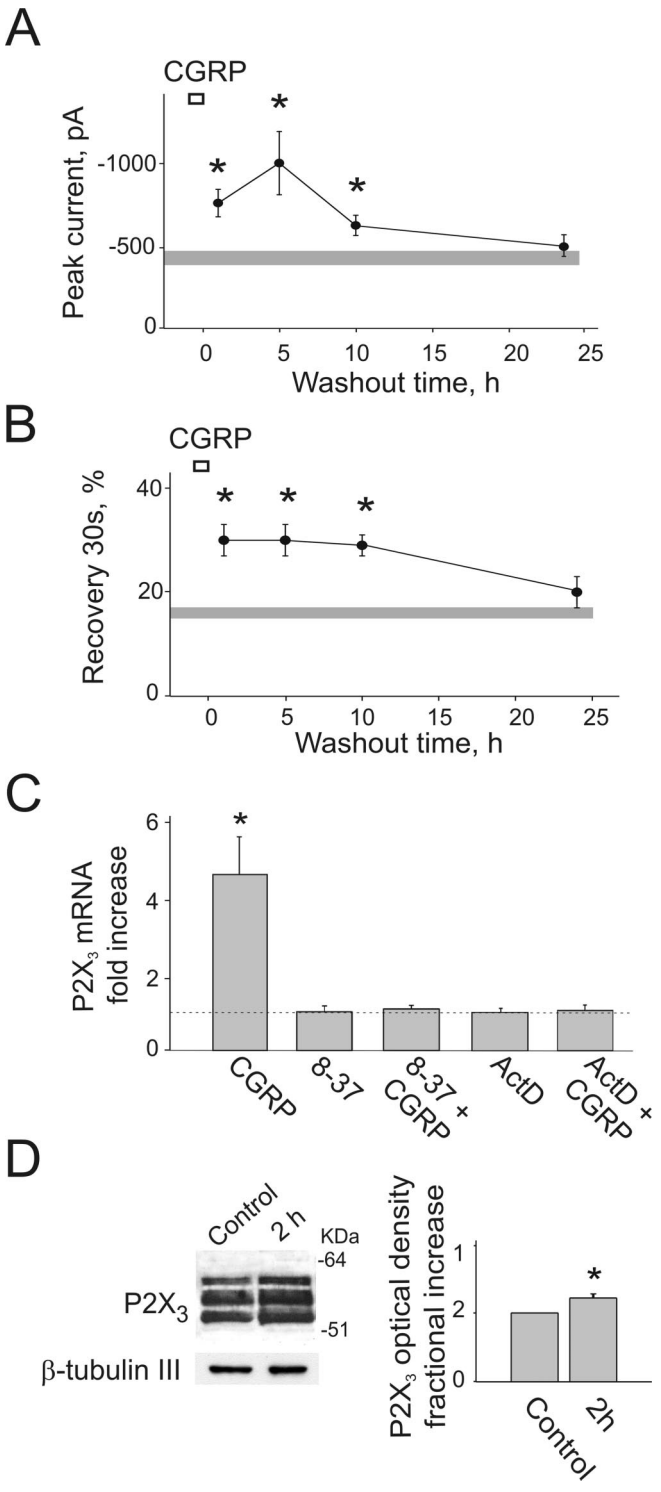

Figure 5. Long-lasting effects of CGRP on $P2X_3$ receptors. ***A***, Time course of potentiation of the $P2X_3$ receptor-mediated responses after 1 h CGRP treatment (1 μM; open bar). Current responses (filled circles) to α,β-meATP application are monitored at different times after CGRP washout. Current amplitude is maximal after 5 h and gradually comes back to the control level at 24 h. The shaded horizontal bar indicates the SEM of values obtained from controls ($n = 81$), whereas data points for CGRP washout range from 14 to 17. *$p < 0.05$. ***B***, Paired pulses of α,β-meATP (spaced by 30 s) are used to study $P2X_3$ recovery from desensitization. The shaded horizontal bar indicates the SEM of values obtained from controls ($n = 52$). CGRP improves recovery for several hours after treatment with a gradual return to control levels; the number of neurons treated with CGRP ranges from 6 to 15; *$p < 0.05$. ***C***, Long-lasting changes in $P2X_3$ receptor potentiation after CGRP are supported by $P2X_3$ receptor neosynthesis. A 1 h application of CGRP (1 μM) upregulates *$P2X_3$* gene transcription ($n = 4$ experiments), an effect prevented by 2 μM $CGRP_{8-37}$ (8-37; $n = 3$ experiments) or by 5 μg ml^{-1} actinomycin D (ActD; $n = 3$ experiments). *$p = 0.01$. ***D***, Left, Example of Western blot analysis of $P2X_3$ in total extracts from TG neurons showing increment of all glycosylated forms of the receptor after 1 h of CGRP treatment (bottom lanes show control loading with β-tubulin III). Right, Histograms for increase in $P2X_3$ receptor expression measured with optical density (arbitrary units; $n = 4$ experiments; *$p < 0.01$).

shorter (seconds, minutes) time course (Paukert et al., 2001). Slow facilitation of $P2X_3$ receptor function by CGRP also differs from the time course of the CGRP-evoked block of nicotinic receptors (Di Angelantonio et al., 2003).

Under the present experimental conditions, we did not observe CGRP-evoked modulation of TRPV1 receptor function, probably because the likelihood of detecting such an interaction was limited by the fact that few TRPV1-expressing neurons coexpressed CGRP binding sites. For analogous reasons, we did not detect potentiation by CGRP of $P2X_3$ receptors on DRG neurons that showed infrequent colocalization of CGRP binding and $P2X_3$ immunoreactivity in accordance with a low expression of CGRP receptors in rat DRG neurons (Natura et al., 2005). The overall outcome of CGRP action therefore appears to bias pain signaling toward purinergic-based rather than vanilloid-based mechanisms, and this phenomenon seems to be specific to TG.

Dynamics of CGRP action

The CGRP receptor consists of a G-protein-coupled complex that operates by increasing intracellular cAMP and activation of downstream effectors (Drissi et al., 1998; Durham and Russo, 1999; Poyner et al., 2002), leading to rapid changes in ion channel activity apparently unrelated to nociception and perhaps responsible for other physiological effects of the peptide (Zona et al., 1991; Di Angelantonio et al., 2003). The present study detected another feature of CGRP, namely delayed and persistent modulation of $P2X_3$ receptors even after washout of the peptide. The reason for the gradual return of $P2X_3$ receptor function to control level remains unclear and requires future studies to identify its mechanisms.

The potentiation of $P2X_3$ receptors was accompanied by significantly larger expression, already after 2 h, of the mature form of these receptors at membrane level. The enhanced trafficking of pre-existing $P2X_3$ receptors and their incorporation into the neuronal membrane was supported by biochemical and confocal microscopy evidence, suggestive of intense translocation of this receptor. Because selective inhibitors of PKA and PKC blocked $P2X_3$ potentiation by CGRP, it is likely that activation of these kinases mediated the action of CGRP. This notion is corroborated by the facilitation of $P2X_3$ receptor function by activators of PKA or PKC, indicating a key role of both enzymes in trafficking of $P2X_3$ receptors. We suggest that PKA and PKC are just a part of a more complex scenario in which each step of $P2X_3$ receptor potentiation is highly regulated and involves activation of several factors.

Real-time reverse transcription (RT)-PCR experiments showed that CGRP could induce *$P2X_3$* gene transcription. Previous reports demonstrated that CGRP activated gene transcription via cAMP-dependent cAMP response element-binding protein (CREB) activation (Seybold et al., 2003; Anderson and Seybold, 2004). It is likely that CREB is involved in *$P2X_3$* gene transcription, because analysis of the genomic $P2X_3$ promoter region (GenBank accession number gi 84781757 ref NM_145526.2) indicates the presence of CRE and CRE-BP elements (nucleotide −92), both equally con-

served in the two parallel strands of the DNA sequence (score 86.1% with TFSEARCH, www.cbrc.jp/research/db/TFSEARCHJ.html). The enhanced $P2X_3$ mRNA synthesis after CGRP could account for a larger, delayed expression of $P2X_3$ receptors at membrane level, providing a molecular mechanism for long-lasting sensitization of $P2X_3$ receptors. Although both RT-PCR and Western blot yielded unidirectional results, we observed a degree of mismatch in the amplitude of RT-PCR and Western blot signals. A discrepancy between transcription and translation is not an uncommon phenomenon, as amply discussed in a recent review (Lewandowski and Small, 2005), because of the complex relationship between mRNA and protein, as transcription and translation are governed by independent mechanisms.

Pathophysiological implications

There is growing interest in the role of CGRP in migraine (Pietrobon and Striessnig, 2003; Goadsby, 2005). *In situ* experiments reveal that CGRP is expressed in human trigeminal neurons (Moreno et al., 1999; Tajti et al., 1999) from which it is released during migraine attacks (Edvinsson and Uddman, 2005). Novel CGRP antagonists are currently investigated for treating migraine pain (Olesen et al., 2004; Rudolf et al., 2005). Nevertheless, the mechanisms responsible for the algogenic action of CGRP remain little understood. The present model study suggests that the action of CGRP is linked to sensitization of $P2X_3$ receptors of trigeminal nociceptors. Behavioral studies on $P2X_3^{-/-}$ mice and the analgesic action of $P2X_3$ receptor antagonists indicate $P2X_3$ receptors to be involved in chronic pain (Cockayne et al., 2000; Jarvis, 2003; North, 2003). Our study showed that 1 h exposure to CGRP was sufficient for a large effect on $P2X_3$ receptors, a timescale that should mimic the development of migraine pain. Hence, trigeminal $P2X_3$ receptors could be potential targets for future analgesics designed to treat chronic pain syndromes such as migraine. Furthermore, our data predict that a CGRP receptor antagonist should be most efficient for analgesia during the early phase of a migraine attack.

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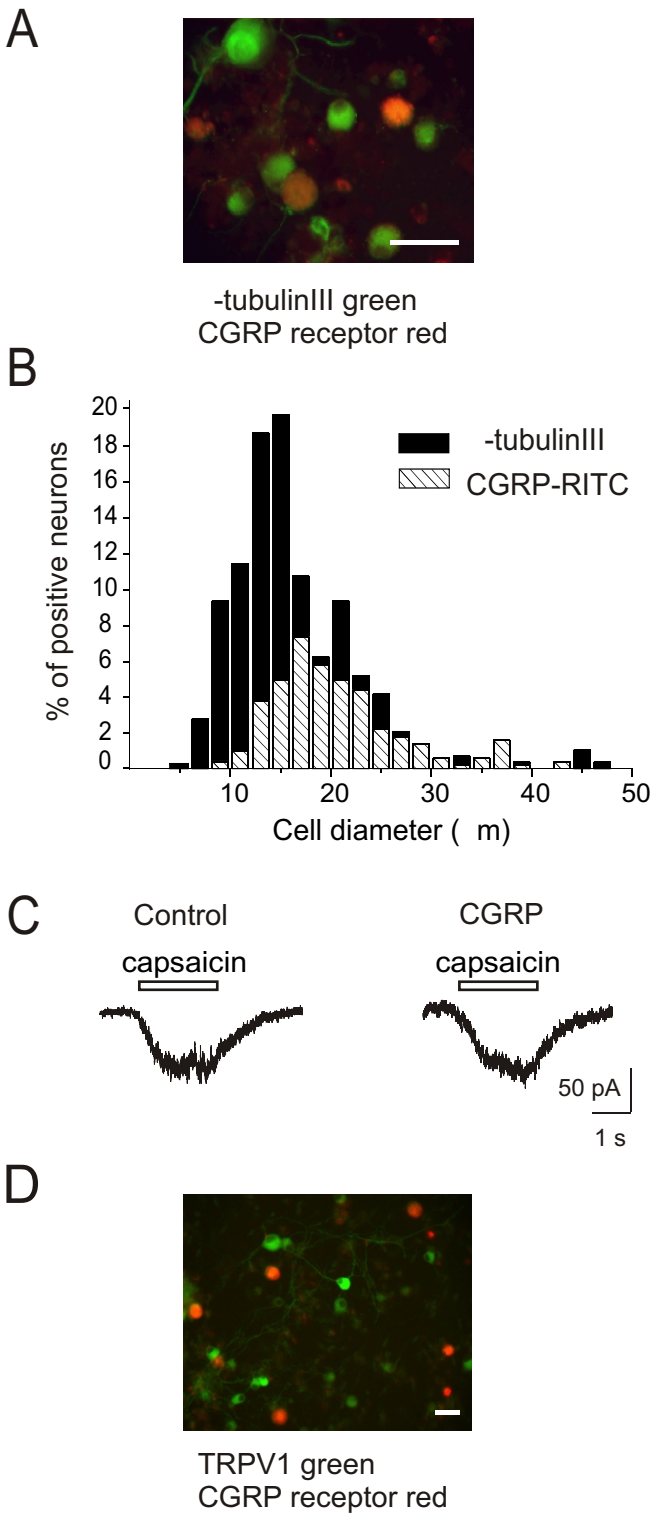
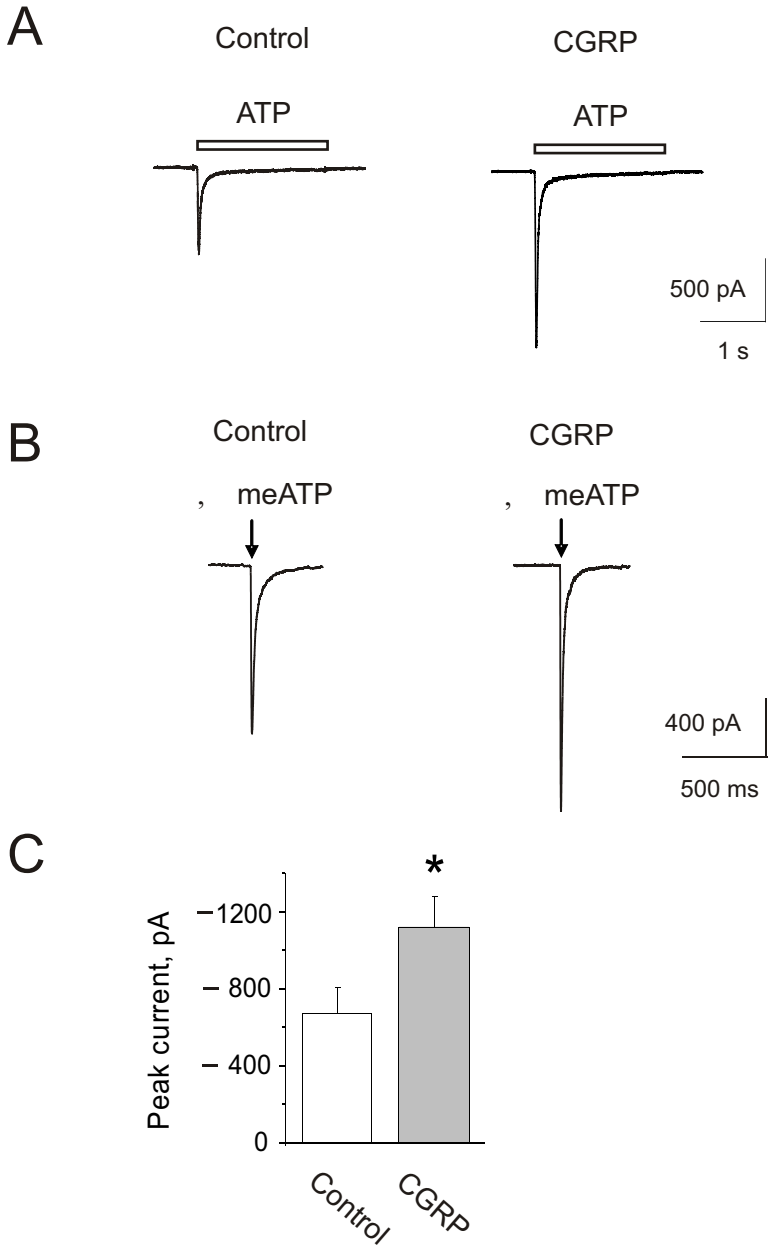


Supplemental Figure 1. CGRP binding to TG neurons. ***A***, microphotograph depicts TG neurons labeled with rhodamine conjugated CGRP (0.5 M; red) and anti- -tubulinIII antibody (green). Calibration bar = 50 m. ***B***, Somatic size distribution of TG neurons immunostained with anti- -tubulinIII antibody (filled columns) and labeled with rhodamine-conjugated CGRP (dashed bars). Data are from approximately 1,500 cells (3 independent experiments). ***C***, CGRP (1 μM, 1 h) has no effect on the amplitude of currents elicited by application of capsaicin (1 μM, 2 s). ***D***, Microphotograph depicts TG neurons labeled with rhodamine conjugated CGRP (0.5 M; red) and anti-TRPV1 antibody (green). Calibration bar = 50 m.



Supplemental Figure 2. CGRP treatment (1 μM, 1 h) induces potentiation of peak currents elicited by ATP or , meATP. ***A*,** Examples of currents evoked in TG neurons by application of ATP (10 μM, 2 s) in control condition (left) or after CGRP treatment (right). The mean peak current amplitude induced by ATP after 1 h CGRP exposure is 180 ± 38 % of control (n = 23), indicating analogous potentiation as observed with , -meATP. ***B*,** CGRP (1 μM, 1 h) increases currents elicited by short (10 ms) pressure application of , -meATP (200 μM). Examples of , meATP-evoked currents in control condition (left) or after 1 h CGRP treatment (right). This result shows that the CGRP-evoked enhancement of $P2X_3$ receptor function is not biased by the method of agonist application. ***C*,** Peak current amplitude evoked by puffer-applied , meATP is significantly increased by CGRP treatment (p = 0.044; n = 11) with respect to control (n = 10). Note that current deactivation is not affected by CGRP treatment because the rapid phase of current monoexponential decay was 23 ± 7 and 21 ± 5 ms for control and treated neurons (n= 9 and 8, respectively).

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Chronic NGF treatment of rat nociceptive DRG neurons in culture facilitates desensitization and deactivation of $GABA_A$ receptor-mediated currents

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1 The present study tested the hypothesis that nerve growth factor (NGF) could affect presynaptic inhibition mediated by $GABA_A$ (GABA-sensitive ionotropic receptors) receptors on the afferents of nociceptive dorsal root ganglia (DRG) neurons, thus reducing the filtering of central nociceptive signals.

2 To investigate this issue, small-diameter, nociceptive DRG neurons were cultured for 48–72 h either in the normal medium or in the presence of NGF (50 ng ml^{-1}). After 15 min washout, cells were patch clamped with Cs^+ containing electrodes to block $GABA_B$ (GABA-sensitive metabotropic receptors) receptor-activated currents.

3 Chronically treated DRG neurons showed no difference in the peak amplitude of GABA-induced currents. However, NGF-treated cells exhibited increased fading of the response to continuous GABA application, with faster desensitization onset, decreased residual current at the end of agonist application and slower recovery from desensitization. Moreover, the deactivation phase after brief agonist pulses was also accelerated.

4 Unlike responses to GABA, chronic NGF treatment had no effect on the desensitization process to the excitatory transmitter ATP, as no difference in peak amplitude, fast and slow time constants of current decay was found.

5 Experimental tests indicated that the observed effects on GABA currents were not a reactive process triggered by washing out NGF after its long application. Acutely applied NGF did not change $GABA_A$ receptor-mediated responses.

6 NGF-treated neurons showed decreased sensitivity to the antagonist picrotoxin. The action of pentobarbitone, midazolam, bicuculline or gabazine was, however, unchanged.

7 These observations suggest that the modulation of $GABA_A$ receptor function of DRG nociceptors by NGF may contribute to the algogenic action of this neurotrophin.
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Keywords: Desensitization; gabazine; bicuculline; picrotoxin; pentobarbitone; midazolam; ATP; pain

Abbreviations: BIC, bicuculline methochloride; DRG, dorsal root ganglion; $GABA_A$, subclass of GABA-sensitive ionotropic receptors; $GABA_B$, GABA-sensitive metabotropic receptors; NGF, nerve growth factor; PAD, primary afferent depolarization; PTX, picrotoxin

Introduction

The neurotrophin nerve growth factor (NGF) has several important effects on morphological and functional properties of neurons. Postnatally, NGF has a major, physiological role in survival, growth and development of ganglion neurons (Crowley *et al*., 1994; Patel *et al*., 2000) as, for example, newborn mice daily injected with anti-NGF antiserum show severe atrophy of sympathetic ganglia (Levi-Montalcini & Cohen, 1960). Although the same cells grown in culture can survive for several days in the absence of NGF, chronic NGF treatment promotes the formation of neuronal processes and allows long-term survival (Levi-Montalcini, 1964). Both high (TrkA)- and low (p75)-affinity receptors for NGF are expressed on dorsal root ganglion (DRG) sensory neurons (Bennett *et al*., 1996).

Another important action of NGF is facilitation of processing of nociceptive signals when levels of NGF grow dramatically in inflamed or damaged tissue (Donnerer *et al*., 1992; Woolf *et al*., 1994; Ueda *et al*., 2002). Furthermore, increased NGF retrograde transport by nociceptive neurons augments the expression of algogenic compounds like substance P and CGRP (Donnerer *et al*., 1992). Administration of NGF can produce hyperalgesia (Lewin *et al*., 1992; Woolf *et al*., 1994; Andreev *et al*., 1995), whereas neutralization of endogenous NGF reduces sensitivity to painful stimuli (McMahon *et al*., 1995; Koltzenburg *et al*., 1999). NGF administration enhances the sensitivity of DRG neurons to capsaicin and noxious heat (Winter *et al*., 1988; Rueff & Mendell, 1996; Bonnington & McNaughton, 2003).

A further action of NGF is concerned with the modulation of synaptic transmission. In fact, in animal models of

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inflammatory pain, NGF can also boost the efficiency of glutamatergic transmission at the spinal cord level, while anti-NGF antibodies decrease spinal glutamate release and attenuate algogenic behavior (Ishikawa *et al*., 1999). Moreover, while ATP is an important messenger in pain signaling in these neurons (Burnstock, 2001; North, 2004), chronic NGF treatment can increase the expression of $P2X_3$ ATP receptors in sensory neurons (Ramer *et al*., 2001).

The processing of nociceptive inputs at the first relay synapse of the pain pathway in the spinal dorsal horn is modulated by GABA, which exerts an analgesic effect (Sivilotti & Woolf, 1994). GABA is believed to be the main transmitter for presynaptic inhibition in the spinal cord (Sivilotti & Nistri, 1991) due to primary afferent depolarization (PAD) that reduces presynaptic impulses and, thus, release of the excitatory transmitter (Rudomin & Schmidt, 1999). Owing to their key role in nociceptive information processing, the $GABA_A$ subclass of GABA-sensitive ionotropic receptors might be an important target in mediating the algogenic action of NGF. However, the difficulty of directly accessing such receptors on the fine central terminals of DRG neurons suggests the study of the action of NGF on somatic $GABA_A$ receptors normally expressed by the soma of these cells (Sivilotti & Nistri, 1991; Rudomin & Schmidt, 1999). On DRG neurons, GABA acts *via* $GABA_A$ receptors (sensitive to bicuculline (BIC) or picrotoxin (PTX)) to mediate a Cl^--dependent depolarization (Sivilotti & Nistri, 1991), as well as on GABA-sensitive metabotropic receptors ($GABA_B$) that mediate a slowly developing K^+ outward current (Gähwiler & Brown, 1985; Newberry & Nicoll, 1985). Since activation of $GABA_B$ receptors appears to play only a minor role in presynaptic inhibition of primary afferent terminals (Stuart & Redman, 1992), we focused on $GABA_A$ receptors. Hence, we used electrophysiological recording from DRG neurons in culture to investigate how acute or chronic NGF treatment might affect $GABA_A$ receptor-induced currents. To find out if chronic NGF treatment might also change the effects of an excitatory transmitter, we used the same cell preparation to examine how DRG neurons responded to ATP.

Methods

Cell culture preparation

Cultures of rat DRG neurons were prepared as described previously (Sokolova *et al*., 2004). In brief, rats (P11–18) of both sexes were deeply anesthetized by slowly raising levels of CO_2 and were killed by decapitation, a procedure (including animal handling and care) in accordance with the Italian Animal Welfare Act and approved by the Local Authority Veterinary Service. Thoracic and lumbar ganglia were excised, enzymatically treated, plated on poly-L-lysine (5 mg ml^{-1}) coated Petri dishes and cultured under an atmosphere containing 5% CO_2. NGF (2.5S NGF; 50 ng ml^{-1}) was added to half of the Petri dishes at the time of DRG neuron plating. Cells were used for recording from the 2nd to the 3rd day after plating. In order to minimize differences in responses between DRG neuron preparations, equivalent numbers of control (from Petri dishes without NGF) and NGF-treated neurons were used on each occasion.

Patch-clamp recording

Control or NGF-treated cells were continuously superfused (2 ml min^{-1}) with physiological solution containing (in mM): 152 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES (pH adjusted to 7.4 with NaOH, osmolarity adjusted to 320 mOsm with glucose). This solution was applied for about 15 min to ensure full washout of the culture medium. Thereafter, single cells were patch clamped in the whole-cell configuration using pipettes with a resistance of $\sim$3–4 MΩ when filled (in mM) with 120 CsCl, 20 HEPES, 1 $MgCl_2$, 3 Mg_2ATP_3 and 5 EGTA (pH adjusted to 7.2 with CsOH). The osmolarity of the pipette solution was 285 mOsm.

Currents were recorded from cells 15–30 μm in diameter (see also Figure 1b), thus considered to be small and medium-sized, nociceptive DRG neurons (North, 2004), which are very sensitive to the pain transmitter ATP (Grubb & Evans, 1999) and bind the nociceptor marker IB4 (Sokolova *et al*., 2004). DRG neurons of diameter up to 25 μm usually express the NK-1 receptor to the pain transmitter substance P (Li & Zhao, 1998), thus further characterizing them as nociceptors, although such expression can be found even in a minority of larger DRG cells. In most cells series resistance was compensated by 80%. Cells were voltage clamped at −70 mV (unless otherwise indicated). After whole-cell configuration was obtained, an equilibration period of 5 min was used for establishing adequate solution exchange between the patch pipette and the cell. Currents were filtered at 1 kHz and acquired by means of pClamp 8.2 software (Axon Instruments, Union City, CA, U.S.A.).

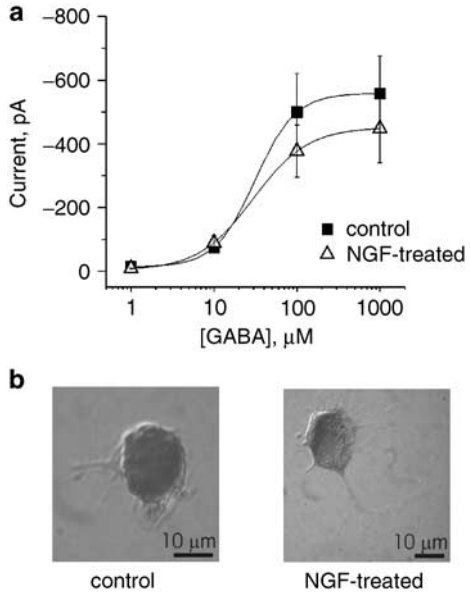

Figure 1 Sensitivity of DRG neurons to GABA. (a) Log concentration–response curves for rapidly superfused GABA in control or chronically NGF-treated DRG neurons. Note the lack of difference between curves ($n = 14$ and 15 for control and NGF-treated, respectively). (b) Example of cresyl violet-stained DRG neurons cultured for 48 h in the medium without (left) or with (right) NGF (50 ng ml^{-1}).

Drug delivery and data analysis

GABA, antagonists and modulators of $GABA_A$ receptors, and ATP were diluted with extracellular solution to final concentration and applied by a rapid superfusion system (Rapid Solution Changer RSC-200, BioLogic Science Instruments, Grenoble, France) consisting in a multibarrel array of capillary tubes 100–150 μm from the recorded cell. The time for solution exchange was about 30 ms. In a separate set of experiments, GABA was applied by pressure application (15–20 psi) for 40 ms from glass micropipettes positioned approximately 15–25 μm away from the recorded cell (Di Angelantonio & Nistri, 2001). On some cells, NGF was acutely administered *via* pressure application (15 psi for 30 s) to pipettes containing a concentration of 150 ng ml^{-1}.

During GABA application, the development of current fade from the early peak was quantified on the basis of its time constant (τ_{ON}), which was best fitted monoexponentially (Clampfit program of the pCLAMP suite), with the exception of a few responses to 100 μM GABA that required two exponents and were discarded from further analysis. Current fade was also measured as the fractional residual current (r) left at the time of switching off GABA, as this value indicated the extent of activated GABA receptors prior to termination of agonist administration. In some experiments, the difference in r values between control and test conditions was expressed as Δr and allowed comparison of degree of fading (r) under various experimental conditions. GABA current deactivation was quantified as the monoexponential time constant of return to baseline (τ_{OFF}).

Paired-pulse experiments in which the second agonist pulse was delivered at different intervals were used to monitor recovery from desensitization. For this purpose, each pulse consisted of 25 μM GABA applied by superfusion for 2 s. The peak of GABA current generated by the second pulse was expressed as a fraction of the peak amplitude of the control response. Data were best fitted with a double hyperbola (SigmaPlot2001, SPSS, Chicago, IL, U.S.A.) and recovery from desensitization was expressed as the time needed to regain 90% of the control peak amplitude (t_{90}).

Log concentration–response curves for GABA were fitted with the logistic equation (Origin 6.0, Microcal, Northampton, MA, U.S.A.). Each concentration of GABA was applied by superfusion for 2 s and repeated two to three times on each cell at a 3-min interval. The strong fading of the GABA responses plus the necessarily limited duration of whole-cell patch clamping (about 40 min) did not allow strictly quantitative tests for characterizing antagonism potency and nature. To circumvent this condition, tests to quantify the action of rapidly superfused blockers (BIC, PTX, gabazine, that is, SR 95531) were usually based on measuring peak currents elicited by a fixed GABA concentration (25 μM) close to the EC_{50} value for this agonist. Note that each antagonist was always present in the GABA solution to avoid sudden dilution of antagonist upon rapid application of the agonist. Several concentrations of each antagonist were cumulatively applied to determine their potency to block GABA-induced currents. GABA currents in the presence of antagonist were expressed as a fraction of the current amplitude obtained in the absence of antagonist. Data were then plotted with the logistic equation to express inhibitory potency in terms of IC_{50} values. When receptor antagonism was rapidly reversible (e.g. bicuculline or gabazine), tests with various antagonist concentrations were also repeated after intermediate washes and gave analogous results.

To assess the potentiating action of pentobarbitone (PB) (sodium salt) on 25 μM GABA, this agent was applied cumulatively in the range of 10–100 μM. The test GABA concentration was prepared in the corresponding PB solution. The GABA current in the presence of PB was expressed as a fraction of the control one and plotted with the logistic equation to obtain the EC_{50} value for PB. The limited availability of the water-soluble benzodiazepine midazolam HCl did not allow its systematic tests at various concentrations applied *via* rapid superfusion. Preliminary tests indicated that maximum facilitation was apparently observed with bath-applied 1 μM midazolam, which was then used in all other experiments. To minimize midazolam dilution by concomitant fast superfusion of GABA, GABA (30 μM) was applied *via* a puffer pipette for 40 ms. Previous experiments have indicated that this method produced an effective dose of the substance at cell membrane level three times lower than the pipette concentration (Di Angelantonio & Nistri, 2001).

In experiments on ATP-induced currents, ATP was rapidly superfused at a concentration of 10 μM for 2 s.

All data are presented as the mean $\pm$ s.e.m. (n = number of cells). The statistical significance of nonparametric data was assessed with Mann–Whitney rank-sum test for comparing two unmatched samples from two populations. For raw data their normal distribution was first assessed with SigmaStat (version 2.0, Jandel Scientific, San Rafael, CA, U.S.A.) and, if data met this requirement, were further analyzed with the unpaired Student's t-test. A P-value of <0.05 was accepted as indicative of a statistically significant difference.

Drugs

Chemicals for cell culture and recording were from Sigma (Milan, Italy); the culture media were obtained from Gibco BRL (Life Technologies, Milan, Italy). GABA and ATP were from Sigma (Milan, Italy), BIC, gabazine and PTX were from Tocris (Bristol, U.K.), PB (sodium salt) was a gift from Dr Laura Ballerini (University of Trieste), while midazolam HCl was donated by Professor J. Lambert (University of Dundee), Dr G. Puja (University of Modena) and Professor G. Biggio (University of Cagliari).

Results

Chronic NGF treatment does not significantly affect GABA sensitivity of DRG neurons

Since DRG neurons do not synthesize NGF as shown by the lack of mRNA signal (Wetmore & Olson, 1995), in the present study control conditions refer to cells bathed with standard culture medium (without NGF addiction). In this case, rapid superfusion of GABA evoked inward currents, the peak of which is plotted in Figure 1a (filled symbols) against varying concentration of GABA. On NGF-treated DRG neurons, peak amplitudes of GABA-evoked currents were slightly lower than in controls (Figure 1a, open symbols), although this difference was not statistically significant. The EC_{50} values (the concentration of GABA eliciting half-maximal current

amplitude) obtained from these average plots were very similar between controls (31.4 μM, $n=14$) and NGF-treated cells (29.7 μM, $n=15$). Analogous results were obtained by averaging the log-transformed EC_{50} values obtained from each cell in control (34.5$\pm$3.9 μM, $n=14$) and after NGF treatment (26.9$\pm$5.1 μM, $n=11$). As all recorded cells had similar size regardless of NGF treatment (see examples in Figure 1b), this observation suggests that unequal GABA current responses were not masked by large differences in cell size. This fact was confirmed by the similar cell membrane capacitance between controls (28.5$\pm$1.4 pF; $n=105$) and NGF-treated cells (31.8$\pm$1.5 pF; $n=68$). Since there was no difference in GABA-induced responses between DRG neurons cultured for 48 and 72 h in the presence of NGF (nor between 48 and 72 h in controls), data from cells at the second and third day from plating were pooled.

NGF-mediated shaping of GABA-induced currents: desensitization degree and onset, deactivation phase and recovery from desensitization

Despite lack of change in peak current amplitude, it appeared that chronic NGF treatment changed the time course of the decay of GABA-evoked currents. Analysis was performed on currents that presented clear fading during application of GABA, namely, those produced by 10, 25 (close to the dose inducing half-maximal responses), 100 and 1000 μM concentrations of this amino acid. Figure 2a and b shows sample traces obtained from control or NGF-treated DRG neurons using 25 or 100 μM GABA, respectively. For each agonist concentration used, care was taken to compare currents of virtually identical peak amplitude. Cells cultured in the presence of NGF showed faster fading of the response and smaller residual current at the end of GABA application. The differences in τ_{ON} value for current fade and in the fractional residual current (r) are quantified in Figure 2c and d, and were statistically significant at 25 and 100 μM agonist concentration. Neither τ_{ON} nor r values were affected by NGF treatment when obtained with 1000 μM GABA, which induced maximal response (Figure 2c and d). This observation suggests that NGF could facilitate current decay, but it could not augment it once it was maximally developed.

The monoexponential time constant of current offset (τ_{OFF}) after interruption of 10, 25, 100 and 1000 μM GABA application was also measured. There was no difference in τ_{OFF} between traces obtained after 10 μM GABA from controls and NGF-treated cells (120$\pm$12 ms, $n=9$; 120$\pm$23 ms, $n=5$, respectively), nor after 25 μM (104$\pm$5 ms, $n=19$ for controls; 102$\pm$7 ms, $n=7$ for treated cells), 100 μM (111$\pm$8 ms, $n=10$ for controls; 104$\pm$10 ms, $n=12$ for treated cells) or 1000 μM GABA (204$\pm$28 ms, $n=7$ for controls; 170$\pm$13 ms, $n=5$ for treated cells).

GABA current return to baseline after termination of the agonist application was a complex phenomenon presumably due to a combination of desensitization recovery, receptor deactivation and agonist removal, since the time course of receptor deactivation can be strongly affected by the duration of agonist application and the consequent desensitization (Jones & Westbrook, 1995). In order to examine deactivation of GABA currents less affected by desensitization, responses were induced with 40 ms pressure application of GABA (30 μM) from a micropipette close to the recorded cell.

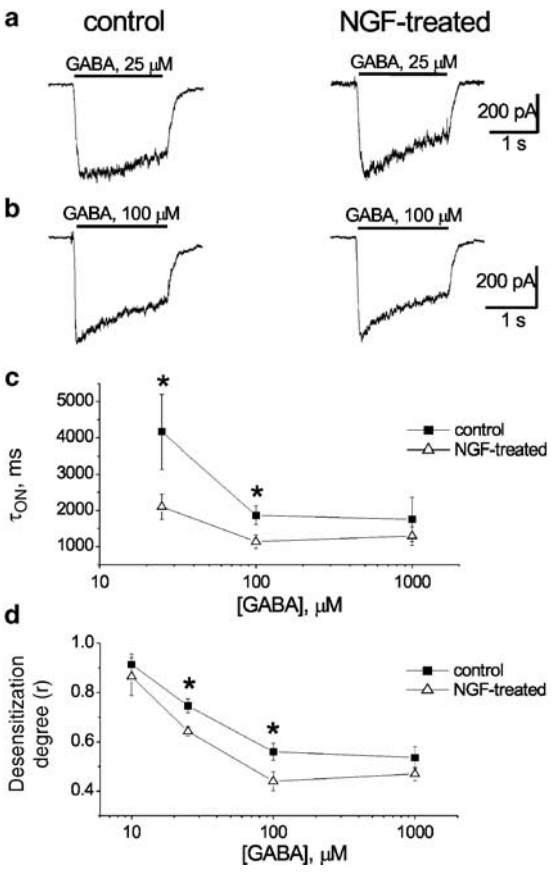

Figure 2 Enhanced desensitization of $GABA_A$ receptor-mediated currents after chronic treatment with NGF. (a) Examples of amplitude-matched currents induced by 25 μM GABA applied for 2 s in control (left) or after NGF treatment (right). (b) Examples of amplitude-matched currents induced by 2 s application of 100 μM GABA as indicated in (a). Note enhanced fading of current with smaller residual response at the end of GABA application. (c) Plot of τ_{on} values for fading of GABA-induced currents *versus* log GABA concentrations from control or NGF-treated neurons. $^*P<0.05$; $n=10$–14 cells. (d) Plot of desensitization degree (r) expressed as a fractional residual current *versus* log GABA concentrations for control or NGF-treated neurons. $^*P<0.05$; $n=11$–13 cells.

Figure 3a shows representative traces of similar amplitude obtained from a control cell and an NGF-treated cell using such an application of GABA. The deactivation phase, best fitted with a monoexponential function, became significantly shorter in NGF-treated neurons as indicated in Figure 3b.

To further analyze the effects of chronic NGF treatment on desensitization of GABA-induced currents, paired-pulse experiments were performed. Figure 3c (upper) shows that fast recovery from desensitization, expressed as fractional amplitude of the second response in the pair, was best fitted by a hyperbolic function for control neurons with calculated t_{90} of 7.6 s. On NGF-treated neurons (Figure 3c, lower), the changes in fractional amplitude of the second response could also be fitted hyperbolically (calculated $t_{90}=19.7$ s). This difference from control was due to the significantly ($P<0.05$) smaller second response at the first pulse interval (1 s), while subsequent data points were indistinguishable from control.

Current fade might be due to a combination of factors including agonist-induced channel block or desensitization. To explore the role of agonist-dependent channel block, 25 μM

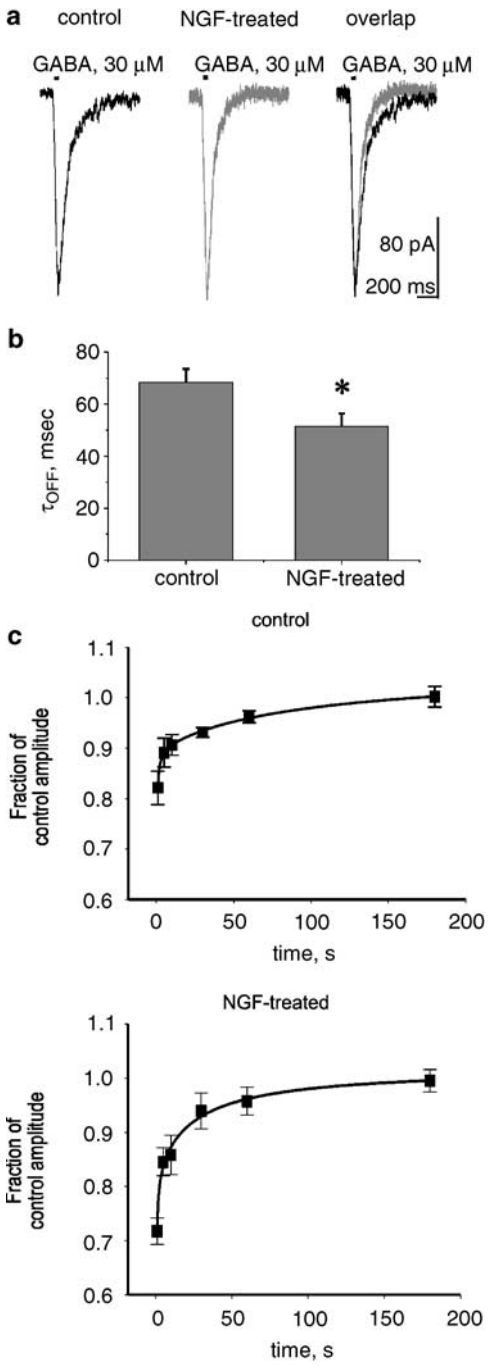

Figure 3 Chronic NGF treatment increases deactivation of $GABA_A$ receptor-mediated currents and modifies their recovery from desensitization. (a) Examples of amplitude-matched, inward currents evoked by brief (40 ms) pressure applications of GABA to single DRG neurons grown in control or NGF-containing medium. Note accelerated current decay (responses superimposed for clarity; right-hand side) after NGF treatment. (b) Histograms showing τ_{OFF} value of current decay for neurons grown in control or NGF-containing medium. $^*P<0.05$; $n=11$–12 cells. (c) Plots of time course of recovery from desensitization of GABA currents for control (upper; $n=10$) and NGF-treated (lower; $n=13$) DRG neurons. Data are presented as fractional amplitude of the second response in the pair of pulses spaced at time intervals indicated on the abscissa. The difference between values at 1 s is statistically significant ($P<0.05$). Note initially slower recovery in chronically treated cells.

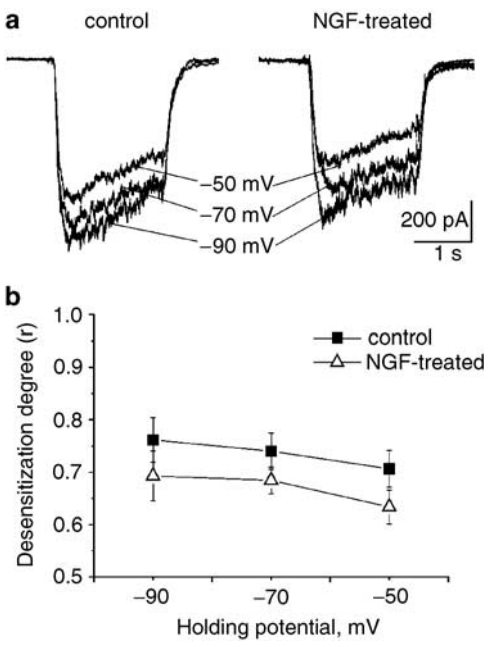

Figure 4 GABA-evoked current decline is voltage insensitive. (a) Examples of GABA-evoked currents from control (left) or NGF-treated (right) neurons. In each panel currents induced by 25 μM GABA on the same cell held at –50 (uppermost), –70 and –90 mV are superimposed for comparison. (b) Plot of desensitization degree expressed as fractional residual current (r) induced by 25 μM GABA *versus* level of holding potential in control or NGF-treated neurons ($n=10$–12 cells).

GABA was applied at three different holding potentials (−50, −70 and −90 mV) to control or NGF-treated cells. As exemplified by raw data in Figure 4a and quantified in Figure 4b, voltage dependence was very weak for both controls and NGF-treated DRG cells, suggesting that the observed fading was not primarily due to agonist-dependent channel block.

Tests to rule out artifactual effects due to NGF

Since all electrophysiological recordings from DRG cells chronically exposed to NGF were performed in physiological solution after NGF washout (see Methods), we considered the possibility that increased current fading during agonist application might have been caused by acute NGF deprivation, rather than due to a long-lasting effect of NGF treatment. To examine this hypothesis, two different tests were performed on NGF-treated DRG neurons. In the first one (deprivation test 1), cells were patch clamped while still in the NGF-containing culture medium and tested for their response to pressure-applied (2 s) GABA (30 μM). The medium was then washed out while preserving patch-clamp recording and replaced with physiological solution. After 3 min, GABA was again applied to the same cell as described above. Comparing currents obtained in NGF-containing culture medium with those in physiological solution gave a good match of the two GABA-induced responses (see example in Figure 5a). To quantify this result, for each cell ($n=5$), we expressed, as Δr, the difference between fractional residual GABA currents (just prior to the end of GABA application) obtained in the culture medium and in physiological solution (Figure 5c). The Δr value for the deprivation test 1 was almost

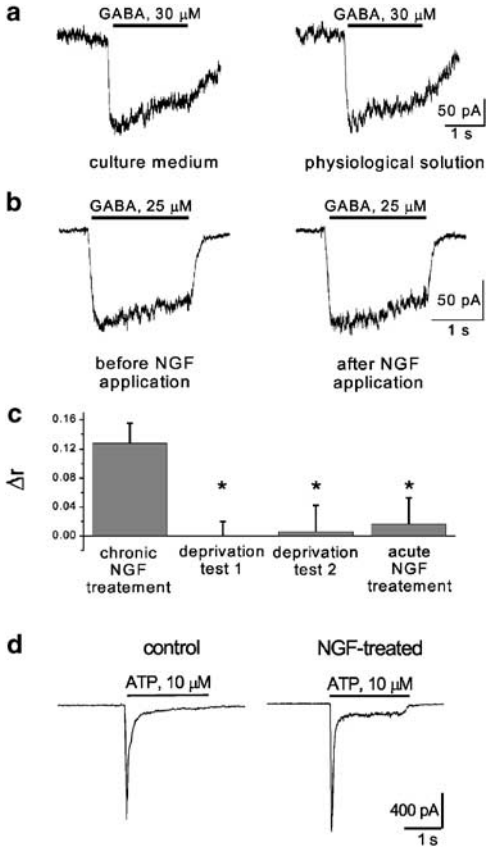

Figure 5 Changes in GABA-evoked current fade are not due to sudden removal of NGF from neurons grown in the presence of this neurotrophin nor are accompanied by alterations in ATP-mediated responses. (a) Example of protocol (test 1) in which the same DRG neuron, grown in NGF-containing medium, is first tested for its response to pressure-applied (30 μM) GABA without washing out NGF (left), and then the same GABA application is repeated 3 min after washing out the NGF-containing medium (right). Note the lack of change in residual current at the end of the GABA response. (b) Example of protocol (test 2) in which the same neuron, grown in NGF-containing medium, is first tested in standard physiological solution for its response to rapidly superfused 25 μM GABA (left) and then the same GABA application is repeated 3 min after washing out NGF (150 ng ml^{-1}) transiently applied from a pressure pipette for 30 s (right). Note again the lack of difference in residual current evoked by GABA. (c) Histograms summarizing differences in desensitization degree following GABA application. Left, values of desensitization degree (r) following 25 μM GABA application are compared between cells grown in control or NGF-containing medium ($n=13$): the difference between r data is expressed as Δr. Middle two histograms refer to Δr values obtained with the protocols exemplified as test 1 ($n=5$) or test 2 ($n=5$) in (a, b). Right histogram refers to Δr obtained by comparing 25 μM GABA-evoked responses before and after acute application of NGF (30 s) to neurons grown in the control medium ($n=5$). *$P<0.05$. (d) Examples of currents induced by 10 μM ATP in control (left) or NGF-treated (right) DRG neurons.

nil and was significantly different ($P<0.05$) from the Δr value obtained when comparing responses from control and chronically treated neurons in the standard protocol.

Furthermore, assuming that the stronger current fade might have been a consequence of NGF washout, a subsequent application of NGF should reverse, in part, this current fade. This possibility was assessed in our second test (deprivation test 2) when NGF was pressure applied for 30 s to chronically NGF-treated cells washed for 5 min with physiological solution. In detail, GABA (25 μM) was applied by rapid superfusion (2 s) thrice (3 min interval), then NGF was pressure applied and the cell retested with repeated GABA applications (see sample traces in Figure 5b). Also in this case, currents obtained from the same cells ($n=5$) before and after acute NGF application were almost identical. These data are quantified in Figure 5c, in which the Δr value from the deprivation test 2 was very small and significantly different when compared with Δr obtained with the standard protocol. Finally, we examined if 30 s NGF application to *naïve*, control cells could change their GABA (25 μM) responses. As shown by the right-hand side histogram of Figure 5c, this experimental condition was accompanied by a small Δr value significantly different ($P<0.05$) from the one obtained with standard protocol (left histogram). This observation demonstrated that short-term exposure of *naïve* DRG neurons to NGF was insufficient to modify their GABA-mediated responses.

In summary, these tests showed that increased fading of GABA-induced responses from NGF-treated DRG neurons was not due to abrupt NGF deprivation.

NGF-treated DRG neurons show differential drug sensitivity

NGF can turn on multiple intracellular messengers, leading to the activation of protein kinases and/or transcription factors (Kaplan & Miller, 2000). Thus, it seemed likely that the observed effects of chronically applied NGF on GABA-induced currents could be mediated by conformational changes of GABA receptors, due to post-translational modifications of native receptors (e.g. phosphorylation; Moss & Smart, 2001) or neosynthesis of receptors with different subunit composition. As a first approach to this issue, we took advantage of the fact that a number of GABA receptor ligands show preferential affinity for certain subunits (Hevers & Lüddens, 1998). Hence, if ligand pharmacology were changed after chronic NGF treatment, one might infer that the receptor subunit(s) binding such ligands were likely altered.

Table 1 shows the IC_{50} values for the competitive antagonists BIC, gabazine and the noncompetitive blocker PTX. While the potency of BIC or gabazine block was similar between NGF-treated cells and controls, PTX was less potent on NGF-treated cells. The potentiating action by PB was the same on control and NGF-treated neurons as indicated by similar EC_{50} values.

Finally, application of 1 μM midazolam to NGF-treated or control DRG neurons indicated that, in both cell groups, GABA currents displayed the same sensitivity to the facilitatory action by this benzodiazepine (Table 1).

Chronic NGF treatment does not affect the amplitude and desensitization of ATP-induced currents

The ATP-sensitive ionotropic receptors P2X are involved in direct excitation of primary afferent DRG neurons (Bland-Ward & Humphrey, 1997; Hamilton & McMahon, 2000). Sensory neurons express different levels of $P2X_2$, $P2X_3$ and $P2X_{2/3}$ receptors, characterized by different time courses of current decay during agonist application (Burgard *et al*., 1999). In the present study, application of 10 μM ATP for 2 s elicited

Table 1 Effect of $GABA_A$ receptor ligands on GABA-mediated responses of control and NGF-treated DRG neurons

	IC_{50} (μM)		*EC_{50} (μM)*		*% enhancement*	
	Control	*NGF-treated*	*Control*	*NGF-treated*	*Control*	*NGF-treated*
BIC	1.77±0.17 ($n=2$)	2.24±0.21 ($n=19$)				
Gabazine	0.55±0.12 ($n=10$)	0.29±0.04 ($n=11$)				
PTX	4.65±1.24 ($n=11$)	9.84±3.07* ($n=8$)				
PB			60.86±9.79 ($n=12$)	50.53±7.50 ($n=19$)		
Midazolam					80±18 ($n=11$)	95±14 ($n=10$)

*$P<0.05$.

inward currents with different decay time courses (see Burgard *et al*., 1999; Sokolova *et al*., 2001), presumably reflecting the presence of various P2X receptor subtypes (see Figure 5d). Since the $P2X_3$ receptor seems to be the main subtype involved in nociception (North, 2004), we focused on cells expressing ATP responses with biphasic current decay, a hallmark of the presence of $P2X_3$ receptors (Burgard *et al*., 1999). In particular, we looked at the fast (τ_{fast}) and slow (τ_{slow}) components of current decay to characterize desensitization of ATP-induced currents in control cells or after chronic NGF application. No statistically significant difference was found in peak amplitude of ATP-evoked currents between NGF-treated neurons (−1226±210 pA, $n=11$) and controls (−935±118 pA, $n=24$). Analysis of ATP-induced desensitization, performed by fitting current decay with a double exponential function, showed no difference in either τ_{fast} (40.5±2.4 ms, $n=25$ controls; 44.7±7.8 ms, $n=11$ NGF-treated neurons) or τ_{slow} (532.9±105.5 ms, $n=25$ controls; 302.8±58.6 ms, $n=11$ treated cells).

Discussion

The principal finding of the present study is the novel demonstration of increased decline of $GABA_A$ receptor-mediated currents of nociceptive DRG neurons grown in the presence of NGF. This phenomenon could be manifested in two ways, namely, neurotrophin-enhanced desensitization of sustained responses mediated by $GABA_A$ receptors, and accelerated deactivation of short responses evoked by brief GABA pulses. This action of NGF might be important for understanding the mechanism of pain induction and/or intensification by NGF, since increased NGF levels during inflammation or injury could change the effectiveness of GABA-mediated inhibition of excitatory transmitter release from afferents of DRG neurons in the spinal cord.

Fading of GABA-evoked currents

In symmetrical Cl^- concentrations, GABA evoked inward currents readily blocked by bicuculline or gabazine. Since these currents reverse at ~0 mV (Sokolova *et al*., 2001), they are clearly due to the activation of $GABA_A$ receptors. Furthermore, inclusion of Cs^+ in the patch pipette solution prevented activation of $GABA_B$ receptors (Otis *et al*., 1993).

A decline in the GABA-evoked currents during continuous exposure to the agonist could have been due to receptor desensitization, agonist-induced channel block or GABA uptake. Our observations that current fade was little sensitive to membrane potential would make channel block unlikely. Furthermore, although DRG neurons can take up GABA applied for several minutes (Hosli & Hosli, 1979), fade of GABA-induced responses during its sustained application is not affected in Na^+-free media (Adams & Brown, 1975; Deschenes *et al*., 1976; Gallagher *et al*., 1978), indicating that Na^+-dependent GABA uptake played a minimal role in the membrane response to this amino acid. Hence, the current fade reported in the present study was probably due to receptor desensitization, which is a prominent property of such receptors (Sivilotti & Nistri 1991; Bormann, 2000).

Current fade following NGF treatment

When DRG neurons were grown in the presence of NGF, applied at the standard concentration (50 ng ml^{-1}) optimized for ganglion culture (Levi-Montalcini & Angeletti, 1963), they generated GABA currents characterized by faster and stronger fade. Although concentrations up to 200 ng ml^{-1} have been used to investigate changes in DRG neuronal responses (Winter *et al*., 1988), the concentration of NGF used in the present study is largely in excess of the one normally present in the extracellular fluid of young rats (about 0.6 ng ml^{-1}; Xia *et al*., 2000), and thus it more closely corresponds to the substantial increases in local NGF levels typically found during tissue inflammation and injury (Ueda *et al*., 2002). Hence, the present experiments suggest that the changes in DRG cells responses to GABA should be considered as a model of what might happen to such cells in the presence of pathological amounts of this neurotrophin.

NGF treatment could not by itself bring about $GABA_A$ receptor desensitization when current responses were small and associated with minimal fade, nor could it intensify the strong fade associated with maximal responses. The role of NGF therefore seemed to be a modulatory one of the desensitization process once it has developed. An earlier report describing reduction in GABA current amplitude only after >1 week of NGF exposure (50–200 ng ml^{-1}; Bevan & Winter 1995) accords with our data on the lack of change in the peak amplitude of GABA currents after 2–3 days of NGF treatment.

Accelerated fading and smaller residual current had no significant influence on the return of the response to baseline after 2 s application, as this process was probably due to a combination of agonist removal, receptor deactivation and recovery from desensitization with complex dependence on NGF treatment. It is, however, worth noting that NGF treatment accelerated the deactivation time course of currents induced by brief pulses of GABA.

Recovery from GABA current desensitization, tested with the paired pulse protocol, was rapid in control cells, enabling

full reattainment of cell responses within 3 min. NGF-treated cells also recovered fully from desensitization within 3 min, although at the earliest measured interval (1 s) their ability to regain responsiveness was significantly less.

The change in GABA current fade took place before the development of neuronal processes and was not associated with apparent morphological or cell capacitance changes in the time frame of 48–72 h. Thus, the effect resulting from NGF treatment was a novel action distinct from the well-known effect of promoting neuronal growth.

Since DRG neurons do not synthesize NGF as they lack mRNA for this substance (Wetmore & Olson, 1995), effects induced by NGF could only be generated by exogenous application of this neurotrophin. It was therefore important to demonstrate in the present study that augmented current fade of GABA currents after NGF chronic exposure was not a consequence of sudden removal of this neurotrophin. In addition, acute application of NGF to *naïve* cells had no effect on GABA-induced currents.

Despite the effects of NGF treatment on the inhibitory GABA-induced currents in DRG neurons, our data suggest that this neurotrophin could not induce any significant change in the response of these neurons to the excitatory transmitter ATP. The lack of difference in peak amplitude of ATP-induced currents is consistent with a previous report based on 1 to 2-week NGF treatment of DRG neurons (Bevan & Winter, 1995). Although it is unknown if the desensitization process is altered by 2-week NGF treatment, the present results can exclude that this neurotrophin might modulate the desensitization of ATP currents in the time frame of 2–3 days.

Mechanisms underlying increased fade of GABA currents by NGF

Desensitization and deactivation kinetics of the current mediated by $GABA_A$ receptors has been shown to depend on $GABA_A$ receptor subunit combination (Gingrich *et al*., 1995; Haas & Macdonald, 1999). While rat DRG neurons at 2–3 postnatal weeks express α_2, α_3, β_2, β_3 and γ_2 subunits (having lost the expression of the embryonic α_5 subunit; Ma *et al*., 1993), the precise combination of $GABA_A$ subunits to form single receptors in DRG neurons remains unclear and cannot exclude the contribution by more recently discovered subunits like θ and ε subunits (Barnard *et al*., 1998; Hevers & Lüddens, 1998). Since chronic NGF treatment did not modify the dose–response curve for GABA, it seems likely that there was no significant change in the interaction of GABA with its binding site at the interface between α and β subunits (Smith & Olsen, 1995). As a first approach to outline potential $GABA_A$ receptor sites modified by NGF, we investigated whether the effect of certain subunit-selective ligands might have been altered by chronic NGF. In particular, we used gabazine or bicuculline to test changes in the competitive antagonist binding sites believed to be partially overlapping the GABA binding one (Sigel *et al*., 1992; Holden & Czajkowski, 2002). This experiment was also advantageous to confirm the observed similarity in GABA sensitivity between control and NGF-treated cells. Furthermore, we tested the potency of PTX that binds a specific site within the Cl^- pore of the transmembrane domain 2 region (Ffrench-Constant *et al*., 1993; Gurley *et al*., 1995), PB that predominantly binds the β_3 subunit (Davies *et al*., 1997; Wooltorton *et al*., 1997) and midazolam that binds the α_2 and α_3 subunits at their interface with the γ_2 subunit (Barnard *et al*., 1998).

The lack of change in the potency of the competitive antagonists gabazine and bicuculline suggests their binding site to be essentially unaltered by NGF. PTX was significantly less potent after NGF treatment, indicating that its binding region was a target for the action of the neurotrophin. In fact, site-directed mutagenesis of motifs corresponding to the PTX binding site can strongly affect desensitization and deactivation of $GABA_A$ receptors (Scheller & Forman, 2002). The lack of change in PB or midazolam activity after NGF treatment suggests that the PB binding site on the β_3 subunit as well as the midazolam binding site at the interface between α_2 and (or) α_3 subunits with the γ_2 subunit were insensitive to neurotrophin exposure. In summary, it seems feasible that chronic application of NGF led to a modification in the $GABA_A$ receptor region inside the Cl^- pore itself. It should be emphasized that validation of the proposed molecular targets on the $GABA_A$ receptor for the action of NGF will require further investigations based on single-cell RT–PCR to identify subunit changes, and on single-channel recordings to characterize the precise mechanisms underlying alterations in GABA-mediated responses. While NGF can upregulate sodium and calcium channels (Baldelli *et al*., 2000; Vidaltamayo *et al*., 2002), its long-term action on $GABA_A$ receptor channels remains unclear.

Pathophysiological implications

By activating $GABA_A$ receptors on sensory neurons endings, GABA generates presynaptic inhibition of primary afferent inputs (Rudomin & Schmidt, 1999). Presynaptic inhibition can be induced only by high-frequency stimulation of GABA-releasing fibers, causing a sustained reduction in excitability lasting hundreds of milliseconds (Frank & Fuortes, 1955). This slow and long-lasting effect of GABA is different from its more rapid transmitter role in other areas of the brain and can be adequately modeled by the method of agonist application employed in the present study.

The results obtained in our work suggest that increased levels of NGF, like those occurring after tissue damage or inflammation (see Introduction), might modify GABA-induced currents in sensory neurons decreasing the total amount of current entering their central terminals, thus reducing local depolarization and its effects on action potential propagation.

Notwithstanding the need for further investigation to clarify the molecular mode of action of NGF, $GABA_A$ receptor modulation by NGF should be viewed within a wide framework of regulatory activity exerted by neurotrophins on afferent inputs, especially during chronic pain states. In particular, since NGF can facilitate BDNF expression in DRG neurons (Michael *et al*., 1997) and BDNF can increase the release of GABA (Pezet *et al*., 2002), the whole process of pain signaling may comprise self-regulating mechanisms to compensate pain (Lever *et al*., 2003) at least in early stages of chronic inflammation.

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