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PURINERGIC TRANSMISSION IN MIGRAINE: ROLE OF P2Y RECEPTORS IN THE SPINAL-TRIGEMINAL SYSTEM IN VIVO AND IN VITRO

BIO/14

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1. INTRODUCTION

1.1 PAIN AND NOCICEPTION

Pain can be defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey & Bogduk, 1994). It is a physical sensation arising from parts of the body, but it is also always unpleasant and therefore become an emotional experience. Experiences which resemble pain but are not unpleasant, e.g., pricking, should not be called pain (Merskey & Bogduk, 1994). Pain is a submodality of somatic sensation like touch and pressure, and serves as an important protective function by warning of injury that should be avoided or treated. However, unlike other somatic submodalities, and unlike vision, hearing, and smell, pain has an urgent and primitive quality, which is responsible for the affective and emotional aspects of pain perception (Basbaum & Jessell, 2000). The concept of pain is further complicated by the fact that its perception is always subjective. In fact, under similar conditions the same stimulus can produce different responses in different individuals (Merskey & Bogduk, 1994). Moreover, many people report pain in the absence of tissue damage or any likely pathophysiological cause; usually this happens for psychological reasons. The highly individual and subjective nature of pain makes difficult to treat it clinically. There are no "painful stimuli" that invariably elicit the perception of pain in all individuals. For example, many wounded soldiers do not feel pain until they are safely removed from battle. Similarly, athletes often do not detect their injuries until their game is over (Basbaum & Jessell, 2000).

Pain can be subdivided in physiological pain and pathological or clinical pain. Physiological pain (also called acute pain, sometimes referred to as ''good'' pain) is adaptive, transient, and has a protective role that warns of potential tissue damage in response to a noxious stimulus. Pathological pain, or clinical pain (also called chronic, ''bad'' pain) is usually maladaptive, persistent, and serves no meaningful defensive, or other helpful purpose (Cao & Zhang, 2008). This kind of pain is mainly subdivided into neuropathic pain, i.e. pain associated with damage or dysfunction of the peripheral nervous system (PNS) and central nervous system (CNS), and inflammatory pain, i.e. pain related to peripheral tissue damage/inflammation (e.g. arthritic pain). In addition, other types of pathological pain, such as cancer pain, and pain elicited by continuous infusion of morphine, share some features with inflammatory and neuropathic pain but also have their distinct characteristics (Brennan et al., 1996; Mantyh et al., 2002).

Pathological pain is typically characterized by hyperalgesia (increased responsiveness to noxious stimuli) and allodynia (painful responses to normally innocuous stimuli), as well as by spontaneous pain. Pain hypersensitivity is not only produced in the injured tissue or territory (innervated by the injured nerve), but also spread to the adjacent non-injured regions or the extraterritory (extraterritorial pain) and to the contralateral body (mirror-image pain). This exaggerated pain is thought to result from peripheral sensitization (increase in sensitivity of nociceptive primary afferent neurons) and central sensitization (hyperexcitability of nociceptive neurons in the CNS (Cao & Zhang, 2008).

In the following paragraphs the basic principles of nociception, as well as the nociceptive neuronal pathways associated to the trigeminal perception of pain, will be discussed. Finally, the emerging role(s) of CNS and PNS glial cells in pain genesis and maintenance will be analyzed.

1.1.1 Molecular basis of nociception

Nociception is the process by which intense thermal, mechanical, or chemical stimuli are detected by a subpopulation of peripheral nerve fibers, called nociceptors (Basbaum et al., 2009). The cell bodies of nociceptors are located in the dorsal root ganglia (DRG) for the body, and the trigeminal ganglia (TG) for the head district, and have both a peripheral and central axonal branch that innervates their target organ and the spinal cord/brainstem, respectively (Lazarov, 2002). There are two major classes of nociceptors (Meyer at al., 2008). The first includes medium diameter myelinated (Aδ) afferents that mediate acute, well-localized "first" or fast pain (conducting signals at about 5-30 m/s). These myelinated afferents differ considerably from the larger diameter and rapidly conducting Aβ fibers that respond to innocuous mechanical stimulation (i.e., light touch). The second class of nociceptor includes small diameter unmyelinated "C" fibers that convey poorly localized, "second" or slow pain (conducting signals at a rate of less than 1.0 m/s).

Neuroanatomical and molecular characterization of nociceptors has further demonstrated their heterogeneity, particularly for the C fibers (Basbaum et al., 2009). For example, the so-called "peptidergic" population of C nociceptors releases neuropeptides, substance P (SP), and calcitonin-gene related peptide (CGRP); they also

express the TrkA neurotrophin receptor, which responds to nerve growth factor (NGF). The nonpeptidergic population of C nociceptors expresses the c-Ret neurotrophin receptor that is targeted by glial-derived neurotrophic factor (GDNF), and a large percentage of the c-Ret-positive population also binds the IB4 isolectin and expresses the specific purinergic $P2X_3$ receptor subtypes (see also *Paragraph 1.4.3*, Ruan & Burnstock, 2003; Basbaum et al., 2009). Nociceptors can also be distinguished according to their differential expression of channels that confer sensitivity to heat (TRPV1), cold (TRPM8), acidic milieu (ASICs), and chemical irritants (TRPA1; Julius & Basbaum, 2001). These functional and molecular heterogeneous classes of nociceptors are associated with specific functions.

Primary nociceptive fibers have a unique morphology, called pseudo-unipolar, where both central and peripheral terminals emanate from a common axonal stalk. The majority of proteins synthesized by the DRG or TG neurons are distributed to both central and peripheral terminals (Basbaum et al., 2009). The biochemical equivalency of central and peripheral terminals means that the nociceptor can send and receive messages from either end. During neurogenic inflammation, in fact, a peripheral release of neuropeptides induces both local vasodilatation and extravasation of plasma proteins, and release of neurotransmitters in the CNS (see *Paragraph 1.2.2*).

1.1.2 The trigeminal nerve and the spinal-trigeminal system

The trigeminal nerve (the fifth cranial nerve) is the largest cranial nerve and functions as the great sensory nerve of the head and face districts, and the motor nerve for muscles involved in mastication (Gray, 2000). The posterior scalp and the neck are instead innerved by spinal nerves between the C2 and C5. The trigeminal nerve emerges from the side of the pons, near to its upper border, by a small motor and a large sensory root (Figure 1.1; Gray, 2000). The fibers of the motor root arise from the brainstem (see also below), while fibers of the sensory root arise from the cells of the trigeminal ganglion, and provide the tactile, proprioceptive, and nociceptive afference of the face and mouth. At a distance of about 1-2 cm from the brainstem, the trigeminal nerve expands, to form the conspicuous trigeminal ganglion (see also below; Castano & Donato, 2001). Three major nerve branches emerge from the ganglion, namely the ophthalmic, the maxillary, and the mandibular nerve (Figure 1.1). The ophthalmic and

maxillary nerves have purely sensory functions, while the mandibular nerve has both sensory and motor functions (Castano & Donato, 2001).

The *Ophthalmic Nerve*, or first (*V1*) division of the trigeminal nerve, supplies branches to the cornea, ciliary body, and iris; to the lacrimal gland and conjunctiva; to the part of the mucous membrane of the nasal cavity; and to the skin of the eyelids, eyebrow, forehead, nose and parts of the meninges (the dura and blood vessels). It is the smallest among the three divisions, and arises from the upper part of the trigeminal ganglion.

The *Maxillary Nerve*, or second (*V2*) division of the trigeminal nerve is intermediate between the ophthalmic and mandibular, both in position and size. It begins at the middle of the semilunar ganglion, and supplies the upper lip, lateral portions of the nose, part of the oral cavity, mucosa of the nasal cavity, maxillary sinus, upper jaw and roof of the mouth, upper dental arch, ethmoid and sphenoid sinuses, and parts of the meninges (Gray, 2000).

The *Mandibular Nerve*, or third (*V3*) division of the trigeminal nerve, supplies teeths and gums of the mandible, the skin of the temporal region, the auricula, the lower lip, the lower part of the face, and the muscles of mastication; it also supplies the mucous membrane of the anterior two-thirds of the tongue. It is the largest of the three divisions, and consists of two roots: a large, sensory root proceeding from the inferior angle of the semilunar ganglion, and a small motor root (the motor part of the trigeminal nerve), which passes beneath the ganglion, and unites with to the sensory root, just after its exit through the foramen ovale (Gray, 2000).

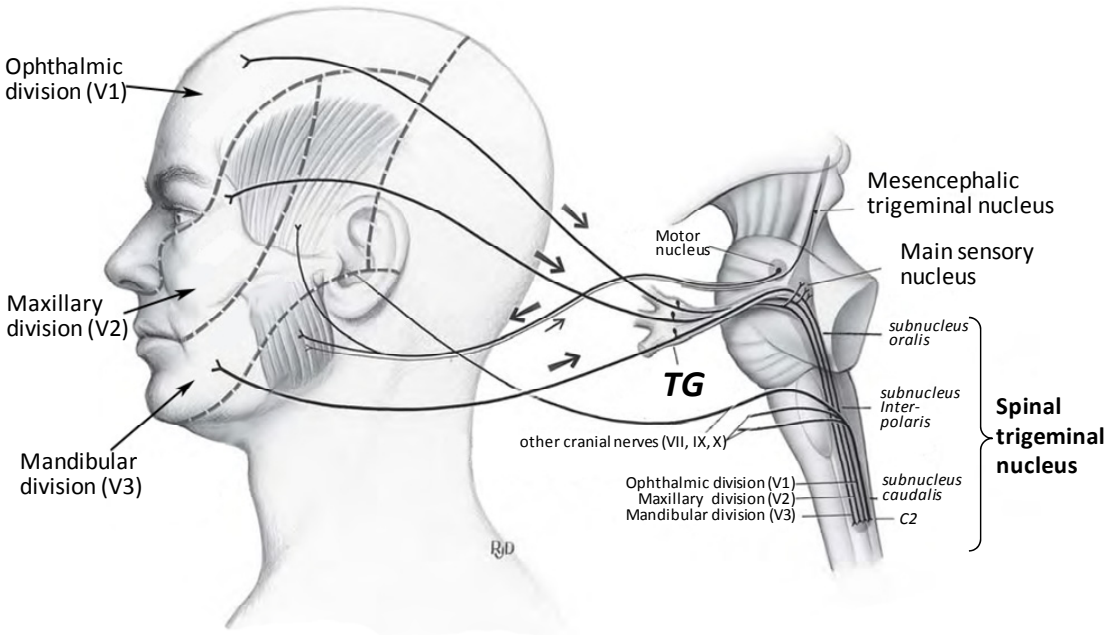


Figure 1.1

Neuroanatomical organization of the spinal-trigeminal system.

The trigeminal nerve consist of three major branches that emerge from the trigeminal ganglion (TG): the ophthalmic (V1), the maxillary (V2), and the mandibular nerve (V3). The trigeminal sensory fibers from the TG enter the pons and terminate in the trigeminal nuclear complex which is formed by three sensitive nuclei: the spinal trigeminal nucleus, the main trigeminal nucleus, and the mesencephalic trigeminal nucleus. The spinal trigeminal nucleus can be further subdivided from caudal to rostral in: subnucleus caudalis, *subnucleus interpolaris* and *subnucleus oralis. Modified from Noback et al., 2005.*

The trigeminal ganglion

The trigeminal ganglion (also called *Semilunar* or *Gasserian* ganglion) occupies a cavity (*cavum Meckelii*) in the dura mater covering the trigeminal impression near the apex of the petrous part of the temporal bone (Figure 1.1; Gray, 2000). It represents the cranial analog of DRGs in the PNS. In the ganglion parenchyma the somata of pseudounipolar neurons are contained, and a single neurite dividing into a central and a peripheral process, which convoy the sensory sensations from the head regions to the trigeminal nuclei of the brainstem, arises from each cell body (Castano & Donato, 2001). On the basis of their morphology, the neuronal cells are traditionally divided into two classes: large light (A) and small dark (B) cells (Lazarov, 2002). It is known that thick myelinated (Aα/β) fibers originate from the large light A-cells and thin myelinated (type Aδ) and unmyelinated (type C) fibers derive from the small dark B-cells (Lazarov,

2002). According to the diameter of their somata, TG neurons can be also divided in three distinct groups: small (20-30 μm), medium-sized (30-50 μm), and large-sized cells (50-80 μm; Lazarov, 2002).

The neuronal somata of sensory neurons are completely enveloped by a layer of small glial cells called satellite glial cells (SGCs; Hanani, 2005). In general, each sensory neuron has its own SGCs sheath (Figure 1.2), and the neuron and its surrounding SGCs form a distinct morphological and functional unit (Hanani, 2005). Originally it was believed that that SGCs provide a mere trophic support to the sensory neurons. However, starting from the observations that SGCs become reactive and increase considerably in number after peripheral nerve injury (Stephenson & Byers, 1995; Hanani et al., 2002), it was suggested, and later demonstrated, that they actively participate in nociception. For this reason, a special discussion on the roles of SGCs in pain transmission will be provided in the next paragraph (*Paragraph 1.1.3*).

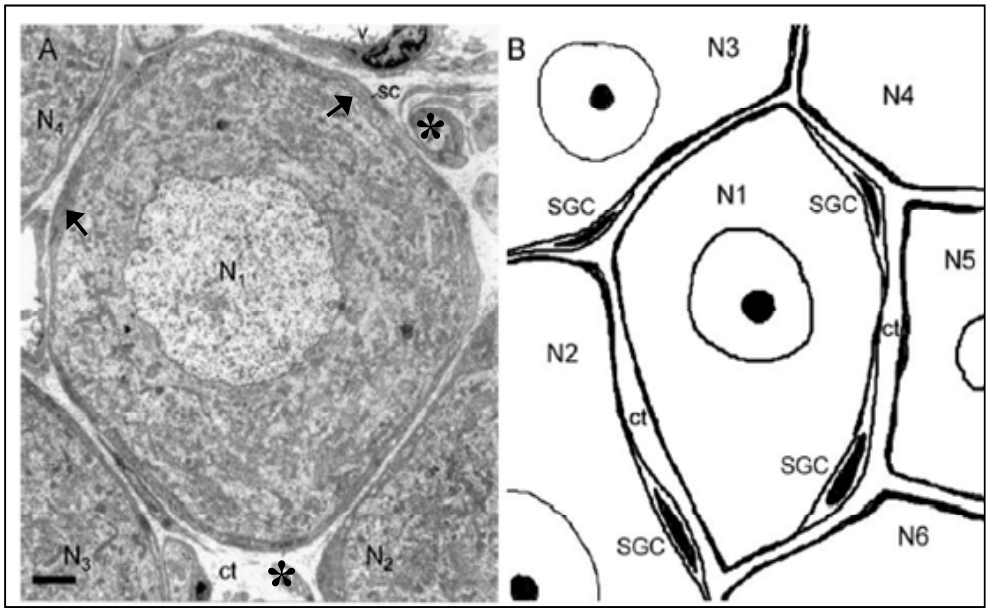


Figure 1.2

The neuron-SGCs morphological unit.

A, *An electron micrograph from the mouse dorsal root ganglion, showing the layer of SGCs (sc) around sensory neurons (N1-4). Neurons with their associated SGCs are separated by a connective tissue space (ct). Asterisks indicate non-myelinated axons surrounded by Schwann cells. Arrows indicate two SCG cell bodies. Calibration bar, 2 μm.* ***B,*** *Schematic drawing of the neuron-SGCs morphological unit. Modified from Hanani, 2005.*

The brainstem nuclei

The central processes of TG neurons enter the pons via the sensory root of the trigeminal nerve, and terminate in the *trigeminal nuclear complex* which is formed by three sensitive nuclei: the *spinal trigeminal nucleus*, the *main trigeminal nucleus*, and the *mesencephalic trigeminal nucleus* (Figure 1.1; Capra & Dessem, 1992). The trigeminal nerve carries most of the sensory information from the face district, even if sensation from certain parts of the mouth, ear and meninges are carried by somatic fibers of the facial, the glossopharyngeal, and the vagus nerves. All sensory fibers from these nerves terminate in the *trigeminal nuclear complex* that contains a complete sensory map of the face and mouth. Near the main trigeminal nucleus the *trigeminal motor nucleus* of the fifth nerve is also located (Figure 1.1), where motor branches of the trigeminal nerve originate and are then distributed through the mandibular branch.

The *spinal trigeminal nucleus* extends throughout the entire brainstem, from the midbrain to the medulla, and overlaps with the dorsal horn of the cervical spinal cord. Three major cytoarchitectonic subdivisions of this nucleus are generally recognized along its rostrocaudal axis. The most caudal is the *subnucleus caudalis*, which extends from C2 or C3 caudally to the obex rostrally; the *subnucleus interpolaris* extends from the obex caudally to the caudal pole of the facial motor nucleus; the *subnucleus oralis* extends from the caudal pole of the facial motor nucleus to the caudal end of the trigeminal motor nucleus (Figure 1.1; Castano & Donato, 2001). The caudal parts of *the spinal trigeminal nucleus* receive pain/temperature sensory sensations, while the rostral parts receive touch/position sensory information. Moreover, a specific dorso-ventral somatotopical organization of the *spinal trigeminal nucleus* can be distinguished: the trigeminal fibers innervating the ipsilateral mandibular division are most dorsally situated, the maxillary fibers are in the middle, and the ophthalmic fibers are in the most ventral part of the tract (Capra & Dessem, 1992).

The *main trigeminal nucleus* receives touch/position sensory fibers from the face. It is located in the pons, close to the entry site of the fifth nerve. The main trigeminal nucleus contains a touch/position sensory map of the face and mouth, just as the spinal trigeminal nucleus contains a complete pain/temperature map. The main nucleus is analogous to the dorsal column nuclei (i.e. the gracile and cuneate nuclei) of the spinal cord, which contain a touch/position map of the rest of the body (Castano & Donato, 2001).

The *trigeminal mesencephalic nucleus* (MTN) is the only known nucleus situated within the CNS that contains the cell bodies of primary afferent neurons. It could be considered a ganglion "embedded" in the brainstem. It is also unique in that its neurons constitute one distinct functional class of trigeminal sensory neurons, i.e. proprioceptive neurons. MTN neurons receive information, via the trigeminal nerve, from the masticatory muscle spindles, and from mechanoreceptors in the periodontal ligament. In turn, they transfer information to the muscles of mastication through their projections to trigeminal motoneurons and premotoneurons for controlling jaw movements, or to the cerebral cortex (via the thalamus) for transmission of sensory feedback (Lazarov, 2002).

The thalamus/cortex nuclei

From the *trigeminal nuclear complex*, the pain/temperature information from the face ascend in the trigeminal lemniscus to the *ventral posteromedial nucleus* (VPM) of the thalamus. From this nucleus, the pain/temperature information is projected to the primary and secondary sensory cortex. Other pain/temperature fibers are alternatively sent: (i) to the medial dorsal thalamic nucleus, which projects to the anterior cingulate cortex, (ii) to the ventromedial nucleus of the thalamus, which projects to the insular cortex, and (iii) to the intralaminar nuclei of the thalamus, which project diffusely to all parts of the cerebral cortex (Castano & Donato, 2001). Other impulses project to the ventrolateral area of the caudal periaqueductal gray region. This latter region is involved in the craniovascular pain not only through ascending projections to the thalamus, but also through descending modulation (mainly inhibitory) of nociceptive afferent information (Figure 1.3, see also below).

1.1.3 Role of non-neuronal cells in pain transmission

Despite great efforts in the last decades towards the understanding of its pathophysiology, and the development of new drugs, chronic pain is a difficult to manage and disabling condition. The reason for this failure may be partially due to the fact that most of the available drugs target neurons (Dworkin et al., 2010), whereas increasing evidence now indicates that glial cells in both sensory ganglia and CNS also play an important role in chronic pain genesis by participating to the development of

hyperalgesia and allodynia (Ren & Dubner, 2008; McMahon & Malcangio, 2009). As already mentioned in the previous paragraph, SGCs wrap around the cell bodies of primary neurons in sensory ganglia, forming a morphological and functional unit (Hanani, 2005). Like other glial cells (see also below), SGCs responds to nerve injury by upregulating glial fibrillary acidic protein (GFAP) expression and undergoing division in response to chronic pain (Takeda et al., 2007). In addition, following nerve injury or inflammation, there is also an unregulated expression of gap junctions, and an increased coupling between SGCs (Ledda et al., 2009; Zhang et al., 2009). It has been speculated that these changes in SGCs coupling might be directly correlated to the generation or maintenance of neuropathic pain. Indeed, in a model of facial neuropathic pain the specific inhibition of Connexin 43 expression levels (one of the major gap junctions subunits expressed by glial cells; Vit et al., 2006) by RNA interference resulted in a strong reduction of pain behavior (Jasmin et al., 2010).

A non-synaptic cross communication between neurons and SGCs is probably at the basis of SGCs activation following chronic pain (Takeda et al., 2009). Indeed, under physiopathological conditions, the somata of sensory neurons release in the extracellular space chemical mediators, such as CGRP, SP, and ATP, which in turn activate their receptors expressed by other neurons or by surrounding SGCs. Activated SGCs in turn release cytokines, such as Interleukin (IL)-1β and tumor necrosis factor α (TNFα), which may further potentiate neuronal excitability, leading to an auto-amplifying loop of neuronal sensitization (Takeda et al., 2009). Noteworthy, the purinergic system participate primarily to this bidirectional neuron-glial communication inside the sensory ganglia (Zhang et al., 2007; Suadicani et al., 2009), and in the *Paragraph 1.4* more details on this subject will be provided.

Since, differently from the CNS, in sensory ganglia there is no blood-brain barrier, immune cells are able to freely move in and out of the ganglion (Ohara et al., 2009). In fact, each sensory ganglion harbors a large number of resident macrophages, representing the first immune cells to respond to nerve injury or viral infection; within days however, circulating macrophages and other immune cells invade the sensory ganglion (Gowrishankar et a., 2010). Inside the ganglion, activated macrophages divide and contribute to the persistence of chronic pain by responding to a mix of molecules including neurotransmitters, growth factors, and cytokines, most of which have been shown to cause increased pain behavior in animal models (Morin et al., 2007; Zhuang et

al., 2007). These data therefore indicate that also macrophages are directly involved in the regulation of chronic pain.

In addition, also glial cells in the CNS (mainly astrocytes and microglia) participate in the development and maintenance of chronic pain, and may as well represent innovative targets for developing new pain killer molecules (McMahon & Malcangio, 2009). In fact, during chronic pain, both microglia and astrocytes become activated and undergo to cell proliferation (Ajami et al., 2007; McMahon & Malcangio, 2009). Compared to resting microglial cells, activated microglia display shorter and thicker ramifications, functional changes (migration, phagocytosis and production/ release of pro-inflammatory substances), and upregulation of cell specific molecules, such as the complement receptor 3 (also known as CD11b), and the ionized calcium-binding adapter molecule (Iba1; Smith, 2010; Vallejo et a., 2010). Astrocytes become activated to a lesser extent than microglia and at later time points after injury; they exhibit hypertrophic cell bodies with thick processes, upregulated GFAP levels, and increased production and release of a variety of pro-inflammatory substances (Raghavendra & Deleo, 2003).

Similar to sensory ganglia, a bidirectional neuron-glia communication is responsible the neuronal sensitization in the CNS. Indeed, glia is first activated by neurotransmitters and other mediators released from primary afferent terminals involved in pain transmission (Ren & Dubner, 2008): these molecules include neurotransmitters, such as SP and CGRP (Guo et al., 2007), purinergic agents (Tsuda et al., 2010; see also *Paragraph 1.4)*, glutamate (Kumar et al., 2010), opioid peptides (Watkins et al., 2007), and chemokines (Zhuang, 2007). Glial cells in turn release a variety of substances including inflammatory cytokines (White et al., 2007), prostaglandins (Zhao et al., 2007), neurotrophic factors (Coull et al., 2005) and nucleotides (Tsuda et al., 2010), that modulate neuronal activity and facilitate pain transmission, eventually contributing to hyperalgesia and allodynia. To further sustain the hypothesis that glial cells are involved in chronic pain development and maintenance, it has been demonstrated that the pharmacological administration of glial cells inhibitors, like pentoxifylline, fluorocitrate and minocycline, effectively attenuates the development of both neuropathic (Mika, 2008) and inflammatory pain (Clark et al., 2007).

1.2 MIGRAINE

Migraine is an episodic neurovascular disorder, typically characterized by spontaneous and recurrent attacks of unilateral headache, with associated autonomic symptoms, like nausea, vomiting, and sensorial hypersensitivity. It belongs to the primary headaches family, which include also the tension-type headache, the cluster headache, trigeminal autonomic cephalalgias, and other headaches (Edvinsson & Uddman, 2005). Migraine is an highly disabling brain disorder that affects about the 15% of the population, and its socio-economic implications are extensive with considerable impact on productivity and quality of life. It has been estimated to be the most costly neurological disorder in the European Community with €27 billion spent per year (Goadsby, 2007). Accordingly, it is now ranked at the 19th place among all world-wide diseases causing disability by the World Health Organization (Lipton et al., 2004).

Migraine can be classified into two major categories: the *migraine without aura* (MO) and the *migraine with aura* (MA). The first one, that occurs in about 80% of patients, is a clinical syndrome characterized by headache with specific features and associated symptoms. The second category, MA, is primarily characterized by focal neurological symptoms that usually precede or sometimes accompany the headache in the remaining 20% of migraineurs (Headache Classification Committee of the international Headache Society, 2004). The diagnostic criteria for these two categories are summarized in Table 1.1.

One of the most important aspects of the pathophysiology of migraine is the inherited nature of the disorder. It is clear from clinical practice that many patients have first degree relatives who also suffer from migraine. However, only in few cases it has been possible to identify specific genes responsible for the inherited component. This is the case of the *Familial Hemiplegic Migraine* (FHM) which is caused by mutations of specific ion channels genes: the α1 subunit of the Cav2.1 (P/Q) type voltage-gated Ca^{2+} channel (CACNA1A), causing the FHM type-I; the ATP1A2 gene, causing the FHM type-II; and the Na^{+} channel SCN1A, causing the FHM type III (see also below; Goadsby, 2007).

Migraine is a complex neurological disorder, and despite recent advances in the comprehension of it pathophysiology, its pharmacological treatment still remains

unsuccessful in a significant number of patients. For this reason, new efforts in clarifying its mechanisms, in the discovery of innovative targets, and in the developing of new drugs are strongly needed.

Table 1.1 ***Diagnostic criteria for migraine.***

Migraine without aura

A. At least five attacks fulfilling criteria B-D.

B. Headache attacks lasting 4 to 72 hours.

C. Headache has at least two of the following characteristics:

    1. Unilateral location

    2. Pulsating quality

    3. Moderate or severe pain intensity

    4. Aggravation by or causing avoidance of routine physical activity

D. During headache at least one of the following:

    1. Nausea and/or vomiting

    2. Photophobia and phonophobia

E. Not attributed to another disorder.

Migraine with aura

A. At least two attacks fulfilling criteria B-D.

B. Aura consisting of at least one of the following, but no motor weakness:

    1. Fully reversible visual symptoms including positive features (e.g, flickering lights, spots, or lines) and/or negative features (i.e, loss of vision)

    2. Fully reversible sensory symptoms including positive features (i.e, pins and needles) and/or negative features (i.e, numbness)

    3. Fully reversible dysphasic speech disturbance

C. At least two of the following:

    1. Homonymous visual symptoms and/or unilateral sensory symptoms

    2. At least one aura symptom develops gradually over ≥ 5 minutes, and/or different aura symptoms occur in succession over ≥ 5 minutes

    3. Each symptom lasts ≥ 5 and ≤ 60 minutes

D. Headache fulfilling criteria B-D for migraine without aura begins during the aura or follows aura within 60 minutes

E. Not attributed to another disorder.

1.2.1 Description of the migraine attack

The migraine attack can consist of four main phases: premonitory, aura, headache, and resolution.

The premonitory phase

Premonitory symptoms occur in 20-60% of patients with migraines, hours to days before headache onset. The most common symptoms are feeling tired or weary (72%), difficulty concentrating (51%), and a stiff neck (50%; Silberstein, 2004). Patients who reported premonitory symptoms usually predicted their incoming headaches.

The aura phase

Migraine aura consists of focal neurological symptoms that precede, accompany, or (rarely) follow an attack. Aura usually develops over 5-20 min, lasts for less than 60 min, can be visual, sensory, or motor, and can involve language or brainstem disturbances (Table 1.1; Lipton et al., 2004). Headache usually follows within 60 min from the end of the aura phase. Patients can have multiple aura types: most patients with a sensory aura also have a visual aura. Auras vary in complexity; simple auras include scotomata, simple flashes (phosphenes), specks, geometric forms, and shimmering in the visual field. More complicated visual auras include teichopsia or fortification spectra (characteristic aura of migraine), metamorphopsia, micropsia, macropsia, zoom vision, and mosaic vision. Paraesthesias are often cheiro-aural, with numbness migrating from the hand up the arm and to the face (Silberstein, 2004). Apraxia, aphasia, and agnosia, states of altered consciousness associated with déjà vu, and elaborate dreamy, nightmarish, trance-like, or delirious states can occur (Silberstein, 2004).

The headache phase

The median frequency of migraine attacks is 1-5 per month The typical headache is unilateral, of gradual onset, throbbing (85%), moderate to marked in severity, and aggravated by movements (Silberstein, 2004). Pain can be unilateral, bilateral, or start on one side and become generalized. It lasts 4-72 h in adults and 1-72 h in children (Lipton et al., 2004). Nausea occurs in almost 90% of patients, while vomiting occurs in

about one third (Lipton et al., 2004). Sensory hypersensitivity results in patients seeking for a dark, quiet room. Blurry vision, nasal stuffiness, anorexia, hunger, diarrhoea, abdominal cramps, polyuria, facial pallor, sensations of heat or cold, and sweating might occur. Depression, fatigue, anxiety, nervousness, irritability, and impairment of concentration are common (Silberstein, 2004).

The resolution phase

After the headache, the patient often feels tired, washed out, irritable, or listless, and can have impaired concentration, scalp tenderness, or mood changes. Some feel unusually refreshed or euphoric after an attack; others experience depression and malaise (Silberstein, 2004).

1.2.2 How and where does the migraine attack originate?

Despite the strong progresses made for a better understanding of migraine pathophysiology, a debate about its origin is still ongoing: some scientists argue for a central origin of the headache pain (for a review see Goadsby et al., 2009), whereas others believe that the headache is triggered by activation of peripheral nociceptors (for a review see Olesen et al., 2009). In the following paragraphs the older and newer theories raise to better explain this complex disorder will be discussed.

For most of the last part of the 20th century, migraine pain was theorized to be due to dilatation of cranial vessels. According to this "vascular theory of migraine", migraine aura would be caused by transient ischemia induced by vasoconstriction, and the headache would arise from rebound abnormal vasodilatation of intracranial arteries, and consequent mechanical activation of perivascular sensory fibers (Wolff, 1948; Pietrobon & Striessnig 2003). Two-thirds of migraineurs experience relief from pain after occlusion of the carotid artery ipsilateral to the side of headache, and distension of major cerebral vessels by balloon dilatation leads to pain referred to the ophthalmic division of the trigeminal nerve (Goadsby et al., 2009). However, more recent studies clearly indicate that vascular changes in the regional cerebral blood flow are totally unrelated to the phase of the attack; indeed, blood flow could be reduced or normal during the headache phase (Olesen et al., 1981; Olesen et al., 1990). Moreover, it was

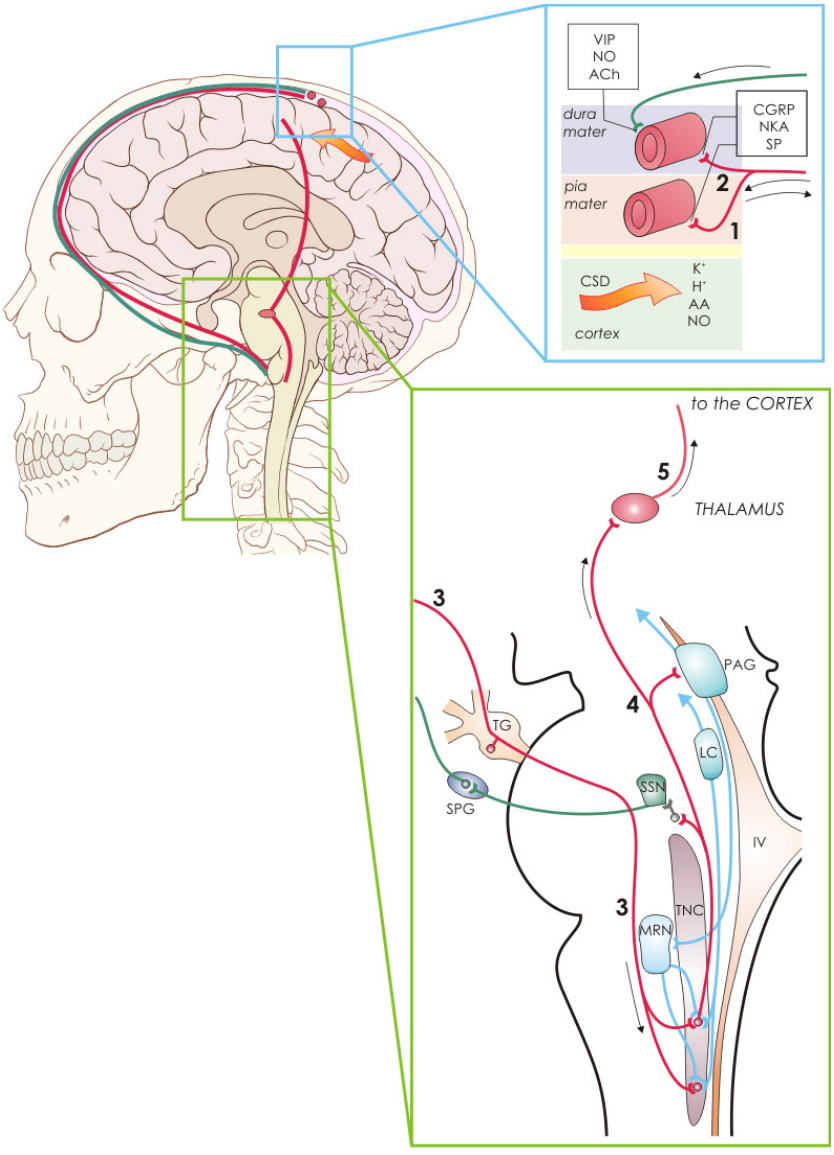
shown that the vasoactive intestinal polypeptide (VIP), can induce vasodilatation without triggering migraine, and migraine can be induced without dilatation in the diameter of the middle cerebral artery, the principal artery of the dura mater (Kruuse et al., 2003). More recently, using high resolution 3T magnetic resonance angiography, it has been reported that migraine triggered by nitroglycerin occurs without any continuing change in intracranial or extracranial vessels (Schoonman et al., 2008). In conclusion, vasodilatation is neither necessary nor sufficient for migraine genesis.

Within the skull, pain sensitivity is primarily restricted to the meningeal blood vessels, which are densely innervated by nociceptive sensory afferent fibers of the ophthalmic division of the trigeminal nerve (Pietrobon, 2005). It has been therefore hypothesized that migraine could arise from a primary dysfunction leading to activation and sensitization of the trigemino-vascular system. According to this view, the sensitization of meningeal nociceptors induced by inflammatory events may be an essential process in migraine. This idea was popular for nearly two decades in form of "the hypothesis of neurogenic inflammation" (Moskowitz, 1993). The key elements of neurogenic inflammation are: (i) vasodilatation, (ii) plasma extravasation and (iii) mast cell degranulation (which can experimentally be induced by stimulation of meningeal afferents) and the subsequent release of pro-inflammatory neuropeptides from these nerve fibers (Figure 1.3; Buzzi et al. 1995; Williamson & Hargreaves 2001). In fact, the neuropeptides SP and neurokinin-A are known to cause mainly plasma extravasation, while CGRP, VIP but also nitric oxide (NO) are powerful vasodilator of intracranial arteries in animals and humans (Pietrobon, 2005). CGRP also induces plasma extravasation and mast cell degranulation (Ottosson & Edvinsson, 1997). The importance of CGRP in migraine neurobiology is also supported by the fact that intravenous infusion of CGRP in migraineurs generates a delayed headache with most of the characteristics of migraine (Tepper & Stillman, 2008). Moreover, during an attack of migraine, plasma levels of CGRP, but not SP, have been found to be increased in the venous outflow from the head (Goadsby et al. 1990; Juhasz et al. 2005). Accordingly, relief of migraine corresponds to reduction of blood CGRP (Tepper & Stillman, 2008). For these reasons, CGRP receptor antagonists have been providing good results in clinical studies for migraine therapy (see below). Activation of the meningeal trigemino-vascular afferents finally leads to activation of the spinal

trigeminal nucleus, and impulses are then carried rostrally to brain structures involved in the perception of pain (see *Paragraph 1.1.2* and Figure 1.3).

Figure 1.3

Neuronal pathways involved in trigeminovascular activation and pain processing.



The development of migraine depends on the activation of nociceptive afferent fibers of trigeminal ganglion (TG) neurons innervating the blood vessels in the meninges and the subsequent activation of second-order dorsal horn neurons in the trigeminal nuclear complex (TNC) and neurons in structures involved in the processing and perception of pain, like the thalamus, the caudal periaqueductal gray region (PAG), and the cortex. The PAG is involved in craniovascular pain not only through ascending projections to the thalamus but also through descending modulation (mainly inhibitory) of nociceptive afferent information via projections to serotonergic neurons in the nucleus raphae magnus (MRN)

IV, fourth ventricle; ACh, acetylcholine; CGRP, calcitonin gene-related peptide; LC, locus coeruleus; NKA, neurokinin A; NO, nitric oxide; SP, substance P; SPG, superior sphenopalatine ganglion; SSN, superior salivatory nucleus; VIP, vasoactive intestinal peptide.

The crucial point of this theory is that neurogenic inflammation depends on a massive activation of meningeal afferents, and this alone would cause the headache. Even if there is no a satisfying idea of which spontaneous process causes the primary activation of meningeal afferents, a complex neurological event taking place in the

cortex during the aura phase, the so called Cortical Spreading Depression (CSD), has been proposed as good trigger candidate for neurogenic inflammation, and possibly for headache pain. CSD is a slow propagating (2-6 mm/min) wave of sustained strong neuronal depolarization that generates a transient intense spike activity in neurons as it progresses into the cortex, followed by a long-lasting neural suppression (Lauritzen, 1994). It has been demonstrated that CSD and the aura phase that precede a migraine attack are strongly correlated events (Eikermann-Haerter & Ayata, 2010). Indeed, aura frequently consists in a visual scotoma (an area of lost vision) with a scintillating border that usually begins near the center of vision as twinkling stars and then develops into an expanding circle that slowly moves across the visual field toward the periphery (Figure 1.4). By analyzing his own visual aura, in 1941 Lashley postulated that the scotoma resulted from a region of depressed neural activity in the visual cerebral cortex and that the scintillations resulted from a bordering region of intense cortical excitation (Lashley, 1941). A few years later, an electrophysiological correlate was reported in the rabbit cerebral cortex and was termed "Cortical Spreading Depression" (Leao & Morison, 1945). In animals, CSD can be triggered by focal (electrical, mechanical, or high K^+) stimulation of the cerebral cortex, and its depolarization phase is associated with an increase in regional cerebral blood flow (rCBF), whereas the phase of reduced neural activity is associated with a reduction in rCBF (Lauritzen, 1994). The similarities between migraine visual aura and CSD led to the hypothesis that CSD was responsible for the migraine aura (Lauritzen, 1994). However, only in 2001 Hadjikhani and colleagues (2001) clearly correlated this two neurological events. In fact, by using blood oxygenation level-dependent functional magnetic resonance imaging (BOLD fMRI), it was demonstrated that several CSD-associated cerebrovascular changes happened in the cortex of migraineurs during a visual aura (Hadjikhani et al., 2001). A clear temporal correlation was established between the initial features of the aura percept (i.e., scintillations beginning in the paracentral left visual field) and the initial increase in the mean BOLD signal, reflecting cortical hyperemia (Figure 1.4). The subsequent decrease in mean BOLD level was temporally correlated with the scotoma following the scintillations. The BOLD signal changes developed first in the occipital cortex, contralateral to the visual changes. It then slowly migrated (3.5 mm/min) toward more anterior regions of the visual cortex, representing peripheral visual fields, in agreement

with the progressive movement of the scintillations and scotoma from the center of vision toward the periphery (Figure 1.4; Hadjikhani et al., 2001).

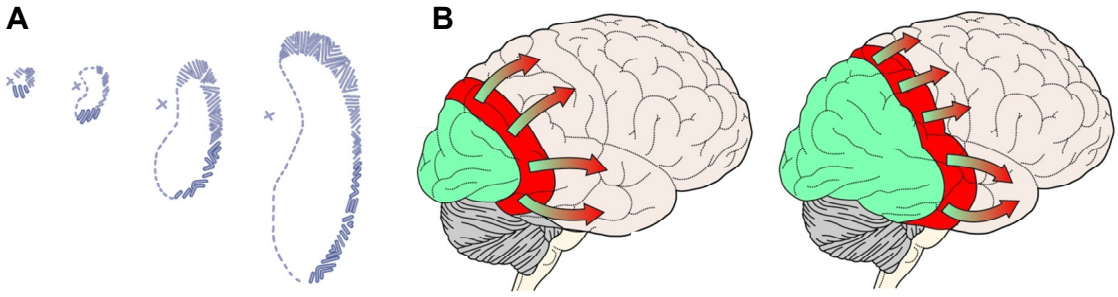


Figure 1.4

Cortical spreading depression during migraine aura.

A, *Drawing illustrating the progression of a visual aura over time, consisting of a scotoma (an area of lost vision; in figure is represented within the dashed line), and scintillating borders. The cross indicates the fixation point.* ***B,*** *Cortical spreading depression that causes migraine aura consists of a wave of depolarization that spreads through the cerebral cortex at a rate of 2-6 mm per min (red area). The depolarization phase is associated with an increase in regional cerebral blood flow (rCBF). Shortly after this, repolarisation takes place and is accompanied by a prolonged decrease in rCBF (green area). A temporal correlation between the initial features of the aura (scintillations beginning in the paracentral visual field) and the rCBF is present. The subsequent decrease in rCBF is temporally correlated with the scotoma. Modified from Lauritzen, 2001.*

This observation was successfully confirmed by other neurological evidence (Bowyer et al., 2001), and therefore it can be concluded that visual aura arises from CSD. Besides causing the aura, CSD also has been proposed as a trigger of headache (Moskowitz, 1984). The CSD can activate the meningeal trigemino-vascular system and downstream pain pathways in rodents leading to meningeal inflammation and plasma extravasation (Moskowitz et al., 1993; Bolay et al., 2002). Although the mechanism of activation remains unknown, it has been assumed that H^+, K^+, NO, and other agents released into the cortical extracellular space during the CSD, depolarize or activate adjacent perivascular trigeminal nerve endings surrounding local blood vessels. This evidence is further supported by data showing that migraine prophylactic drugs, which

are efficacious in preventing migraine attacks with or without a perceived aura, decreased CSD susceptibility (see P*aragraph 1.2.4*; Ayata et al., 2006).

The facts that only about the 20% of migraineurs experience the aura phase (see above) conflicts with the idea of CSD as primary event in migraine pathogenesis. It could be hypothesized that in patients suffering of *migraine without aura*, CSD could occur in clinically silent area of the cortex, thus it would not give rise to aura symptoms (Woods et al., 1994). An alternative view considers migraine aura and headache as parallel processes and proposes that the primary cause of headache as an episodic dysfunction in brainstem nuclei involved in the central control of nociception. To support this idea two evidence can be considered. First, placement of electrodes in PAG for the treatment of chronic pain can produce migraine-like headaches in non-migraineurs (Raskin et al., 1987). Second, rCBF increases in several areas of the dorsal rostral brainstem during migraine attacks (Bahra et al., 2001). Although the spatial resolution of the imaging techniques does not allow the distinction of most brainstem nuclei, the foci of maximum rCBF increase, as measured by Positron Emission Tomography (PET), coincided with the dorsal raphe nucleus and locus coeruleus in patients with MO, and with the red nucleus and substantia nigra in a patient with MA, during a spontaneous attack (Weiller et al., 1995). Animal studies indicate that these brainstem centers might be involved in the central control of nociception and, in particular, in descending mechanisms of pain inhibition. Compared to other hypothesis on migraine neurobiology, dysfunction of such brainstem structures and networks could not only account for the somatosensory component of migraine (headache) but also for the auditory, olfactory and visual components. Moreover, a locus coeruleus dysfunction could also explain the distractibility and anxiety which is often observed in migraineurs (Goadsby et al., 2009). On the other hand several criticisms can be pointed out to this brainstem generator theory (Pietrobon, 2005; Olesen et al., 2009), and unfortunately, we have to conclude that no clear answers to the crucial question of how and where the migraine pain arises are available so far. Migraine is a complex pathology, and different clinical manifestations can be observed among the migraineurs. It may be well that the primary dysfunction leading to headache could be different among patients, depending on either genotypical and/or phenotypical features. To provide an ultimate answer to this question, additional studies in this field are strongly needed.

1.2.3 Familial hemiplegic migraine

As mentioned before, migraine has a strong genetic component, and an example of a monogenic subtype of migraine is familial hemiplegic migraine (FHM; Pietrobon, 2007; de Vries et al., 2009). This form of migraine is a rare autosomal dominant subtype of MA, whose aura symptoms include motor weakness or paralysis (often, but not always, unilateral). Apart from the motor aura, typical FHM attacks resemble MA attacks. Usually at least three aura symptoms are present in FHM (typically in the temporal order: visual, sensory, motor, aphasic; see Table 1.1), and they last longer than in MA. Also the headache phase last longer in FHM than MA, while all other headache characteristics are similar (Pietrobon, 2007). In addition to typical FHM attacks, some FHM patients can experience atypical severe attacks with signs of diffuse encephalopathy, impairment of consciousness (coma) or confusion, prolonged hemiplegia lasting several days, and in a few cases seizures (Ducros et al., 2001). Moreover, about 20% of FHM families show permanent cerebellar symptoms consisting of progressive cerebellar ataxia with or without nystagmus. Emotional stress and minor head trauma are among the most common triggers of FHM attacks (Ducros et al., 2001). Three genes have been successively identified in FHM families, and, accordingly, three types of FHM are recognized.

Familial hemiplegic migraine type 1 (FHM1)

The first FHM gene identified is CACNA1A, which is located on chromosome 19p13 and encodes for the α1 subunit of neuronal $Ca_V2.1$ (P/Q-type) voltage-gated calcium channel that is widely expressed throughout the CNS (de Vries et al., 2009). All the 21 FHM1 mutations known so far are missense mutations associated with a broad spectrum of clinical features besides hemiplegic migraine, including cerebellar ataxia and epilepsy (de Vries et al., 2009). The $Ca_V2.1$ channels are located in presynaptic terminals and somatodendritic membranes throughout the brain, where they control neurotransmitter release particularly at central excitatory synapses (Pietrobon, 2005). All the FHM1 mutants show an enhanced single channel Ca^{2+} influx in a wide range of mild depolarizations, reflecting an increased channel open probability, mainly due to a shift to lower voltages of channel activation (Tottene et al., 2002). In fact, the analysis of mutant channels in neurons from knock-in mice revealed that FHM1 mutations

produce gain of function of $Ca_V2.1$ channels. Mutant human $Ca_V2.1$ channels also open at lower voltages and more readily than WT channels, and Ca^{2+} influx through mutant channels can occur in response to small depolarizations insufficient to open WT channels. As a consequence, FHM1 mutations produce an increase of $Ca_V2.1$-dependent neurotransmitter release at synapses. Moreover, recent evidence indicates that FHM1 knock-in mice are more susceptible to CSD than wild-type mice. Indeed, by using animal models of FHM1 (the R192Q and S218L knock-in mice) a lower threshold for CSD induction and an increased velocity of propagation of CSD induced by electrical stimulation of the visual cortex *in vivo* were demonstrated (van den Maagdenberg et al., 2004). The CSD threshold is reached when the regulatory mechanisms that keep the local K^+ ion concentrations in the physiological range are overwhelmed by the build-up of K^+ via positive feedback loops. A lower increase of extracellular K^+ can activate the hyper-functional $Ca_V2.1$ channels, leading to an abnormal release of glutamate which in turn initiate the positive feedback cycle leading to CSD. Thus, a relatively weak depolarizing stimulus, as a minor head trauma, which is without consequences in healthy individuals, may release enough glutamate to initiate the positive feedback cycle, therefore initiating the CSD (van den Maagdenberg et al., 2004; Pietrobon, 2007). Altogether, these findings support a key role of the CSD in the pathogenesis of FHM1.

Familial hemiplegic migraine type 2 (FHM2)

The second FHM gene was identified in 2003 in two Italian families (De Fusco et al. 2003). This gene, located on chromosome 1q23, encodes for the α2 subunit of the Na^+,K^+-ATPase pump that utilizes the energy of ATP to actively transport Na^+ ions out of and K^+ ions into the cell. Over 30 FHM2 mutations have been now recognized and, with a few exceptions, are all found in single families (De Fusco et al., 2003). The Na^+,K^+-ATPase pump generates the ion gradients that maintain resting membrane potential and cell volume, and provide the driving force for nutrient and neurotransmitter uptake. Glial and neuronal Na^+,K^+-ATPase pumps play an important role in the clearance of K^+ from the extracellular space during neuronal activity and are also fundamental for the clearance of released glutamate from the synaptic cleft, because the active transport of glutamate into astrocytes and neurons is driven by both Na^+ and K^+ gradients (D'Ambrosio et al., 2002). Impaired clearance of K^+ and glutamate by astrocytes during cortical neuronal activity, consequent to a decreased

number of functional Na^+,K^+-ATPase pumps, would depolarize neurons and enhance glutamate concentration in the synaptic cleft, thus impairing the recovery from neuronal excitation. On the basis of this mechanism, it could be predicted that also FHM2 patients have enhanced susceptibility to CSD: a weak depolarizing stimulus, which would be without consequences in healthy individuals, may be able to produce a local buildup of K^+ concentration above the critical value, leading to CSD in FHM2 patients (Pietrobon et al., 2007).

Familial hemiplegic migraine type 3 (FHM3)

The most recently identifed FHM gene is the SCN1A gene, which is located on chromosome 2q24, and was identified in 2005 in three German families (Dichgans et al. 2005). SCN1A encodes for the α1 subunit of neuronal $Na_V1.1$ voltage-gated sodium channels and represents an already well-known gene with over 100 truncating and missense mutations that are associated with several kind of epilepsy (de Vries et al., 2009). Five FHM3 mutations have been identified so far (Castro et al. 2009; Vahedi et al. 2009), and for three of them their functional consequences have been investigated. When the FHM3 Q1489K and L1649Q mutations have been inserted in the cardiac $Na_V1.5$ cDNAs, various gain-of-function effects were revealed (Vanmolkot et al. 2007). However, when these mutations were expressed in the more appropriate $Na_V1.1$ protein, clear loss-of-function effects were detected (Kahlig et al. 2008). The third FHM3 mutation, L263V, that causes FHM3, and in the majority of carriers also generalized tonic-clonic epilepsy, has essentially a gain-of-function effect (Kahlig et al. 2008). It was hypothesized that loss of sodium channel activity primarily disturbs the functioning of inhibitory neurons where the $Na_V1.1$ are expressed normally, whereas gain-of-activity has a predominant effect on excitatory neurons. The functional consequences of FHM3 mutations can be very complex; indeed, depending on the test paradigm the Q1489K mutation resulted in either hyperexcitability or hypoexcitability of cultured neurons (Cestele et al. 2008). The generation of FHM3 SCNA1a knock-in mice will allow to make the situation clearer, and to predict if the FHM3 mutation leads to enhanced susceptibility to CSD, similarly to the other FHM types.

1.2.4 Current and future pharmacological treatment of migraine

Migraine pharmacotherapy begins with a correct diagnosis, and the choice of treatment depends on the severity and frequency of the attack, associated symptoms, coexistent disorders, as well as previous treatment responses, drugs' efficacy, and adverse events (Bartleson & Cutrer, 2010). Pharmacological treatment can be *acute* (abortive) or *preventive* (prophylactic), and patients might need both approaches. Acute treatment attempts to reverse or stop the headache progressing once it has started. Preventive treatment is designed to reduce the frequency and severity of the attack. Acute treatment is appropriate for most attacks and should be restricted to 2-3 days a week; it can be *specific* (with ergotamine derivatives or triptans), or *non-specific* (with nonsteroidal anti-inflammatory drugs, NSAIDs, or opioids). Nonspecific drugs control migraine pain or other pain disorders, whereas specific drugs are effective in migraine headache attacks, but are not useful for non-headache pain disorders (Silberstein et al., 2004). Analgesics are used for mild to moderate headaches while triptans or ergotamine derivates are first-line drugs for severe attacks and for less severe attacks not responding to analgesics (Goadsby & Sprenger, 2010). A list of the common used drugs is listed in Table 1.2.

Ergot alkaloids and triptans are potent 5-HT1B/1D agonists and in some cases 5-HT1F receptor agonists. Ergots alkaloids have much greater receptor affinity at 5-HT1A, 5-HT2, adrenergic, and dopaminergic receptors than triptans, leading to more adverse events. Contraindications include documented or suspected ischaemic heart disease, Prinzmetal's angina, uncontrolled hypertension, basilar or hemiplegic migraine, and pregnancy (Silberstein et al., 2004).

Triptans, whose progenitor is sumatriptan, are more effective than ergotamine derivates. They are safe (for patients without cardiovascular risk factors), effective, and appropriate first-line treatment for patients who have a moderate to severe migraine headache or for whom analgesics have failed to provide adequate relief. Subcutaneous sumatriptan is the fastest and most effective. Sumatriptan or zolmitriptan nasal spray sometimes provide a faster onset of action than oral triptans. Triptans were originally thought to provide relief from migraine by causing cranial vasoconstriction, through their action at postsynaptic 5-HT1B receptors on the smooth-muscle cells of blood vessels. It is now theorized that triptans also block the release of vasoactive peptides from the perivascular trigeminal neurons through their action at presynaptic 5-HT1D

receptors on the nerve terminals. In addition, triptans are thought to have a central activity by blocking the release of neurotransmitters that activate second-order neurons ascending to the thalamus, and facilitating descending pain inhibitory systems (Loder, 2010). All triptans have the same contraindications and safety concerns. None is safer than another; however, the response to triptans is often idiosyncratic. One triptan might work for one patient and cause no adverse events, and a different triptan might work for another patient. The triptan of choice is the one that restores the patient's ability to function by swiftly and consistently relieving pain and associated symptoms with minimum adverse events and without recurrence of symptoms. Triptans also relieve from nausea and vomiting (Loder, 2010). Before deciding that a drug is ineffective, at least two attacks should be treated. It might be necessary to change the dose, formulation or route of administration or add an adjuvant. When the response is inadequate, the headache recurs, or adverse events are bothersome, changing the drug might be needed. If all treatments fail, rescue drugs (opioids, neuroleptics, and corticosteroids) are needed. They provide relief, but they have important adverse effects (Silberstein et al., 2004)

Preventive drugs reduce attack frequency, duration, or severity. Indications for preventive treatment include: migraine that substantially interferes with the patient's daily routine despite acute treatment; failure of, contraindication to, or troublesome adverse events from acute drugs; very frequent headaches with risk of drug overuse; special circumstances, such as hemiplegic migraine or attacks with a risk of permanent neurologic injury. Preventive drugs include β-adrenergic blockers, antidepressants, calcium-channel antagonists, serotonin antagonists, anticonvulsants, and non-steroidal anti-inflammatory drugs (Table 1.2). The choice is based on effectiveness, adverse events, and coexistent and comorbid conditions (Goadsby & Sprenger, 2010). If headache is well controlled, treatment can be lessened and discontinued, in order to provide a better risk-to-benefit ratio. Behavioral and psychological interventions can be also used for prevention, and they include relaxation training, thermal biofeedback combined with relaxation training, electromyography biofeedback, and cognitive-behavioral treatment (Silberstein et al., 2004).

Table 1.2 ***Current pharmacological treatments for migraine.***

Acute treatments

Specific drugs

- NSAIDS: aspirin, ibuprofen, tolfenamic acid, naproxen sodium and acetaminophen
- Neuroleptics and antiemetics: prochlorperazine and droperidol
- Corticosteroids: hydrocortisone, methylprednisolone, and dexamethasone
- Opioids

Non-specific drugs

- Ergotamine and dihydroergotamine
- Triptans: sumatriptan, zolmitriptan, naratriptan, rizatriptan, almotriptan, frovatriptan, and eletriptan.

Preventive treatments

- β-blockers: propranolol, nadolol, atenolol, metoprolol, and timolol
- Antidepressants: amitriptyline, fluoxetine and venlafaxine
- Calcium-channel blockers: flunarizine
- Anticonvulsant drugs: valproate, gabapentin and topiramate
- Serotonin antagonists: methysergide
- Nutraceuticals: riboflavin, coenzyme Q, butterbur (Petasites hybridus) and feverfew (Tanacetum parthenium)

Recent and future pharmacological options

Although triptans remain the most effective option for acute attack therapy, not all patients respond to treatment, and of those who do respond, about one out of three experience headache recurrences within 24 hours. Moreover, despite their relative safety, the fact that triptans constrict cranial and coronary blood vessels is an important issue for patients with cardiovascular diseases (Loder, 2010). For this reason, substantial efforts for discovering more effective, and safer, treatments are strongly needed. Possible future candidates for migraine treatment include CGRP receptor antagonists, nitric-oxide synthase inhibitors, vanilloid receptor antagonists, glutamate (AMPA, kainate) receptor antagonists, and 5-HT1F receptor agonists (Goadsby & Sprenger, 2010). The purinergic P2 receptors have been proven as important mediators in acute and chronic pain (Burnstock et al., 2009b; Jarvis, 2010; Villa et al., 2010), and

we strongly believe that this receptor family has a role also in migraine. Indeed, as discussed in *Chapter 2*, our research is based on the study of this field, in order to find new molecular targets for migraine therapy. In the meantime, some prominent pharmacological approaches are currently studied in clinical trials; in the next section they will be briefly discussed.

CGRP receptor antagonists: CGRP is one of the most important molecules involved in migraine neurobiology (see *Paragraph 1.2.2*). CGRP receptor antagonists are the most advanced class in clinical for migraine therapy. The intravenous antagonist olcegepant and the orally available antagonist telcagepant have shown clear effects in phase 1 and 2 studies. Telcagepant has recently undergone two phase 3 studies without cardiovascular liability. A phase 2 trial for another CGRP receptor antagonist, BI44370, has been completed and results are awaited. CGRP receptor antagonists have shown some positive effects in terms of reduced headache recurrence. The clinical tolerability of these drugs in terms of CNS and vascular side-effects seems to be more favorable than for triptans. However, concerns about possible liver toxicity remain (Tepper & Stillman, 2008). The development program of one compound, MK3207, which was undergoing phase 2 evaluation, has been discontinued because of delayed liver test abnormalities. Moreover, in a migraine prevention study of daily telcagepant over 3 months, increases in transaminases were observed in a few patients. FDA filing for telcagepant has thus been delayed to allow a review of additional safety data (Goadsby & Sprenger, 2010).

Serotonin receptor agonists: The announcement of the success of a phase 2 study with the 5-HT1F receptor agonist COL-144 offers another prospect for acute anti-migraine therapy. Unlike triptans, COL-144 does not act at 5-HT1B/D receptors and therefore does not cause vasoconstriction. In clinical studies it was shown than the drug was well tolerated and did not produce triptan-like chest symptoms. At the effective doses of COL-144, a higher proportion of patients showed a headache response at 2 h than those on placebo. An orally bioavailable formulation of COL-144 is now in phase 2 testing (Neeb et al., 2010).

CSD inhibitors: Similarly to the already available preventive agents, tonabersat, a novel potential preventive agent that has recently been investigated in three phase 2 studies, is a specific CSD inhibitor. During clinical studies investigators found a significant reduction of the number of aura attacks in tonabersat-treated patients,

although the number of migraine headache days was unaffected. This result is in agreement with the view that CSD is the experimental equivalent of aura, and substances effective in reducing CSD are also effective in preventing aura symptoms. Unfortunately, three different clinical studies were negative in terms of prevention of migraine without aura (Hauge et al., 2009). Together, these results suggest that, although tonabersat is unlikely to be a major step forward for the treatment of patients with migraine without aura, it could be a valid option for the management of patients with aura.

Botulinum toxin type A: Botulinum toxin type A (onabotulinum toxin A; BTA) is a reversible inhibitor of presynaptic acetylcholine release at motor nerve terminals. Open-label experience from its cosmetic use suggested its potential benefits in headache after injection into glabellar, frontalis, and temporalis muscles. Its effectiveness in pain disorders is probably due to inhibition of the release of neurotransmitters, such as SP and CGRP, and effects on muscle spasm and nerve transmission. Despite positive open-label studies and case reports, BTX has not proven to be effective for many patients with chronic tension-type headache or episodic migraine based on double-blind placebo-controlled trials. There is increasing evidence, however, that BTX is effective in the treatment of chronic migraine and chronic daily headache. More recently, a large multicenter randomized, double-blind, placebo-controlled trial of more than 1300 patients with chronic migraine demonstrated significant reductions in headache symptoms, frequency, disability and triptan utilization along with improvements in measures of health-related quality of life. Compared with other preventive treatments, BTX has a rapid onset of action (less than 2 weeks) with few serious adverse events and does not require daily medication use or titration (Goadsby & Sprenger, 2010).

1.3 THE PURINERGIC SYSTEM

ATP has long been recognized only as an intracellular energy molecule, and its acceptance as an extracellular signaling molecule has taken a considerably long period of time. The potent effects of ATP on the heart and blood vessels were first described in 1929 (Drury & Szent-Györgyi, 1929), while in 1972 Burnstock proposed new roles for adenosine-5'-triphosphate (ATP) as neurotransmitter in non-adrenergic, non-cholinergic nerves in the gut and bladder (Burnstock, 1972). In the following years ATP metabolites, like the nucleotide adenosine-5'-diphosphate (ADP) and the nucleoside adenosine (Ado), obtained by its enzymatic hydrolysis, as well as other extracellular nucleotides like the uridine-5'-triphosphate (UTP), uridine-5'-diphosphate (UDP), and sugar nucleotides, were progressively proposed as transmitters not only in sensory nerves, but also in motor nerves and CNS neurons (Fields & Burnstock, 2006; Inoue et al., 2007). The actions of these molecules in the extracellular environment implicated the existence of post-junctional receptors. Accordingly, numerous subtypes of these receptors were progressively cloned, and scientists gradually coined and accepted the term "*purinergic system*" for describing the system composed by extracellular nucleotides/nucleosides and their receptors. The first classification of purinergic receptors family dates back to 1978, when Burnstock proposed some criteria for differentiating these receptors in two families: the P1 receptors activated by Ado and antagonized by methylxantinic compounds, and the P2 receptors, responding to ATP and ADP.

Nowadays, P2 purinoceptors are divided into two families: the ionotropic receptors P2X and the metabotropic P2Y receptors (Burnstock & Knight, 2004). P2X receptors are ligand-activated cationic channels, specifically activated by ATP (Burnstock & Knight, 2004), while P2Y receptors are activated by purine or pyrimidine nucleotides, or by sugar-nucleotides, and couple to intracellular second-messenger systems through heteromeric G proteins (Abbracchio et al., 2006). In 1994, the IUPHAR Subcommittee for Purinoceptor Nomenclature and Classification has approved the new classification and has proposed to substitute the term "*P2 purinoceptors*" with "*P2 receptors*", in order to take into account the observation that some P2 receptors are preferentially activated by uridine nucleotides (Fredholm et al., 1997). To date, at least seven P2X subtypes ($P2X_{1-7}$) and eight P2Y members

($P2Y_{1,2,4,6,11,12,13,14}$) have been cloned from different animal species (Abbracchio et al., 2006). The missing numbers in the P2Y series correspond to receptors cloned from vertebrates different from mammals and for which no mammalian orthologs have been identified so far, or to receptors that have not been functionally characterized yet.

The concept that ATP is an extracellular signalling molecule has been established not only in the rapid signalling involved in neurotransmission, but also in a wide range of other biological processes, including release of cytokines, neurotransmitters and hormones, cell proliferation, differentiation and apoptosis in tissues as diverse as the skin, skeletal muscle, bone, nervous and immune system (Fields & Burnstock, 2006; Inoue et al., 2007). Thus, alterations in purinergic signalling may contribute to the development of disorders of the immune system, inflammation, neurodegeneration, osteoporosis and cancer. On this basis, a better understanding of the roles of purinergic signalling may help identifying novel therapeutic targets for several human diseases.

1.3.1 Purinergic signalling

The first clues on the role of extracellular nucleotides in signal transduction came from the observation that neurons and neuroendocrine cells released ATP copackaged with other neurotransmitters (Burnstock, 1972). Moreover, it has been demonstrated that, following mechanical stress, also non secretory tissues can release nucleotides (Lazarowski et al., 2000) which may signal to the same secretory cell (autocrine stimulation) as well as to adjacent cells (paracrine stimulation). Following injury or inflammation, nucleotides can also be released as a consequence of cell lysis. There is ongoing debate, however, about the transport mechanisms involved in nucleotide release. There are hints for exocytotic release from endothelial and urothelial cells, osteoblasts, astrocytes, and mast and chromaffin cells, but other transport mechanisms have been proposed, including ATP binding cassette transporters, connexin hemichannels and plasmalemmal voltage-dependent anion channels (Fields & Burnstock, 2006). More recently, pyrimidine nucleotides release has been described (Lazarowski et al., 2003).

Once released in the extracellular environment, nucleotides are rapidly degraded by ubiquitous ecto-nucleotidases (Zimmermann, 2000), a family of phosphatases expressed on the cell surface that are able to dephosphorylate different nucleotides. ATP

hydrolysis sequentially produces ADP, AMP and adenosine, whether UTP is degraded to UDP, UMP and uridine; many of these metabolites can act as extracellular signalling molecules The local response to a specific nucleotide is thus the result of the effects of the nucleotide itself and of its degradation products. Distinct classes of ecto-nucleotidases with different properties and different substrate specificities have been identified so far (Yegutkin, 2008). Some are membrane proteins with an extracellular catalytic domain, but soluble forms released in the extracellular space have also been described. Ecto-nucleotidases can be also released with ATP from sympathetic nerve terminals, representing one of the mechanisms to turn off neurotransmitter signalling (Todorov et al., 1997). A general scheme of nucleotide-hydrolyzing enzymes include: (i) the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, (ii) the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family, (iii) ecto-5'-nucleotidase, and (iv) alkaline phosphatases (AP; Yegutkin, 2008). This various enzymatic family demonstrates that ATP and other nucleotides can be considered classical neurotransmitters whose signal can be regulated by enzymatic degradation.

Nucleotides released or leaked from both glial cells and neurons play a role in cell-to-cell communication under physiological and pathophysiological conditions (Burnstock & Knight, 2004). Accordingly, in CNS both neuronal cells and the major classes of glia (i.e. astrocytes, microglia and oligodendrocytes), express a broad range of purinergic receptors (Fields & Burnstock, 2006). For instance, astrocytes release ATP in response to various stimuli or even spontaneously, and communicate with neurons, microglial cells, and even vascular cells at capillaries (Inoue et al., 2007). Also microglial cells respond to a wide range of ATP receptor agonists, through increases in intracellular calcium, secretion of cytokines, and rapid changes in their morphology and migration (Inoue, 2008). Extracellular nucleotides represent therefore a class of signalling molecules that functionally “unite” glia and neurons together. Not by chance neuron-glia or glia-glia communications participate in the control of several pathophysiological mechanisms, including regulation of synaptic transmission (Wieraszko & Ehrlich, 1994), neuroimmune interactions (Inoue et al., 2007), processing of information through the retina (Newman, 2006), Schwann cell proliferation and myelination (Fields, 2006; Stevens, 2006), cell proliferation (Neary et al., 2006), inflammation (Di Virgilio et al., 2009), and pain (Burnsock, 2009a,b; see also *Paragraph 1.4*)

1.3.2 P2X receptors

P2X receptors are membrane ion channels that open in response to the binding of extracellular ATP. ATP can elicit rapid responses (<10ms) via these ion channels, resulting in selective permeability to Na^+, K^+, and Ca^{2+} cations (North, 2002). In vertebrates, seven genes encode P2X receptor subunits, which are 40-50% identical in their aminoacidic sequence. Each subunit has two transmembrane domains, separated by an extracellular domain (~280 aminoacids). Channels function as trimers of several subunits (Figure 1.5). As of today, seven homomeric channels ($P2X_{1-7}$) have been identified, but functional expression studies have also highlighted the existence of heteromeric $P2X_{1/5}$, $P2X_{2/3}$, $P2X_{2/6}$, $P2X_{4/6}$, $P2X_{4/7}$ and $P2X_5$ receptors which assemble with any others, except $P2X_7$ (North, 2002).

Table 1.4 ***Pharmacological properties of rodent P2X receptors.***

Receptor	n° of amminoacids	Agonist potency	Antagonists
$P2X_1$	399	BzATP >> 2MeSATP > ATP > ,αβmeATP	TNP-ATP, Suramin, PPADS, MRS2159
$P2X_2$	472	2MeSATP > ATP	PPADS
$P2X_3$	397	BzATP >> 2MeSATP > ATP = α,βmeATP	TNP-ATP
$P2X_4$	388	ATP > 2MeSATP >> α,βmeATP	TNP-ATP
$P2X_5$	455	ATP > 2MeSATP > ADP	-
$P2X_6$	379	ATP > 2MeSATP > ADP	PPADS
$P2X_7$	595	BzATP >> ATP^{4-}	KN-62, PPADS, oATP

The lengths of P2X subunit differ from 384 ($P2X_4$) to 595 ($P2X_7$) aminoacids. Each subunit has two hydrophobic regions of sufficient length to cross the plasma membrane, placing most of the protein extracellularly. These hydrophobic regions are

separated by the bulk of the polypeptide. The NH_2 and COOH terminals are both cytoplasmic (North, 2002). The extracellular loop contains the ATP binding site as well as sites for antagonists and modulators (Khakh et al., 2001). The aminoacidic identity between P2X receptor subunits is distributed throughout the extracellular domain, a striking feature of which is the conservation of 10 cysteine residues among all known receptors which form disulfide bonds to give the correct conformation to the receptor (Vial et al., 2004). All the P2X receptor subunits have consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr), and some glycosylations are essential for trafficking to the cell surface. The extracellular domain carries few conserved glycine (G) and proline (P) residues which are involved in conformational changes subsequent to ligand-receptor binding. Extracellular protons, bivalent cations and some metals are P2X receptors allosteric modulators. In addition, P2X receptors can be modulated via phosphorylation of serine (S) and threonine (T) residues. A list of the main nucleotide analogues acting as agonists or antagonists at the P2X receptors is summarized in Table 1.3.

1.3.3 P2Y receptors

P2Y receptors belong to the superfamily of G protein-coupled receptors (GPCR). GPCR are a family of membrane receptors responding to a wide variety of ligands such as nucleotides, biogenic amines, peptides and other small molecules (Marchese et al., 1999). The binding of the GPCR to its specific ligand results in the activation of the associated heterotrimeric G protein (α, β and γ subunits) that mediate a number of intracellular responses. In particular, ligand binding to its receptor results in a decreased affinity of the α subunit of the G protein for GDP, that is thus exchanged for GTP. This binding causes a conformational modification in the G protein and the dissociation of the α subunit from the βγ complex. Both α and βγ subunits can then activate signal transduction pathways (Rebois et al., 1997).

From a phylogenetical point of view, the eight human P2Y receptors can be subdivided into two distinct subgroups characterized by a relatively high level of sequence divergence. The first subgroup encompasses $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$, whereas the second subgroup encompasses $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ (Abbracchio et al., 2006). Site-directed mutagenesis of $P2Y_1$ and $P2Y_2$ receptors has shown that some

positively charged residues in transmembrane domains 3, 6 and 7 are crucial for receptor activation by nucleotides (Abbracchio et al., 2006). They probably interact with the negative charges of the phosphate groups of nucleotides.

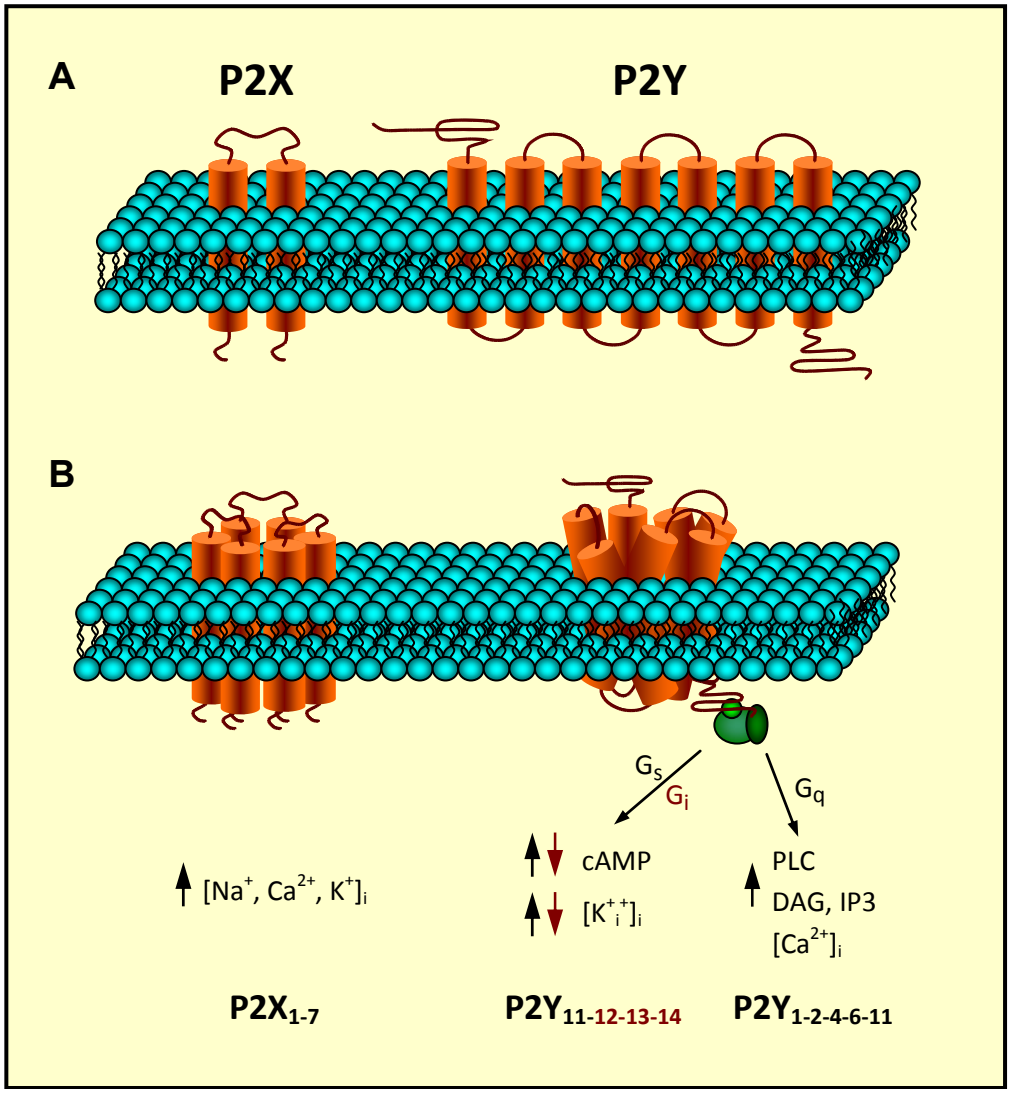


Figure 1.5

Organization of P2X and P2Y receptors

A, *Schematic representation of a typical P2X receptor, and a P2Y receptor.* ***B,*** *Representation of P2X and P2Y receptor organization in the plasma membrane, and of their intracellular signalling pathways. While activation of P2X receptors lead to increase in permeability to Na^+, K^+, and Ca^{2+}, nucleotides acting at P2Y receptors cause activation of specific G proteins: G_q/G_{11} for the $P2Y_{1,2,4,6,11}$, leading to the recruitment of the PLC pathway with increases in intracellular calcium; G_S for $P2Y_{11}$, leading to increases in cAMP levels; $G_{i/o}$ for $P2Y_{12,13,14}$, leading to decrease in cAMP levels.*

The eight P2Y receptors identified so far have a H-x-x-R/K motif in TM6 that might be important for agonist activity. Receptors in the first subgroup also share a YQ/K-x-x-R motif in TM7 (proposed to participate in ligand binding), whereas in receptors of the second subgroup, another motif (K-E-x-x-L) which might affect ligand binding characteristics, is observed (Abbracchio et al., 2006). These two P2Y receptor subgroups also differ in their primary coupling to transductional G proteins. In particular, all receptors in the first subgroup (i.e., $P2Y_{1,2,4,6,11}$) couple to G_q/G_{11} to activate PLC/IP_3 pathway and release intracellular calcium, whereas receptors in the second subgroup (i.e., $P2Y_{12,13,14}$) almost exclusively use the $G_{i/o}$ class of G proteins to lower cAMP levels. Secondary couplings have been also reported, especially for receptors of the first subgroup in heterologous expression systems (Abbracchio et al., 2006). Among receptors of the second group, $P2Y_{13}$ has been also reported to couple to $G_{\alpha 16}$ and to stimulate PLC in recombinant systems overexpressing this G protein (Fumagalli et al., 2004). Such "promiscuity" of G protein coupling may depend on the indirect activation of additional G protein subtypes within protein complexes containing the P2Y receptor.

From a pharmacological point of view, P2Y receptors can be broadly subdivided in four groups based on their responsivity to nucleotides: i) adenine nucleotide-preferring receptors, mainly responding to ADP and ATP. This group includes human and rodent $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$, and human $P2Y_{11}$; ii) uracil nucleotide-preferring receptors, including the human $P2Y_4$ and $P2Y_6$ responding to either UTP or UDP; iii) receptors of mixed selectivity, the human and rodent $P2Y_2$, the rodent $P2Y_4$ and, possibly, $P2Y_{11}$; and iv) the $P2Y_{14}$ receptor, responding to both UDP and sugar nucleotides (mainly UDP-glucose and UDP-galactose; Abbracchio et al., 2006; Carter et al., 2009). In this latter group it could be possibly included a receptor phylogenetically and structurally related to already known P2Y receptors, named GPR17, which has been recently reported to respond to both cysteinyl-leukotrienes and uracil nucleotides (Ciana et al., 2006). A list of the main nucleotide analogues, which can act both as agonists, antagonists or partial agonists at the P2Y receptor subtypes, is summarized in Table 1.4. Unfortunately, although important progress in exploring structure-activity relationships has been achieved, most of the P2Y receptor subtypes are still lacking potent and selective synthetic agonists and antagonists (Abbracchio et al., 2006).

Table 1.4 ***Pharmacological properties of rodent P2Y receptors.***

Receptor	n° of amminoacids	G-protein coupling	Agonist potency	Antagonists
$P2Y_1$	373	Gq/G_{11}	2MeSADP > MRS2365 > ADP > ATP	MRS2500, MRS2179
$P2Y_2$	377	Gq/G_{11}	UTP = ATP = MRS2698	Suramin, PSB-716 AR-C126313
$P2Y_4$	365	Gq	UTP > ATP	PPADS
$P2Y_6$	328	Gq/G_{11}	PSB-0474 > 5-iodo-UTP > UDP > UTP	MRS2567, MRS2578, MRS2575
$P2Y_{11}$	374	Gq/G_{11} and Gs	NF546 > ATP > 2 MeSATP	Suramin, Reactive blue 2, NF340
$P2Y_{12}$	342	Gi	2MeSADP = 2MeSATP > ADP >ATP > UTP	Cangrelor, PSB-0739 AZD6140
$P2Y_{13}$	333	Gi	ADP = 2MeSADP	Cangrelor MRS2211
$P2Y_{14}$	338	Gi/Go	UDPglucose > UDPgalactose > UDP	-

1.3.4 Pathophysiological roles of extracellular nucleotides in the nervous system

The hypothesis that ATP plays a central role in modulating cerebral functions rises from the broad distribution of P2 receptors in the CNS (Abbracchio et al., 2006). ATP is released from nerve terminals and has rapid and direct actions, generally associated to activation, whether Ado has inhibitory effects with a negative feed-back action on the effects of ATP and of other neurotransmitters released at the synapse. The first evidence suggesting a role of ATP as a neurotransmitter in the brain was obtained in 1992, with the demonstration that its stable analog, α,β-meATP, provokes a current of excitation in rat neurons which is antagonized by P2 antagonists (Edwards et al., 1992). In some brain areas, ATP regulates neurotransmission through the modulation of glutamate release from nerve endings. This effect is mediated by P2 receptors located

on the presynaptic as well as on the post-synaptic element (Motin & Bennett, 1995). In particular, it has been suggested that ATP can amplify glutamate post-synaptic action following interaction with a P2Y receptor, or by activating an ecto-kinase responsible for the phosphorylation of membrane proteins associated to glutamate receptors (Chen et al., 1996). These observations have led to the hypothesis that ATP, and maybe also UTP, might participate in long-term synaptic potentiation, memory, and learning processes (Price et al., 2003, Fujii, 2004). According to this hypothesis there is the experimental evidence indicating colocalization of ATP with neurotransmitters and neuropeptides in secretory vesicles of most synapses. Noradrenaline (NA) and ATP are released from sympathetic nervous system endings in variable ratios according to the tissue and to the animal species considered (Burnstock & Verkhratsky, 2010). ATP co-transmission has been also observed in isolated blood vessels as well as in skeletal muscle, kidney and intestine vasculature (Burnstock, 2009a). Despite the demonstration that ATP is stored in the nerve endings together with NA, prejunctional neuromodulation studies demonstrate that ATP and NA are released independently following different stimuli (Starke et al., 1996). It can thus be hypothesized that different nerve populations containing different ratios of NA and ATP exist in the sympathetic nervous system. Finally, indirect evidence demonstrates that ATP and acetylcholine co-localize in central and peripheral cholinergic terminals and that a co-transmission with peptides such as SP, CGRP, neuropeptide Y and somatostatin also exists (Burnstock, 1997; Zimmermann, 2008).

Recent studies also highlighted a direct involvement of extracellular nucleotides in neurogenesis and gliogenesis. In the nervous system ATP has been identified as a mitogen for v-myc immortalized neural progenitor cells (Ryu et al., 2003), and ATP-mediated purinergic signalling through the $P2Y_1$ receptor has been associated with developmental neurogenesis (Weissman et al., 2004). Moreover, neurospheres cultured from the adult SVZ express functional nucleotide receptors as well as enzymes for their degradation (Milosevic et al., 2006; Mishra et al., 2006). Interestingly ATP release and purinergic signalling may be required not only for the developmental expansion of progenitor cell and neurogenesis, but also to control the proliferative and differentiative potential of the progenitor cells in the adult brain. The colocalization of both P2Y receptors and ecto-nucleotidases activity to regions of active mitotic progenitor cell expansion and neurogenesis in the adult brain (Braun et al., 2003; Shukla et al., 2005) is

particularly significant in this regard, given the apparent necessity of purinergic signalling to neural progenitor cell expansion *in vitro* (Mishra et al., 2006).

Following pathological/traumatic events a massive release of purines and pyrimidines at the site of injury is observed and they seem to have a dualistic effect: on one hand, they contribute to lesion worsening, but on the other side they are involved in tissue regeneration and repair. In the next sections the main roles of these molecules during pathological events will be briefly discussed, whereas a detailed dissertation of their role in pain transmission will be provided in the following paragraphs.

Brain ischemia and spinal cord injury

During ischemia or hypoxia in the CNS a massive release of nucleotides is observed (Neary et al., 1996; Melani et al., 2005). ATP participate to cell death induction immediately (minutes to hours) following tissue injury, probably as a consequence of a diffuse dysregulation of the release mechanisms and of a pathologic activation of P2 receptors. For instance, it has been demonstrated that ATP has a cytotoxic effect on cerebellar neurons (Amadio et al., 2002) and potentiates hypoglycemia-induced tissue injury (Cavaliere et al., 2002). In the weeks following ischemic injury, when homeostatic control is re-established in the injured area, ATP seems to contribute to differentiation and to promote the long-term functional repair of the damaged area (Abbracchio & Ceruti, 2006; Abbracchio & Verderio, 2006). During ischemic and hypoxic events, high extracellular concentrations of adenosine are also reached, in part as a consequence of ATP degradation. It has been demonstrated that adenosine has a protective role in cerebral ischemia in several experimental models. Accordingly, both adenosine administration as well as inhibition of its degradation reduces injury associated to cerebral or cardiac ischemia and protects from organ loss of function (Phillis & O'Regan, 1996; Mentzer et al., 1996; Fredholm, 2010).

Extracellular nucleotides can also induce cell death, through the formation of $P2X_7$ receptor-associated pore (Di Virgilio et al., 1996; Ferrari et al., 1997). This mechanism could influence neuronal remodeling through the elimination of critically injured cells, in particular during acute brain ischemic events, thus limiting the area of damaged tissue (Neary et al., 1996). Brain damage caused by a transient MCAo results in a sustained pathologically high ATP outflow; while in the infarct region the release of ATP is low because of disturbed cellular synthesis, in the peri-infarct area its

concentration is increased, due both to the deregulation of its enzymatic degradation, and to the concomitantly enhanced outflow of the nucleotide from damaged cells (Melani et al., 2005).

An abnormal purinergic signaling has been also observed following spinal cord injury (SCI). In the peritraumatic spinal cord regions, ATP is released at high levels and causes neuronal cell death through $P2X_7$ receptors. In this respect, $P2X_7$ receptor blockade is associated to improved functional recovery and diminished cell death in the peritraumatic zone (Wang et al., 2004). Analysis of $P2X_4$ receptor expression following SCI also demonstrated a significant accumulation of $P2X_4$-positive microglia/macrophages as early as 24 h after SCI, peaking on day 7 (Schwab et al., 2005). Taken together, these observations confirm the role of the purinergic system in the traumatic degeneration, and possibly in tissue remodeling.

P2 receptors in reactive astrogliosis

Astrocytes respond to traumatic and inflammatory CNS insults through major and rapid modifications of their morphology and cell structure and with an increased proliferation, a process known as "reactive astrogliosis" (Eddleston & Mucke, 1993). Reactive astrogliosis occurs in several pathologies, including ischemia and chronic pain, and is characterized by hypertrophy and proliferation of astrocytes around the injured region (Marchetti & Abbracchio, 2005). Reactive astrocytes produce and release inflammatory mediators such as cytokines and chemokines, as well as various growth factors. In addition, reactive astrocytes upregulate the expression of nestin, GFAP, and vimentin markers (Clarke et al., 1994). The functional role of reactive astrogliosis has not been clearly delineated yet; some studies suggest a protective role of the glial scar that protects nervous tissue and limits damage progression, whereas others indicate that it could interfere with axonal growth and regeneration (Sofroniew & Vinters, 2010). Despite these discordant experimental evidence, there is agreement on the fact that an excessive and long-lasting reactive gliosis can be deleterious for functional repair of the damaged area. Probably this mechanism is initially activated as a protective response to tissue injury, but a prolonged activation can result in a pathological event and contribute to neurodegeneration. Importantly, astroglial cell activation has been correlated to P2 activation (Neary et al., 2006). It has been demonstrated that ATP induces pro-inflammatory enzymes and arachidonic acid release (Bolego et al., 1997; Brambilla et

al., 1999), therefore worsening the neurodegenerative damage through the formation of radical oxygen species, prostaglandins, leukotrienes and the blockade of glutamate reuptake (Volterra et al., 1994). These data suggest that in pathological conditions, P2 receptor activation on both neurons and astrocytes can contribute to neurodegenerative damage, and thus P2 antagonists might represent an interesting approach to neuroprotection in acute cerebral damage.

Chronic neurodegenerative diseases

P2 receptor ligands have been proposed as potential neuroprotective agents following neuronal death associated with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and multiple sclerosis (Burnstock, 2008). For instance, different P2 receptor subtypes are involved the development and growth of dopaminegic neurons in CNS (Heine et al., 2007), and $P2X_7$ receptors are involved in ATP-mediated necrotic volume increase in substantia nigra, which has been implicated in the pathogenesis of Parkinson's disease (Jun & Kim, 2004). $P2X_7$ receptors have been also found upregulated in the brain of patients with Alzheimer's disease (McLarnon et al., 2006), and ATP released during neuronal excitation or injury enhances the inflammatory effects of cytokines and prostaglandin E2 in astrocytes, thus contributing to the chronic inflammation seen in Alzheimer's disease (Xu, et al., 2003). $P2X_7$ receptor antagonism therefore represents a therapeutic strategy for blocking inflammatory responses associated to neurodegenerative disorders.

In the brain, stress or damage causes the release of nucleotides and activation of $P2Y_2$ receptors, leading to pro-inflammatory responses including the stimulation and recruitment of glial cells, which can protect neurons from injury (Peterson et al., 2010). Indeed, $P2Y_2$ receptor expression is increased in glial cells by stimulation with IL1β, a pro-inflammatory cytokine whose levels are elevated in Alzheimer's disease, and this effect has been proposed to be neuroprotective, since it increases the non-amyloidogenic cleavage of the amyloid precursor protein (Camden et al., 2005). Thus, activation of $P2Y_2$ receptors in glial cells can promote neuroprotective responses, therefore indicating this receptor subunit as a novel pharmacological target for the treatment of neurodegenerative diseases.

1.4 PURINES AND PAIN

The first hints about the involvement of ATP in pain transmission came from the observation that its injection into the human skin elicited pain (Bleehen and Keele, 1977; Krishtal et al., 1983). A significant breakthrough in this field was made in 1995, when the $P2X_3$ receptor channel was cloned from rat DRG, and it was shown to be expressed on small nociceptive sensory neurons together with $P2X_{2/3}$ heteromultimer receptors (Chen et al., 1995; Lewis et al., 1995). The next year Burnstock proposed a unified hypothesis on the role of ATP in pain, suggesting that: (i) ATP released as a cotransmitter with noradrenaline and neuropeptide Y from sympathetic nerve terminal varicosities is involved in sympathetic pain; (ii) ATP released from vascular endothelial cells of microvessels during reactive hyperaemia is associated with pain in migraine, angina and ischemia; (iii) ATP released from tumor or damaged cells activates $P2X_3$ receptors on nociceptive sensory nerves (Burnstock, 1996). This hypothesis has been followed by an increasing number of papers that confirmed and expanded this concept (see also below).

Although ATP is one of the key mediators in nociception, emerging evidence indicates that also other extracellular nucleotides (namely ADP, UTP and UDP), and nucleosides (adenosine) participate in the modulation of pain transmission (Liu & Salter, 2005; Burnstock, 2009a; Jarvis, 2010). Indeed, several P1 and P2 receptors have been found to be expressed by both neurons and glial cells involved in nociceptive transmission (Donnelly-Roberts et al., 2008; Burnstock, 2009a), and the effects of extracellular nucleotides on nociception are extremely complex and related to the specific receptor subtypes and/or their cellular localization. For instance, both P2X (e.g., $P2X_4$) and P2Y (e.g., $P2Y_{6,12}$) purinergic receptors are upregulated in activated microglia following nerve injury, and their pharmacological or biotechnological inhibition has been demonstrated to critically modulate the development and maintenance of neuropathic pain (Inoue, 2008; Tsuda et al., 2010). Another interesting pharmacological target involved in pain pathophysiology is the $P2X_7$ channel, specifically expressed by SGCs in sensory ganglia. The purinergic P2 receptors therefore represent interesting targets in nociception, and the development of clinically administrable drugs will be of primary importance for successfully treating chronic pain diseases like migraine.

1.4.1 Role of P2X receptors in pain transmission

Since the publication of early works demonstrating ATP involvement in pain (see above), the expression and function of P2X receptors in sensory neurons have been characterized. To date the most important P2X receptors subtypes implied in pain transmission are the $P2X_3$ and $P2X_{2/3}$ receptors subtypes, expressed by sensory neurons; the $P2X_4$ receptor, expressed by CNS microglia; and the $P2X_7$ receptor, expressed in sensory ganglia by SGCs (Burnstock, 2009b).

$P2X_3$ and $P2X_{2/3}$ receptors

The $P2X_3$ receptor was originally cloned from the rat DRG, and was shown to be expressed by about the 40% of small to medium-sized nociceptive sensory neurons, together with $P2X_{2/3}$ heteromultimer receptors. $P2X_3$ receptors are expressed by IB4-positive sensory neurons, colocalize with the vanilloid receptor subtype 1 (VR1), and only partially with the neuropeptide CGRP (Bradbury et al., 1998; Guo et al., 1999). $P2X_3$ receptor subunits are synthesized in the cell bodies of sensory neurons and then transported both peripherally and centrally: peripheral expression contribute to ATP nociceptive sensory transmission, while the central projections of $P2X_3$-positive neurons terminate in inner lamina II of the spinal cord (Vulchanova et al., 1998).

Expression level and functional properties of $P2X_3$ and $P2X_{2/3}$ are dynamically modulated in different pain models. For instance, chronic inflammation induced by complete Freund's adjuvant (CFA) results in the upregulation of $P2X_3$ or $P2X_{2/3}$ receptor expression and functionality in both in DRG and TG neurons (Xu & Huang, 2002; Shinoda et al., 2005). Moreover, injury produced by the chronic constriction of the sciatic nerve produces a general increase of $P2X_3$ receptor-positive DRG cells, in both small- and medium-sized neurons (Novakovic et al., 1999). By using an experimental model of trigeminal neuropathic pain, produced by the partial ligation of the infra orbital nerve, it was shown that the number of $P2X_3$-immunoreactive neurons was significantly increased ipsillaterally, and the heat-related hyperalgesia development was inhibited by the peripheral injection of P2 receptors antagonists pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (TNP-ATP; Shinoda et al., 2007). This evidence, together with others indicating a strong expression of $P2X_3$ receptors in deep craniofacial nerve

terminals (Ambalavanar & Dessem, 2009), indicates this receptor as a potential target for developing novel therapies for trigeminal neuropathic pain, and possibly for migraine.

The subcutaneous or intrathecal injection of the highly selective, stable and competitive $P2X_3/P2X_{2/3}$ antagonist, A-317491, effectively attenuated both formalin-induced acute pain, and thermal hyperalgesia induced by intra-plantar CFA injection. Spinal delivery of A-317491 also reduces chronic neuropathic pain induced by the chronic constriction of the sciatic nerve, or by the L5/L6 spinal nerve ligation (Jarvis et al., 2002; McGaraughty et al., 2003). The selective knock down of $P2X_3$ receptors expression by using antisense oligonucleotides (ASO) or RNA interference demonstrated a significant role for $P2X_3$ receptor in both inflammatory and neuropathic pain. Indeed, the nociceptive behaviors observed after injection of formalin or α,β-methylene-ATP (α,β-meATP) into the rat hind paw, the mechanical and thermal hyperalgesia induced by CFA, as well as the mechanical hyperalgesia and allodynia induced by nerve injury were significantly reduced by acute $P2X_3$ gene knock down (Honore et al., 2002; Barclay et al., 2002; Dorn et al., 2004). Finally, the physiological role of $P2X_3$ receptor subunits in sensory transmission has been also studied using $P2X_3$ null mutant mice (Cockayne et al., 2000; Souslova et al., 2000). These animals respond normally to acute noxious thermal and mechanical stimuli, but display attenuated responses to non-noxious warm stimulation. Formalin-induced nociceptive behavior is attenuated in $P2X_3$ null mice, thus providing a genetic evidence for the requirement of this receptor in acute inflammatory pain. However, in chronic inflammation induced by CFA, there is a paradoxical enhancement of thermal hyperalgesia in $P2X_3$ null mice, which may be due to some unknown developmental compensatory changes (Souslova et al., 2000). Moreover, ATP-induced hyperalgesia is reduced but not eliminated in $P2X_3$ null mice, thus indicating that other P2 receptors are probably involved in these mechanisms (Cockayne et al., 2000; Souslova et al., 2000).

A role for the $P2X_3$ receptor during migraine has been also proposed. In fact application of migraine mediators, like NGF and the neuropeptide CGRP, enhanced $P2X_3$ receptor conductance in cultured trigeminal sensory neurons (Fabbretti et al., 2006; Giniatullin et al., 2008). Moreover, by using a mouse genetic model of FHM1 (knockin for the R192Q mutation of the CACAN1A gene, see *Paragraph 1.2.3*), a significant increase in $P2X_3$ receptor activity was found (Nair et al., 2010). The

molecular analysis of this mechanism revealed that $P2X_3$ potentiation in these animals is due to increased calcium/calmodulin kinase II (CaMKII)-dependent activation of the phosphatase Calcineurin, and subsequent impairment of the phosphorylation state of the receptor. Although these studies require further investigations, it has been proposed that a strong $P2X_3$ receptor activation might facilitate ATP-dependent migraine and sensitize trigeminal nociceptors (Nair et al., 2010).

$P2X_4$ receptors

An initial clue indicating a role for $P2X_4$ receptors in pain came from pharmacological investigations of pain behavior after nerve injury. Indeed, the pharmacological blockade of spinal $P2X_4$ receptors reversed tactile allodynia caused by peripheral nerve injury, without affecting acute pain behaviors (Tsuda et al., 2003). Following nerve injury, the expression of $P2X_4$ receptors increased specifically in spinal cord microglia, indicating that a tonic activation of this receptor is necessary for sustaining allodynia (Tsuda et al., 2003). Moreover, intraspinal administration of $P2X_4$-specific ASO decreased the induction of receptor expression, and suppressed tactile allodynia. Conversely, intraspinal administration of microglia in which $P2X_4$ receptors had been induced and stimulated, produced tactile allodynia in naive rats. Therefore, $P2X_4$ receptor activation in microglia is not only necessary but also sufficient to cause tactile allodynia (Tsuda et al., 2003). This hypothesis is substantially supported by recent findings showing that both mice treated spinally with a $P2X_4$ ASO and mice lacking $P2X_4$ show attenuated tactile allodynia after nerve injury (Tsuda et al., 2010).

The mechanisms by which microglia are crucial for producing neuropathic pain involve signaling pathways from activated microglia to dorsal horn neurons. In this respect, studies on rats spinal cord slices revealed that spinal microglia stimulated by $P2X_4$ receptors agonists cause neuropathic pain through a rise in intracellular $[Cl^-]$ in spinal lamina I neurons (Coull et al., 2005). This effect was shown to be mediated by activation of $P2X_4$ receptors on microglial cells and the subsequent release of brain derived neurotrophic factor (BDNF; Trang et al., 2009). Accordingly, interference of signaling between BDNF and its receptor (TrkB) prevented tactile allodynia caused by peripheral nerve injury or by intrathecal administration of $P2X_4$-stimulated microglia. Thus, these results indicate that $P2X_4$-stimulated microglia release BDNF as a crucial

factor to signal to dorsal horn neurons, causing a collapse of their transmembrane anion gradient with a subsequent neuronal hyperexcitability (Trang et al., 2009).

The above evidence indicates that the $P2X_4$ subtype might be a potential therapeutic target to treat neuropathic pain. However, there are currently no antagonists to potently and selectively inhibit $P2X_4$ receptors. It has been proposed that some antidepressants and anticonvulsants clinically used in patients with neuropathic pain have inhibitory effects on ATP-evoked Ca^{2+} response in cells expressing recombinant $P2X_4$ receptors and primary cultured microglial cells (Nagata et al., 2009). Among the drugs used, paroxetine and fluvoxamine, but not citalopram, produced an anti-allodynic effect in an animal model of neuropathic pain, and this effect correlated with the potency of inhibition of rat $P2X_4$ receptors. Interestingly, the anti-allodynic action of paroxetine is insensitive to 5-HT receptor antagonists, suggesting that this effect could be independent from the spinal 5-HT system, and could be mediated through a direct inhibition of $P2X_4$ receptors (Nagata et al., 2009). It remains to be tested whether novel selective $P2X_4$ antagonists will elicit analgesic effects in neuropathic and inflammatory pain states.

$P2X_7$ receptors

In sensory ganglia, the $P2X_7$ subunit has been demonstrated to be selectively expressed by SGCs (Kobayashi et al., 2005; Zhang et al., 2005; Chessell et al., 2005; Chen, et al., 2008). In non-neuronal cells from DRG, the pharmacological characterization of cell responses showed a rank order of potency for known P2X receptor agonists as follows: BzATP > ATP > α,β-meATP, and the inhibition of 100μM BzATP-evoked currents by pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS) and Mg^{2+} (Zhang et al., 2005). This pharmacological profile was consistent with the involvement of $P2X_7$ receptors (Bianchi et al., 1999).

In 2005, Chessell and coll. have shown that the $P2X_7$ knock out mouse did not develop either mechanical allodynia or thermal hyperalgesia after inflammatory and neuropathic injury, while normal nociceptive processing was preserved (Chessell et al., 2005). Interestingly, this receptor was found upregulated in DRGs and injured nerves from chronic neuropathic pain patients (Chessell et al., 2005). Given that $P2X_7$ stimulation is known to induce IL-1β release from macrophages/microglia and reactive astrocytes (Bianco et al., 2005; Sanz et al., 2009) and IL-1β levels were also strongly

upregulated in the inflamed paws following CFA intraplantar injection (Chessel et al., 2005), it has been suggested that the $P2X_7$ receptor subtype might play a pivotal role in the molecular cascade of events leading to inflammatory and neuropathic pain. In this context, growing evidence supports a role for $P2X_7$ receptors as a "sensor of danger" that, by monitoring ATP extracellular levels, may participate to the modulation of IL-1β processing and release (Bianco et al., 2005; Ferrari et al., 2006). To further confirm this issue, systemic administration of selective $P2X_7$ receptor antagonists, such as A-740003 (Honore et al., 2006), and A-438079 (McGaraughty et al., 2007), was associated with a significant dose-dependent reduction of both allodynia and hyperalgesia in several animal models of neuropathic and inflammatory pain. The antinociceptive actions of A-438079 were correlated to its ability to reduce both the evoked and the spontaneous firing of different classes of spinal neurons in neuropathic animals, and to inhibit the release of IL-1β (McGaraughty et al., 2007). Thus, these results would suggest a specific role for glial $P2X_7$ receptors in neuron to glial cell interaction associated with ongoing pain (Zhang et al., 2005; Jarvis, 2010).

1.4.2 Role of P2Y receptors in pain transmission: sensory ganglia

In contrast to the abundance of data on the roles of P2X receptors in pain, only little and controversial information about the involvement of P2Y receptors in nociceptive transmission is known. However, these receptors are widely expressed in both neurons and glial cells of the PNS and CNS and therefore the comprehension of their nociceptive functions is of paramount importance for discovering new potential targets for pain therapy.

In sensory ganglion neurons, P2Y receptors are located not only at the cell body, but also at the peripheral and central terminals (Gerevich & Illes, 2004; Burnstock, 2009a). Here, their activity is integrated within the complex molecular network associated to the transmission of nociceptive signals to the CNS. For example, the preferential $P2Y_1$ agonist ADP was found to inhibit the N-type voltage-activated calcium channels in rat DRG neurons. The outcome of this channels inhibition could be a decrease of sensory transmitters release from DRG terminals in the spinal cord, therefore diminishing the spinal pain transmission (Gerevich et al., 2004). P2Y receptor

expression on sensory terminals could also control ATP actions on P2X subtypes. In fact, $P2Y_1$ and $P2X_3$ receptors are often coexpressed in the same neuron (Ruan & Burnstock, 2003), and functional studies in DRG showed that $P2Y_1$ receptor activation leads to $P2X_3$ receptor inhibition (Gerevich et al., 2005; Chen et al., 2008). P2Y receptors can also modulate neuropeptide release; indeed, the $P2Y_{2\text{-}4}$ preferential agonist UTP was found to induce release of CGRP from the rat DRG neurons (Sanada et al., 2002). Others studies failed to demonstrate a direct release of CGRP by UTP, but rather an enhancement of acid buffer solutions and capsaicin evoked neuropeptide release (Zimmerman et al., 2002; Huang et al., 2003).

In sensory ganglia, P2Y receptors undergo significant changes on their expression levels after neuropathic or inflammatory conditions. It has been shown that after peripheral axotomy of the sciatic nerve in rat, $P2Y_1$ mRNA is upregulated in DRGs (Xiao et al., 2002). Additionally, $P2Y_2$ mRNA levels were strong upregulated after CFA injection in the hindpaw, while $P2Y_{2\text{-}4\text{-}6}$ RNAs were downregulated (Malin et al., 2008). After CFA-induced inflammation, only WT mice showed hyperensivity to noxious heat, while $P2Y_2^{-/-}$ mice failed to develop thermal hyperalgesia. On the contrary, there were no differences in the extent of mechanical allodynia between WT and $P2Y_2^{-/-}$ mice (Malin et al., 2008). These results demonstrate that neuronal $P2Y_2$ receptor subtype is specifically involved in thermal nociceptive transmission. It has been also demonstrated that receptor subtypes that are expressed but not functional upon basal conditions (e.g., $P2Y_{14}$), are upregulated under specific chronic pathological painful situations (Vega-Avelaira et al., 2009). These receptors could therefore represent innovative drug targets with an ideal therapeutic profile, since their pharmacological manipulation should not interfere with physiological transmission.

Very few data are available on the role of the P2Y receptor family in SGCs. In DRG, in situ hybridization histochemistry (ISHH) studies detected mRNA expression only for the $P2Y_{12}$ and $P2Y_{14}$ receptor subtypes on these cells (Kobayashi et al., 2006). However, immunofluorecence studies showed staining for $P2Y_1$ on both neurons and SGCs (Chen et al., 2008). Immunofluorescence staining also indicated the selective expression of $P2Y_4$ on SGCs from both mouse and rat TG (Weick et al., 2003; Vit et al., 2006). Unfortunately no functional studies, which could help clarifying this issue, are available for DRG-derived SGCs. Calcium imaging experiments on intact trigeminal ganglia revealed that application of ATP and UTP caused functional raises in $[Ca^{2+}]_i$ in

almost all SGCs (Weick et al., 2003). This effect persisted in nominally Ca^{2+}-free extracellular solution, but was blocked by cyclopiazonic acid, an inhibitor of intracellular Ca^{2+} release from the endoplasmic reticulum. These experiments confirmed that ATP/UTP responses on SGCs were due to functional P2Y receptors, likely the $P2Y_2$/$P2Y_4$ receptor subtypes (Weick et al., 2003). Moreover, $[Ca^{2+}]_i$ increases were observed in SGCs after application of adenosine 5'-[β-thio]-diphosphate (ADPβS) and 2-methylthio adenosine 5'-diphosphate (2MeSADP), two $P2Y_1$ receptor selective agonists (Abbracchio et al., 2006), therefore suggesting that $P2Y_1$ receptors are also expressed and functional in these cells (Weick et al., 2003). In conclusion, also SGCs from TG bear a wide panel of functional P2Y receptors, thus suggesting a primary role for nucleotides in modulating cell-to-cell communication in sensory ganglia.

As already mentioned in *Paragraph 1.1.3*, SGCs form a morphological and functional unit together with sensory neurons (Hanani, 2005). Recent evidence suggests that, within this functional unit, a non-synaptic cross communication between neurons and SGCs can increase the excitability of both primary afferents and centrally projecting neurons, leading to the development of hyperalgesia and allodynia (Takeda et al., 2009). The P2 purinergic receptors expressed by SGCs participate primarily to this bidirectional neuron-glial communication inside the sensory ganglia. Indeed, a seminal work by Zhang and coll. (Zhang et al., 2007) reported that the electrical stimulation of DRG neurons elicited robust vesicular ATP release from their somata. The rate of ATP release was dependent on the frequency of nerve stimulation, and on the entry of external Ca^{2+} into the neuron. In addition, released ATP could activate $P2X_7$ receptors on SGCs, leading to the release of TNFα, which in turn potentiated $P2X_3$-mediated ATP currents in neurons (Zhang et al., 2007). The purinergic signaling pathways activated in SGCs might therefore represent interesting pharmacological targets to be exploited for the development of new analgesic strategies.

1.4.3 Role of P2Y receptors in pain transmission: CNS

There are only few and relatively controversial data on the presence and functions of P2Y receptors in spinal cord (Jarvis, 2009; Tsuda et al., 2009). For instance, intrathecal administration of UTP and UDP elevated the mechanical nociceptive threshold and prolonged the thermal nociceptive latency in uninjured rats. Moreover,

following partial ligation of the sciatic nerve UTP and UDP produced significant anti-allodynic effects, thus suggesting an antinociceptive role for the $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor subtypes (Okada et al., 2002). Surprisingly, this study contrasts with a more recent data showing that the intrathecal delivery of suramin (a non-selective $P2Y_2$ and $P2Y_4$ receptor antagonist) blocks microglia activation and long-term hyperalgesia induced by formalin injection, therefore suggesting that a block, rather than an activation, of P2Y receptors con provide pain relief following inflammatory conditions (Wu et al., 2004).

Contrasting data notwithstanding, it seems clear that purinergic receptors expressed by microglial cells critically participate to the genesis and maintenance of neuropathic pain. Several purinoceptors are expressed by microglial cells, including the $P2X_4$, $P2X_7$, $P2Y_6$, and $P2Y_{12}$ receptor subtypes (Inoue & Tsuda, 2009). Besides the prominent roles of the $P2X_4$ subtype (see previous paragraph), recent studies have revealed that the $P2Y_{12}$ receptor is also crucial for neuropathic pain. Both the expression of $P2Y_{12}$ receptor mRNA and protein are markedly enhanced in microglial cells of the ipsilateral spinal cord following nerve injury (Tozaki-Saitoh et al., 2008) or partial ligation of the sciatic nerve (Kobayashi et al., 2008). Interestingly, administration of $P2Y_{12}$ receptor antagonists, such as Cangrelor (intrathecally) or Clopidogrel (orally), as well as antisense oligonucleotide for $P2Y_{12}$ receptors, significantly suppressed the development of neuropathic pain after spinal nerve injury (Tozaki-Saitoh et al., 2008) and partial sciatic nerve injury (Kobayashi et al., 2008). Furthermore, mice lacking $P2Y_{12}$ receptors displayed impaired tactile allodynia after nerve injury without any change in basal mechanical sensitivity (Tozaki-Saitoh et al., 2008). The $P2Y_{12}$ and $P2X_4$ receptor subtypes have been identified as primary sensors for ATP-mediated chemotaxis in microglia (Inoue & Tsuda, 2009), and $P2Y_{12}$ receptors are also implicated in the motility of microglial cells (Haynes et al., 2006). The strategic importance in $P2Y_{12}$ receptor targeting is due to its restricted expression in the CNS. In fact, this receptor is specifically localized to brain and spinal cord resident microglia, but not in peripheral macrophages (Haynes et al., 2006). Therefore, the $P2Y_{12}$ receptor subtype could be considered as a molecular switch to specifically inhibit microglial cell activation, and possibly, chronic pain.

Microglial cells also express $P2Y_6$ receptors, and their expression markedly increases in the spinal cord after peripheral nerve injury (Koizumi et al., 2007). It has

been also found that UDP, an agonist for $P2Y_6$ receptors, facilitated phagocytosis in primary cultured microglial cells. The $P2Y_6$ receptor can be thus considered a sensor for microglial cell phagocytosis by sensing diffusible UDP signals (Koizumi et al., 2007); unfortunately its role under neuropathic pain conditions still remains unknown.

2. AIM OF THE STUDY

Migraine is a highly disabling neurovascular disorder, affecting about 15% of adults in the Western World, which is characterized by spontaneous and recurrent attacks of unilateral headache with associated autonomic symptoms. Its socio-economic implications have a considerable impact on both productivity and quality of life, and it has been estimated to be the most costly neurological disorder in the European Community (Goadsby, 2007). Accordingly, it is now ranked at the 19th place among all worldwide diseases causing disability by the World Health Organization (Lipton et al., 2004). Despite the strong progresses made in recent years, there is still ongoing debate about the mechanisms leading to the generation of migraine pain. Consistently, the pharmacological treatment of migraine still remains unsuccessful for a significant number of patients. For this reason, new efforts in the study of its pathophysiological mechanisms, in the discovery of innovative pharmacological targets, and in the developing of new effective drugs are strongly needed.

As mentioned in *Paragraph 1.1.2*, the trigeminal ganglia (TG) are the location of primary afferent neurons for sensing and relaying nociceptive sensations associated with painful conditions, such as dental pain, trigeminal neuralgia, and temporomandibular disorders (Fried et al., 2001). Importantly, the peripheral sensitization of sensory neurons in the TG and the concomitant increased release of neurovascular mediators, like the Calcitonin Gene-Related Peptide (CGRP), are also thought to play an important role in migraine (Messlinger, 2009). Sensory neurons in the TG act in strict synergy with non-neuronal satellite glial cells (SGCs), which envelop neuronal bodies to constitute a functional unit within the ganglion (Hanani, 2005). Recent evidence suggests that, within this functional unit, a non-synaptic cross communication between neurons and SGCs can increase the excitability of both primary and CNS neurons, leading to the development of hyperalgesia and allodynia (Takeda et al., 2009). Although various pro-inflammatory mediators are released in the TG (Takeda et al., 2009), the whole molecular network at the basis of this neuron-to-SGCs interplay, and its involvement in migraine pain mechanisms, is still largely unknown. Therefore, the identification of the signalling molecules controlling neuron-to-glia communication could help finding new potential strategies to modulate pain transmission and, possibly, to yield new pharmacological targets to prevent or abort migraine attacks.

Starting from the first observations that ATP injection into the human skin elicited pain (Bleehen and Keele, 1977; Krishtal et al., 1983), several studies progressively indicated that the purinergic system plays a crucial role in the transmission and integration of pain sensation. Besides ATP, emerging evidence indicates that also other extracellular nucleotides (ADP, UTP and UDP), and nucleosides (adenosine) participate in the modulation of pain transmission (Liu & Salter, 2005; Burnstock, 2009a; Jarvis, 2010). The most studied and characterized receptors involved in nociception are the $P2X_3$ and the $P2X_{2/3}$ receptor channels, which are expressed by neurons in sensory ganglia, and play a fundamental role in transducing ATP-mediated painful signals (Burnstock, 2009a). However, a role for G protein-coupled P2Y receptor subtypes in nociception is also emerging (Jarvis, 2010). These receptors are widely expressed by both neurons and glial cells of the PNS and CNS, and undergo significant changes in their expression levels after neuropathic or inflammatory conditions (Jarvis, 2010). However, most of the currently available information on P2Y receptors in nociception is based on results obtained from dorsal root ganglia (Burnstock, 2009b), or from the spinal cord (Tsuda et al., 2010). Therefore, deciphering the nociceptive functions of P2Y receptors in TG is of paramount importance for discovering new potential targets for migraine therapy.

On this basis, the first objectives of my study have been aimed at setting up an *in vitro* model of primary mixed neuron-glia cultures from mouse TG, and at evaluating the presence and functionality of P2 receptors in both neurons and SGCs. To evaluate the presence of the specific P2 receptor subtypes, I have utilized RT-PCR analysis and single cell calcium imaging by applying subtype-selective P2 ligands. Since very few data are available on the role of the P2Y receptor family in SGCs (Villa et al., 2010), peculiar attention has been given to the characterization of P2Y receptors in this cell population. I have also evaluated changes in P2Y receptors calcium transients *in vitro* after acute or long-term treatments with Bradykinin and CGRP, two mediators involved in migraine neurobiology (See *Paragraph 1.2.2*; Pietrobon, 2005).

One of the most important features of the pathophysiology of migraine is its inherited nature. However, only few specific genes have been correlated with migraine. This is the case of the Familial Hemiplegic Migraine (FHM) which is caused by mutations of specific genes: the α1 subunit of the Cav2.1 (P/Q) type voltage-gated Ca^{2+} channel (CACNA1A), causing the FHM type-I; the ATP1A2 gene, causing the FHM

type-II; and the Na^+ channel SCN1A, causing the FHM type III (Goadsby, 2007). We have taken advantage of a genetic mouse model of FHM1, carrying the R192Q missense mutation in the α1 subunit of $Ca_V2.1$ calcium channels (van den Maagdenberg et al., 2004), and we have looked for possible alterations in purinergic signaling in TG cultures that could have implications for migraine pathophysiology.

The last part of my PhD project has been aimed at setting up *in vivo* models of pain, as tools for evaluating the roles of specific P2Y receptors expressed by glial cells. I have initially set up a model of acute pain, based on the injection of formalin into the upper lip of mice, and I have looked for changes in pain behavior after double-stranded RNAs mediated silencing of the $P2Y_4$ receptor subtype, which is selectively expressed by SGCs in the TG.

As for migraine pain, chronic pain is still a difficult to manage and disabling condition, despite great efforts in the last decades towards the understanding of its pathophysiology, and the development of new drugs. The reason for this failure may be partially due to the fact that most of the available drugs target neurons (Dworkin et al., 2010), whereas increasing evidence now indicates that glial cells in both sensory ganglia and CNS also play an important role in chronic pain genesis (McMahon & Malcangio, 2009). A well-established model of inflammatory pain, which shares several characteristics with migraine-associated TG sensitization (Ballegaard et al., 2008; Taub et al., 2008), is based on the injection of pro-inflammatory mediators (e.g., the Complete Freund's Adjuvant, CFA) in the temporomandibular joint (TMJ). TMJ inflammation potentiates the excitability of both primary and secondary neurons, and leads to increased neuron-to-SGC communication within the TG (Iwata et al., 1999; Thalakoti et al., 2007). However, no studies have explored the reaction of glial cells in the whole spinal-trigeminal system following induction of TMJ inflammation so far. Therefore, we have characterized the reaction of PNS and CNS glial cells to the injection of CFA into the rat TMJ and, finally, we have evaluated expression changes for the $P2Y_{12}$ receptor subtype, which is selectively expressed by CNS microglia, and participates actively in chronic pain development (see *Paragraph 1.4.3*). This model of sub-chronic inflammation will allow us to evaluate the role of specific P2Y receptor subtypes, expressed by glial cells, in the development and maintenance of chronic trigeminal pain and migraine-associated pain.

3. METHODS

3.1 CELL CULTURES

Primary mixed neuron-satellite glial cells (SGCs) cultures were prepared from trigeminal ganglia (TG) of wild type C57Bl/6J mice (Charles River lab, Calco, Italy) or of transgenic $Ca_V2.1$ $\alpha1$ R192Q mutant KI mice at postnatal day 11 (P11; van den Maagdenberg et al., 2004), as previously described (Ceruti et al., 2008) (see Figure 3.1A for a schematic protocol). Briefly, after decapitation TG were rapidly excised and dissociated in 0.25 mg/ml trypsin, 1 mg/ml collagenase and 0.2 mg/ml DNAse (Sigma-Aldrich, Milan, Italy) in F12 medium (Invitrogen, Gibco, Italy) at 37°C. Enzymes were then inactivated by adding 10% fetal calf serum and 0.125 mg/ml trypsin inhibitor (Sigma-Aldrich). Cells were centrifuged at 1,000 rpm for 5 min, resuspended in F12 medium +10% fetal calf serum and plated onto poly-L-lysine-coated 24 mm-diameter glass coverslips. Experiments involving primary mixed neuron-SGCs cultures were performed 48 h after plating. A representative field of primary mixed neuron-SGCs cultures is shown in Figure 3.1B.

SGCs purified cultures were prepared from mixed cultures at day 6 in culture, after replacing culture medium at days 1 and 3 (Figure 3.1A). At day 6, cells were detached from the Petri dish by a 5-min treatment with 0.5%-trypsin/0.2%-EDTA (Sigma-Aldrich, Milan, Italy) at 37°C, resuspended in fresh culture medium, and replated onto uncoated wells or 24 mm diameter coverslips. This procedure completely removed all neurons without affecting SGCs adhesion and growth (England et al., 2001; Capuano et al., 2009). The culture medium was replaced 24 hours later, and experiments were performed after additional 24 hours. A representative field of purified SGCs cultures is shown in Figure 3.1C.

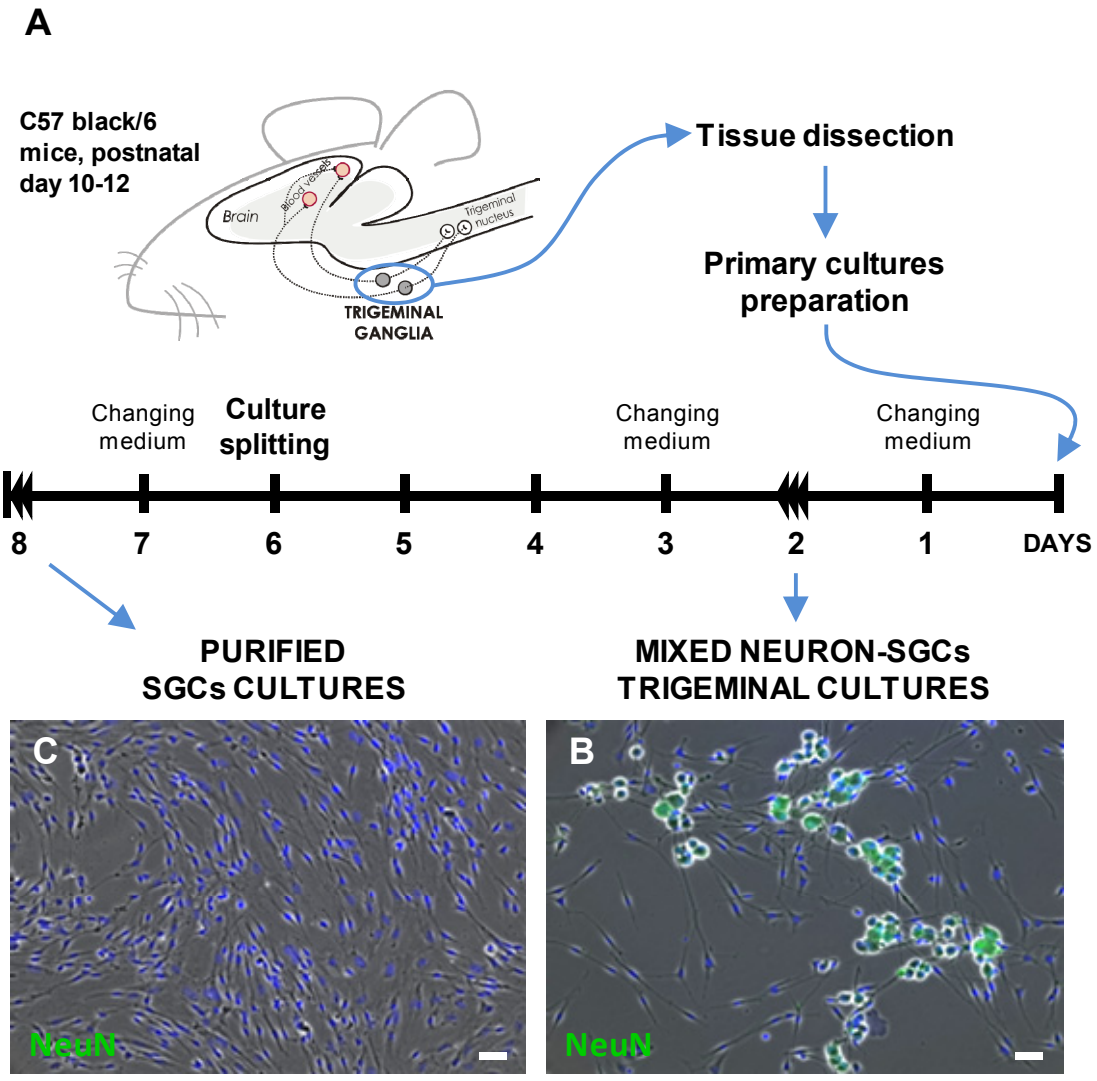


Figure 3.1

Schematic experimental protocol for trigeminal cultures preparation.

A, *Primary mixed neuron-SGCs cultures were prepared from trigeminal ganglia of P11 wild type C57Bl/6 mice or of transgenic* $Ca_V2.1$ *α1 R192Q mutant KI mice (see text for more details).* ***B,*** *Representative picture showing primary mixed neuron-glia trigeminal cultures 48h after plating. NeuN-positive neurons are shown in green.* ***C,*** *Representative picture showing purified SGCs cultures 8 days after plating. Using our purification protocol (see text for more details), neurons were completely removed, as demonstrated by the lack of NeuN staining. In all pictures, nuclei were stained with the Hoechst 33258 dye. Scale bars: 50 μm.*

3.2 PHARMACOLOGICAL TREATMENTS

Both primary mixed and purified SGCs cultures were exposed to 100 nM BK (Sigma-Aldrich), or 1 μM CGRP (Tocris Bioscience, Bristol, UK) for the indicated time periods (see Results and Figures). In selected experiments, the CGRP receptor antagonist, $CGRP_{8-37}$ (2 μM; Tocris Bioscience), or the ERK1/2 inhibitor PD98059 (50 μM; Sigma-Aldrich), were added to the culture medium 30 min before CGRP or BK application. Parallel control cells were treated with vehicle (Ham's F-12) alone.

For single cell calcium measurements, the following pharmacological agents were tested: αβ-methylene-ATP (αβ-meATP; 100 μM), ADP (10 and 100 μM), UTP (10 and 100 μM), UDP (100 μM), UDP-glucose (100 μM), N^6-methyl-2-deoxyadenosine 3',5'-bisphosphate (MRS2179; 100 μM), Cangrelor (10 μM), Reactive-blue-2 (RB-2, 100 μM), Suramin (100 μM). All reagents were obtained from Sigma-Aldrich, except for Cangrelor that was a kind gift of The Medicines Company, Parsippany, NJ, USA.

3.3 IMMUNOCYTOCHEMISTRY

Cell cultures were fixed at room temperature for 25 min with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; Euroclone, Milan, Italy) containing 0.12 M sucrose. Cells were subsequently incubated for 20 min at room temperature with Goat Serum Dilution Buffer (GSDB; 450 mM sodium chloride and 20 mM sodium phosphate buffer, pH 7.4, 15% goat serum, and 0.3% Triton X-100), before exposure to the primary antibodies diluted in GSDB. The following primary antibodies were utilized: rabbit anti-CGRP (1:800; Enzo Life Sciences AG, Lausen, Switzerland), mouse anti-β-Tubulin-III (β-TubIII; 1:500; Promega, Milan, Italy), mouse anti-glutamine synthetase (GS, 1:100; Millipore, Vimodrone, Italy), mouse anti-vesicle associated membrane protein 2 (VAMP2; 1:300; Synaptic Systems, Göttingen, Germany), rabbit anti-$P2X_3$ (1:250 o/n at 4°C; Alomone Labs, Jerusalem, Israel), mouse anti-2'3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase, 1:100, 1 hour room temperature; Chemicon). *Bandeireae simplicifolia* isolectin B4 directly conjugated to fluorescein isothiocyanate (FITC-IB4, 1:100; Sigma-Aldrich) was also utilized. After an overnight incubation at 4°C, cells were rinsed three times for 10 min in a high salt

buffer solution (500 mM sodium chloride and 20 mM sodium phosphate buffer, pH 7.4), and then incubated (1 hour, room temperature) with secondary goat anti-rabbit and goat anti-mouse antibodies conjugated to AlexaFluor®488 or AlexaFluor®555 (1:600 in GSDB; Molecular Probes, Invitrogen, Milan, Italy). Subsequently, nuclei were labeled with the fluorescent dye Hoechst-33258 (1:10,000 in PBS, Molecular Probes, Invitrogen). Cells were rinsed three times in high salt buffer, once in PBS, and finally once in 5 mM sodium phosphate buffer, pH 7.4. Coverslips were mounted with Fluorescent Mounting Medium (Dako, Milan, Italy), and analyzed using an inverted fluorescence microscope (200M; Zeiss, Milan, Italy) connected to a PC computer equipped with the Axiovision software (Zeiss). The same software also enabled us to measure neuron diameters. Non-specific staining was evaluated on coverlips where the primary antibodies were omitted from the staining procedure.

3.4 IMMUNOHISTOCHEMISTRY

3.4.1 Tissue processing

Naïve mice or rats injected with saline or CFA (see *Paragraph 3.9*) were anesthetized with intraperitoneal injection of 400 mg/kg chloral hydrate and transcardially perfused with 4% formalin fixative. Intact brains and TGs were excised, postfixed in 4% formalin for 60-90 min, and cryoprotected in 30% sucrose for at least 48 hours. The left and right TG from each animal were embedded together in mounting medium (OCT; Tissue Tek, Sakura Finetek, Zoeterwoude, The Netherlands), and cut longitudinally on a cryostat at 15 μm thickness. Brainstems were separate from the rest of the brain, and marked ventrally on the contralateral side to subsequently identify tissue orientation. Transverse 40 μm thick free-floating sections were then cut on a cryostat.

3.4.2 Immunostaining of tissues

Free-floating brainstem sections or on-slide TG sections were incubated for 45 min at room temperature in PBS containing 10% normal goat serum (Sigma-Aldrich, Milan, Italy) and 0.1% Triton X-100 (Sigma-Aldrich), and then overnight at room temperature with the primary antibodies listed in *Paragraph 3.3* with the addition of:

rabbit anti-glial fibrillary acidic protein (GFAP, 1:600; Dako, Milan, Italy), mouse anti-NeuN (1:500; Millipore, Vimodrone, Italy), rabbit anti-ionized calcium binding adaptor molecule 1 (Iba1, 1:800; Biocare Medical, Space Import-Export, Milan, Italy), rabbit anti-$P2Y_{12}$ receptor polyclonal antiserum (1:1,500; a generous gift by Prof. David Julius, University of California San Francisco, CA, USA), and mouse anti-ED1 (1:200; Serotec, Space Import-Export).

For fluorescence analysis, sections were then rinsed three times with PBS, and incubated for 1 h at room temperature with goat anti-rabbit and goat anti-mouse secondary antibodies conjugated to AlexaFluor®488 or AlexaFluor®555 fluorochromes (1:600; Molecular Probes, Invitrogen, Milan, Italy). Nuclei were subsequently labeled with the fluorescent dye Hoechst 33258 (1:10,000 in PBS; Molecular Probes). Slides were finally washed, mounted with Fluorescent Mounting Medium (Dako), and examined with a laser scanning confocal microscope (LSM 510; Zeiss, Jena, Germany). Images were acquired and analyzed using the LSM Image Browser software (Zeiss).

For light microscopy, sections were incubated for 1h at room temperature with an anti-rabbit biotinylated secondary antibody (1:500; PerkinElmer, Monza, Italy), and then with horseradish peroxidase (HRP)-conjugated streptavidin (1:400, 45 min at room temperature; PerkinElmer). To visualize the antibody-antigen complex, the nickel- 3,3'-diaminobenzidine (Sigma-Aldrich) protocol was used. Sections were mounted with the DPX mountant for histology (Sigma-Aldrich), and analyzed with an inverted microscope (Axiovert 200; Zeiss) equipped with a color CCD camera (AxioCam HRc; Zeiss), connected to a PC computer equipped with the software Axiovision (Zeiss).

All antibodies were diluted in PBS containing 0.1% Triton X-100 and 1% normal goat serum.

3.4.3 Quantification of results and data analysis

Quantitative analysis of the number of $Iba1^+$ macrophages in TGs was performed by using the NIH Image-J software on digital images of immunolabeled sections, captured at 10x magnification. A stack of all acquired images was created, and the threshold was set to a level that included all Iba1 immunopositive pixels but not the lighter background pixels. Both the number and the size of $Iba1^+$ cells, distributed in the V1, V2 and V3 divisions, were then automatically measured. The number of positive cells has been normalized to the area of measurement.

ED1-immunoreactivity in the TG, and GFAP-, Iba1- and $P2Y_{12}$ receptor-immunoreactivity in the brainstem was assessed by densitometric analysis. A digital image of the immunolabeled sections was acquired at 20x or 10x magnification for TG or brainstem, respectively, and the threshold for the positive staining was set as described above. The number of immunostained pixels was then automatically counted by using the NIH Image-J software. For GFAP, Iba1 and $P2Y_{12}$ receptor immunostaining, the mean pixel intensity values were expressed as fold increase compared to the contralateral side of saline injected animals set to 1.0.

Two sections for each TG or brainstem and for each antibody were analyzed. To avoid variability in the staining procedure, all the sections to be compared were immunostained together, and images were acquired under the same exposure conditions. The anatomical structures in the brainstem and spinal cord were identified with reference to a rat brain atlas (Paxinos & Watson, 1986).

3.5 INTRACELLULAR CALCIUM MEASUREMENTS

Cultures were loaded for 45 min at 37°C with 2 μM Fura-2 pentacetoxy methylester in Krebs-Ringer solution buffered with HEPES (KRH; 125 mM NaCl, 5 mM KCl, 1.2 mM $MgS0_4$, 2 mM $CaC1_2$, 10 mM glucose, and 25 mM HEPES/NaOH, pH 7.4), as previously described (Ceruti et al., 2008), and transferred to the recording chamber of an inverted microscope (Axiovert 100TV; Zeiss) equipped with a calcium imaging unit. Polychrome IV (TILL Photonics, Germany) was used as light source. Fura-2 fluorescence images were collected with a CCD camera Imago-QE (Till Photonics), and analyzed with the Tillvision 4.0.1 software.

Fura-2 is a ratiometric fluorescent dye which binds to free intracellular calcium. The Fura-2-Ca^{2+} complex adsorbs the light at 340nm, while the free form of Fura-2 adsorbs at 380nm. Application of stimuli increasing the intracellular calcium concentration ($[Ca^{2+}]_i$), evoke an increase in light absorption at 340nm and a decrease at 380nm. Calcium concentrations are estimated by measuring the emitted light after excitation at 340 and 380 nm wavelengths, and by calculating the F340/380 fluorescence ratio. A representative calcium imaging experiment, showing the $[Ca^{2+}]_i$ increase after application of 100μM ATP or 50mM KCl, is shown in Figure 3.2. In our

settings, recordings were usually made at 1 ratio/s, and were increased up to 8 ratios/s upon stimulation with αβ-meATP. Neurons were identified by their peculiar responsiveness to 50 mM KCl. The total number of cells analyzed for any given condition is indicated as "*n*".

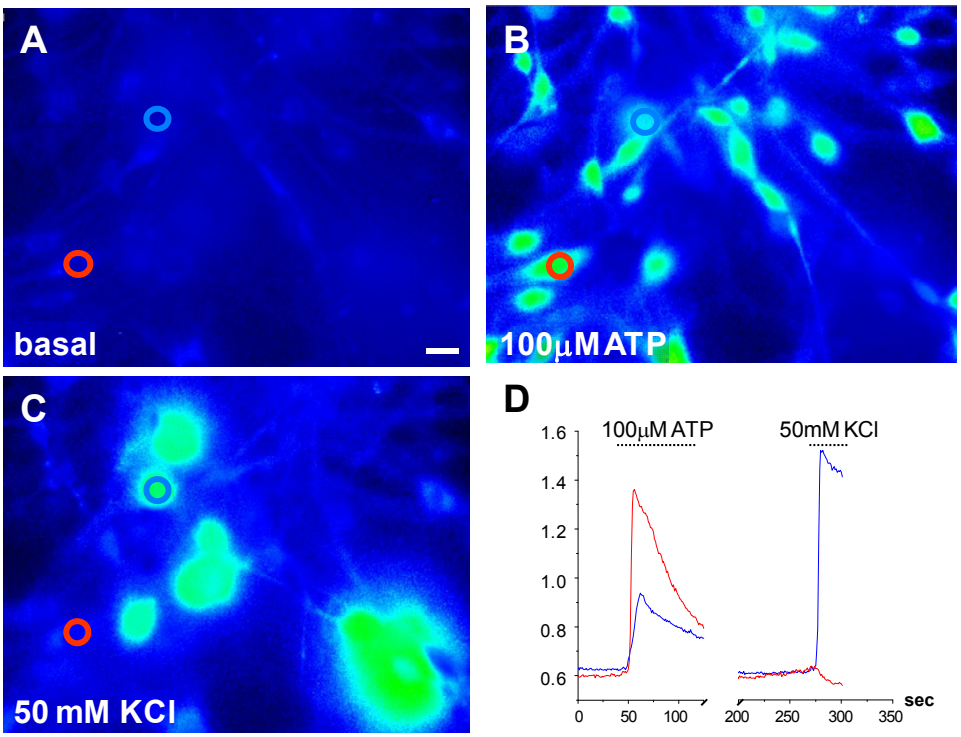


Figure 3.2

Representative series of pictures from a Calcium imaging experiment.

A-C, *representative series of pseudocolor images of Fura-2-loaded cells under basal conditions (basal,* ***A****), and after application of 100µM ATP (****B****) or 50mM KCl (****C****). The latter is utilized as a depolarizing agent to identify neurons. Graded colors from blue to green and yellow indicate the increase in F340/380 ratio. Scale bar: 15 µm.* ***D,*** *representative temporal plot of* $[Ca^{2+}]_i$ *changes recorded from the two cells circled in* ***A-C****, and stimulated with 100 µM ATP followed by 50mM (blue circle: neuron, indicated by the blue trace, and responding to KCl****;*** *red circle: SGC, indicated by the red trace).*

3.6 TOTAL RNA ISOLATION AND RT-PCR ANALYSIS

Total RNA was extracted using the TRIZOL® reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was then pre-treated with RQ1 DNase (Promega) for eliminating genomic DNA contamination. Retrotranscription of 1µg RNA was performed with Superscript II RNaseH Reverse Transcriptase (200U per sample; Invitrogen) using 100 pmoles of random hexamers or OligodT (Applied Biosystems) as primers. Aliquots of total cDNA (1-2µl) were amplified in each PCR assay with Platinum Taq DNA polymerase (1.25U per sample; Invitrogen) in a 25µl reaction mixture containing 20 pmoles of primer pair, in a standard PCR buffer (50mM KCl, 1.5mM MgCl2, 20mM Tris–HCl, pH 8.4). For cDNA, control samples which did not undergo reverse transcription (indicated as -RT) were processed in parallel with the same experimental protocol to check for contamination of RNA with genomic DNA. Amplifications were performed in a GeneAmp 9700 thermal cycler (Applied Biosystems, Milan, Italy) for 35 cycles (typically 94 °C/45 sec; 45 sec at an the optimal annealing temperature for each primer pair, see Table 3.1; 72 °C/45 sec), after an initial denaturation at 94 °C for 2 min. The primer pairs used for detecting the RNAs of interest via PCR amplification, and designed with the software Oligo 4.0, are reported in Table 3.1. Amplified products were size-separated by electrophoresis on a 1.5% agarose gel with ethidium bromide, and visualized under an UV light.

Table 3.1: Primer sequences, annealing temperatures and expected molecular weight of the detected PCR products.

Gene	Primers	Ta (°C)	cDNA (bp)	Accession number (Gene Bank)
β-Actin	S 5'-TGACGGGGTCACCCACACTGTGCCCATCTAC-3' A 5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'	55.0	661	NM_007393
β-TubIII	S 5'-CTTCCAGCTGACACACTCAC-3' A 5'-AGACACAAGGTGGTTGAGGT-3'	56.5	295	NM_023279
B1	S 5'- TCCTGTCCTTCTTCCTTTTG -3' A 5'- CCATTGTCTTGCTGTCCTTG -3'	57.4	600	NM_007539.2
B2	S 5'- TTCACCAACGTGCTGCTGAAC -3' A 5'- ACTGTTTCTTCCCTGCCCAGTC -3'	60.0	508	NM_009747.2
RAMP1	S 5'- GACGCTATGGTGTGACT -3' A 5'- AGTGCAGTCATGAGCAG -3'	57,4	249	NM_007588.2
CLR	S 5'- TGCTGGAATGACGTTGCAGC -3' A 5'- GCCTTCACAGAGCATCCAGA -3'	54,1	483	NM_016894.3
$P2X_1$	S 5'-GTTTGGGATTCGCTTTGA-3' A 5'-TCAGGAAGGGAAGTGTGG -3'	55.8	452	NM_008771
$P2X_2$	S 5'-GGTGGAGGATGGGACTTC-3' A 5'-ATGGTGGGAATGAGACTG-3'	53.1	498	AB094664 AB094663
$P2X_3$	S 5'-ACTTTGTGGGGTGGGTTT-3' A 5'-GCTGCCATTCTCCATCTT-3'	55.6	767	NM_145526
$P2Y_1$	S 5'-CCTGCGAAGTTATTTCATCTA-3' A 5'-GTTGAGACTTGCTAGACCTCT-3'	51.6	319	NM_008772
$P2Y_2$	S 5'-GCAGCATCCTCTTCCTCACCT-3' A 5'-CATGTTGATGGCGTTGAGGGT-3'	60.2	503	NM_008773
$P2Y_4$	S 5'- CTTTGGCTTTCCCTTCTTGA -3' A 5'- GTCCGCCCACCTGCTGATGC -3'	57.2	492	NM_020621
$P2Y_6$	S 5'-CGCTTCCTCTTCTATGCCAA-3' A 5'-GTAGGCTGTCTTGGTGATGTG-3'	59.6	480	AF298899
$P2Y_{12}$	S 5'-CCGCTACCTGAAGACCACCA-3' A 5'-GTTCGCCACCTTCTTGTCCTT-3'	55.1	641	NM_027571
$P2Y_{13}$	S 5'-CAGGGACACTCGGATGACA-3' A 5'-CACCGCATAAAACAGAAGC-3'	55.4	577	NM_028808
$P2Y_{14}$	S 5'-GTCTCTGCCGTCATCTTCT-3' A 5'-GGGTCCAGACACACATTG-3'	54.3	591	NM_133200

3.7 WESTERN-BLOTTING ANALYSIS

Whole-cell lysates were prepared and analyzed by Western blotting as previously described (Bianco et al., 2005). Briefly, approximately 30 μg aliquots from each protein sample were loaded on 11% sodium-dodecylsulphate polyacrylamide gels, and blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Milan, Italy). Filters were then saturated with 10% non-fat dry milk in Tris-buffered saline (TBS; 1 mM Tris-HCl, 15 mM NaCl, pH 8) for 1 h at RT, and incubated overnight at 4°C with mouse anti-phospho-ERK1/2 (p-ERK1/2) and rabbit anti-ERK1/2 primary antibodies (1:500 and 1:1,000 in 5% non-fat dry milk in TBS respectively; Cell Signaling, Danvers, MA, USA). Filters were then washed in TBS-T (TBS plus 0.1% Tween20®), incubated for 1 h with goat anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (1:4,000 or 1:2,000 in 5% non-fat dry milk in TBS respectively; Sigma-Aldrich). Detection of proteins was performed by enhanced chemiluminescence (ECL, Amersham Biosciences, Milan, Italy) and autoradiography. Non-specific reactions were evaluated by in the presence of the secondary antibodies alone.

3.8 ANALYSIS OF CGRP RELEASE BY ENZYME IMMUNO-ASSAY

Basal and stimulated extracellular CGRP concentrations were evaluated in primary mixed cultures after 48 h *in vitro*. For each sample, basal CGRP levels were measured after a 15-min incubation with fresh culture medium (see above), which was then removed, stored for subsequent analysis, and replaced with culture media alone (CTR), or containing 100 nM BK. After 1 hour at 37°C, culture medium was collected, samples were centrifuged at 1,200 rpm for 5 min, and the supernatants processed for CGRP evaluation. Maximal CGRP released was evaluated by a 15 min incubation with 50 mM KCl. A commercial Enzyme Immunometric Assay (EIA) kit for rat CGRP (SPIbio, Montigny-le-Bretonneux, France) was utilized, following manufacturer's instructions. We validated the use of this EIA kit for the measurements of mouse CGRP by verifying the homology between the mouse and rat mature α-CGRP peptide sequences (UniProtKB database; www.uniprot.org), which was found to be 100%. Samples were analyzed at a 415 nm wavelength with a microplate reader (iMark™,

Bio-Rad Laboratories). CGRP concentrations in pg/ml were extrapolated from a best-fit line calculated from serial dilutions of a CGRP sample standard.

3.9 IN VIVO EXPERIMENTS

3.9.1 Animals

Experiments were performed on adult male Sprague-Dawley rats (200 - 250 g) or adult C57Bl/6J mice (30 g; Charles River Lab, Calco, Milan, Italy). Animals were housed under controlled conditions (temperature 22 ± 2 °C; relative humidity 50 ± 10%; artificial light 12 h light/dark cycle, lights on at 7 A.M.). All animals had access to both distilled water and standard diet ad libitum. The study has been approved by the Council of the Department of Pharmacological Sciences of the Università degli Studi di Milano, Milan, Italy, and was carried out in accordance with National and European regulations regarding the protection of animals used for experimental and other scientific purposes (D.M. 116192; 86/609/EEC), as well as following the ethical guidelines of the International Association for the Study of Pain (IASP; Zimmermann, 1983).

3.9.2 Orofacial formalin test

A 29-gauge needle was used to inject subcutaneously 10µL of 4% formalin or saline (0.9% NaCl), into the center of the right vibrissae pad of adult mice. Injections were performed as quickly as possible, with minimal animal restraint. Following the injection, animals were placed in a test box, and video recorded for 30 min. The total recording time was divided into 6 blocks of 5 min, and the nociceptive behaviour was determined for each block by measuring the number of seconds that the animals spent grooming the injected area with the ipsilateral fore- or hindpaw. Movements of the ipsilateral forepaw were accompanied by movements of the contralateral forepaw. Analysis of the behaviour was made by an investigator who was blinded to the animal's group assignment.

3.9.3 Synthesis of dsRNAs and their injection into the trigeminal ganglion

To reduce $P2Y_4$ receptor expression, long double-stranded RNAs (dsRNAs) sequences that have been shown to produce robust, specific, and reversible gene silencing were used (Bhargava et al., 2004). A BLAST (basic local alignment search tool) search of the non-redundant mouse database identified a 564 bp sequence that was not homologous to any other sequences, and therefore targeting only $P2Y_4$ receptor. cDNA of $P2Y_4$ was produced by reverse transcription of total RNA followed by a 30-cycle PCR using specific primers. These cDNAs were then cloned into a pTOPO vector (Invitrogen). The specific forward and reverse primer sequences used to make the dsRNAs corresponded to nucleotides 481-500 and 1114-1145, respectively (GenBank accession number NM_020621). MalE sequences were used as nonspecific dsRNA control as previously described (Bhargava et al., 2004). Sense and antisense RNAs were synthesized from cDNA inserts by using MegaScript RNA kit (Ambion, Monza, Italy) according to the manufacturer's specifications. Before the injection in the trigeminal ganglion, 11 μg of dsRNAs of either $P2Y_4$ or MalE dsRNA were mixed with 1μL lipofectamine (Invitrogen) in a final volume of 5μl, and let stand at room temperature for 30 min.

Injections were performed under xylazine-ketamine anesthesia by placing the mice in a stereotaxic instrument. The skull was exposed, and a burr hole was drilled above the location of the trigeminal nerve of the right trigeminal ganglion, at 1.6 mm posterior to the bregma, and 1.6 mm lateral to the midline. A pulled pipette, filled with dsRNAs, was then inserted through the hole, at 6.6 mm below the cortical surface. The pipette was connected to a Hamilton syringe attached to a microinjection pump, set to deliver the 5μL dsRNAs over a 2 min period.

3.9.4 Induction of TMJ inflammation

The sub-chronic TMJ inflammation was induced by injecting 50 μl of CFA (Sigma, Milan, Italy) oil/saline (1:1) emulsion into the left TMJ capsule, under isoflurane anesthesia. Control rats were injected with saline (0.9% NaCl). The TMJ capsule was identified by palpating the zygomatic arch and condyle, and the injection was delivered by advancing a 27-gauge needle medioanteriorly through the skin immediately below to the posteroinferior border of the zygomatic arch until it entered

into the joint capsule (Bereiter et al., 2005). Then, CFA or saline was injected slowly over 2 minutes.

3.9.5 Behavioral tests

Mechanical allodynia was measured by a previously described protocol with some minor modifications (Ren et al. 1999). Unrestrained rats were trained to stay in position and to be probed with von Frey filaments (North Coast Medical, Morgan Hill, CA, USA) at least one week before the injection of CFA. The left and right orofacial skin regions were tested, near the center of the vibrissa pad. An ascending series of von Frey filaments was used. The starting filaments corresponded to log unit 4.31 (force: 2 grams) and 3.22 (force: 0.16 grams) for control and inflamed animals, respectively. Each filament was tested five times with an interval of a few seconds. The response threshold was defined as the lowest force required eliciting at least three head withdrawal responses out of five tests. The elapsed time between the applications of a new filament was 2 minutes. All experiments were carried out in a quiet room between the 8.30 AM and 1.00 PM, in order to avoid diurnal variations.

3.9.6 Measurement of Evans' blue dye extravasation

Evans' blue dye (5 mg/kg, 0.3% solution) was injected into the tail vein (Zhou et al., 1999), 10 minutes before animal perfusion with PFA (see *Paragraph 3.4*) . Ipsi- and contra-lateral (with respect to the side of CFA injection) TMJs were then dissected, cut into small blocks and incubated overnight at room temperature in a 7:3 (vol/vol) mixture of acetone and 35.2 mM sodium sulphate on a shaking table. Samples were then centrifuged, the supernatant separated, and dye absorbance determined in a spectrophotometer at 620 nm. Evans' blue dye concentrations, in μg/ml, were extrapolated from a best-fit line calculated from a standard curve, prepared from a series of supernatants extracted from the TMJs of naïve animals, and mixed with serial dilutions of the Evans' blue dye.

3.10 STATISTICAL ANALYSIS

All results are expressed as mean±s.e.m. of at least three independent experiments. Statistical significance between groups was derived from one-way ANOVA followed by Scheffe's analysis, performed with the SPSS software. Three degrees of significance were considered: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

4. RESULTS

Section I: in vitro studies

4.1. SET UP OF PRIMARY MIXED TRIGEMINAL CULTURES AS AN IN VITRO MODEL FOR STUDYING CELL TO CELL COMMUNICATION IN THE TRIGEMINAL GANGLIA

As mentioned in Chapter 2, the first part of my PhD experimental work has been aimed at evaluating the presence and function of P2 receptors in an *in vitro* model of primary mixed cultures from mouse trigeminal ganglia (TG). This culture model was previously set up and validated as a reliable experimental model to study the purinergic regulation of trigeminal functions (Fabbretti et., 2006; Simonetti et al., 2006). However, these studies were focussed on studying only the neuronal $P2X_3$ receptor subtype, while no evidence on the role of other purinergic receptors, or on the contribution of glial cells to purinergic signalling, was provided.

As a first step, we have characterized our primary TG cultures, 48h after their preparation, in terms of the presence and organization of the different cell populations. As discussed in *Paragraph 1.1.2*, the cellular organization of the intact TG consists of neuronal cell bodies surrounded by satellite glial cells (SGCs; Hanani, 2005). These cells can be identified by the expression of the marker Glutamine Synthetase (GS), as shown by immunohistochemistry on sections from intact undissociated ganglia (Figure 4.1A). Since SGCs share some common characteristics with oligodendrocytes, a subpopulation of SGCs was also stained with an antibody against 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; Figure 4.1B). Panels D and E of Figure 4.1, show the typical appearance of primary mixed TG cultures 48 hours after plating, consisting of neurons, identified by staining with the neuronal marker β-tubulin-III (β-tubIII; Figure 4.1D-F), surrounded by GS- (Figure 4.1D) and CNPase-positive (Figure 4.1E) glial-like cells. Interestingly, several neurons sat on and were wrapped by glial cells, likely belonging to the SGCs population (Figure 4.1D,E). Cultured neurons displayed the typical pseudo-unipolar morphology of sensory neurons (Figure 4.1D) and accounted for the 11.1±0.97% of total cell population (n=8340; 12 coverslips from 8 independent experiments). As shown in Figure 4.1H and in line with previous data (Fabbretti et., 2006; Simonetti et al., 2006), the 41.5±5.8% of total neuronal cells were small neurons with a diameter of the soma lower than 15 μm, the 53.4±4.1% were medium neurons with a diameter between 15 and 25 μm and only the 5.1±0.7% were large neurons with a diameter greater than 25 μm.

We also characterized the molecular phenotype of the cultured sensory neurons. Most of the nociceptive neurons that have unmyelinated C-fibre axons can be subdivided into two overlapping groups, on both anatomical and functional c (see *Paragraph 1.1.2*; Ruan & Burnstock, 2003): the first group contains peptidergic neuropeptides, such as the Calcitonin gene-related peptide (CGRP), the other can be distinguished by the binding of the Isolectin B4 (IB4). By immunofluorescence analysis we showed that in intact ganglia CGRP was expressed by 34.2±0.80% of β-tubIII-positive neurons (n=3251; Figure 4.1C). Interestingly, primary mixed TG cultures retained the neuronal location of CGRP observed in the intact tissue (35.0±1.85%, n=588; Figure 4.1F). Moreover, in our cultures the 37.3±1.5% of the total β-tubIII-positive neurons were also positive for IB4 (Figure 4.1G,G'). These cells were exclusively small- to medium-sized, as shown in Figure 4.1H.

In conclusion, in our primary mixed TG cultures the presence of neurons surrounded by SGCs resembles the morphological and probably functional unit observed in the intact ganglia. Moreover, cultured sensory neurons retain most of the phenotypical characteristics observed *in vivo*. Taken together, these data validate the use of dissociated primary cultures as an adequate model to study the nociceptive functions of the various TG cell populations and the associated cross talk between neurons and SGCs.

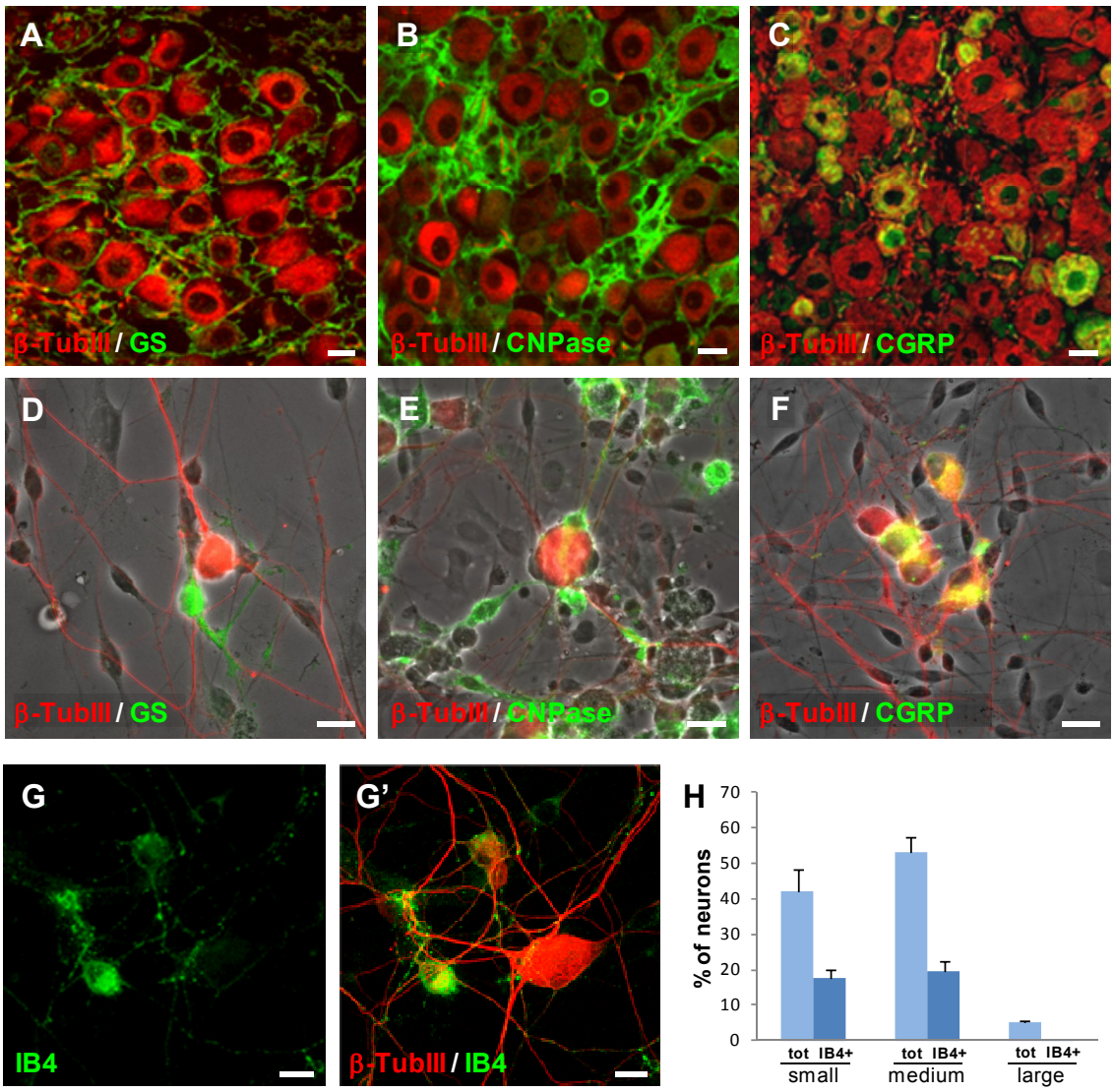


Figure 4.1

Primary mixed neuron-SGCs trigeminal cultures partially maintain the cellular and anatomical organization of the trigeminal ganglia *in vivo*.

A, B, C, *Representative confocal microscopy pictures from intact trigeminal ganglia after double immunofluorescence staining for the neuronal marker β-tubIII (red) together with glutamine synthetase (**A**), CNPase (**B**) and CGRP (**C**) expression (green).* ***D, E, F,*** *Double immunostainings, with the same antibodies as above, of primary mixed trigeminal cultures after 48h in vitro.* ***G,*** *Confocal microscopy image of trigeminal sensory neurons labeled with fluorescein-conjugated isolectin B4 (IB4, green).* ***G',*** *Same field as in **G,** after double immunostaining for IB4 (green) and β-tubIII (red), demonstrating IB4 colocalization with β-tubIII only in medium/small size neurons.* ***H,*** *Histograms showing the distribution of β-tubIII-positive neurons according to the size (diameter) of their soma (indicated as "tot", light-blue columns). The size distribution of IB4-positive neurons was also calculated and expressed as percentage of the total neuronal population ("IB4+" blue columns). To rank the diameters of neuronal cells, a classification previously proposed by Simonetti and coll. (Simonetti et al., 2006) was used (small neurons: diameter < 15 μm; medium neurons: diameter between 15 μm and 25 μm; large neurons: diameter > 25 μm). In all pictures, nuclei were labeled with the Hoechst 33258 dye (blue). Scale bars: 15 μm.*

4.2. BOTH TRIGEMINAL NEURONS AND SATELLITE GLIAL CELLS BEAR FUNCTIONAL P2 RECEPTORS

Previous studies have highlighted a role for ATP in cell-to-cell signalling in intact TG and have indicated the presence of various P2 receptor subtypes on both neurons and glia (Weick et al., 2003; Ceruti et al., 2008; Burnstock, 2009a,b). Among the ATP-responding receptors, the $P2X_3$ receptor subunit represents the most characterized in TG sensory neurons (Simonetti et al., 2006; Teixeira et al., 2010; Villa et al., 2010). To assess the presence and function of this receptor in our experimental setting, we performed double immunostaining experiments with an anti-β-tubIII and an anti-$P2X_3$ antibody. Most neurons (i.e., 73.6±2.7% of β-tubIII-positive cells, n=440) expressed $P2X_3$; Figure 4.2A). Moreover, this receptor was found in almost all (95.3±0.9%, n=324) IB4-positive sensory neurons (Figure 4.2B). The presence of $P2X_3$ in our cultures was also confirmed by RT-PCR (Figure 4.2C), and by western blot analysis (data not shown). Expression of the $P2X_2$ receptor (that can assemble in heterodimers with $P2X_3$) was also found, whereas the $P2X_1$ receptor subtype was not expressed (Figure 4.2C). Calcium transients induced by the $P2X_1/P2X_3$ receptor agonist α,β-meATP indicated the presence of functional receptor in trigeminal neurons (Figure 4.2D), with 58.0±4.7% of responding neurons The mean calcium increase (evaluated as the change in the 340/380 fluorescence ratio; ΔF340/380) was 0.12±0.01 (n=115). The $P2X_3$ receptor was never found in glial cells, as assessed by both immunostaining and calcium imaging analysis (data not shown, n=105).

Trigeminal cells have been demonstrated to express several P2Y receptors (Ruan & Burnstock, 2003; Burostock, 2009b; Villa et al., 2010), but a systematic analysis of their role in TG cultures has never been performed. Thus, we analyzed the presence of all P2Y receptors cloned from rodent tissues in both intact ganglia and mouse trigeminal cultures. RT-PCR analysis showed that all known rodent P2Y receptors (i.e., the $P2Y_{1,2,4,6,12,13,14}$ subtypes) were expressed with no appreciable differences in their expression profile between the intact tissue and the dissociated cultures (Figure 4.3). We next analyzed the responses of TG neurons to the most commonly utilized P2Y agonists by single cell calcium imaging. A small percentage of neurons responded to either ADP (8.5±3.9% of total, n=142) or UTP (13.0±4.1% of total, n=142; Figure 4.4C,E) application, with a mean calcium response of 0.44±0.06 ΔF340/380 and

0.34±0.09 ΔF340/380, respectively (Figure 4.4D). No neuronal cell showed responses to either UDP or UDPglucose (n=83 and n=43, respectively; Figure 4.4C). Response to ADP is likely mediated by $P2Y_1$ receptors, as demonstrated by the almost complete blockade exerted by the $P2Y_1$ receptor antagonist MRS2179 (Figure 4.4F,G). In some experiments, ADP response was also partially antagonized by the $P2Y_{12}/P2Y_{13}$ antagonist Cangrelor, suggesting that some neurons may also express these receptor subtypes together with $P2Y_1$ (data not shown).

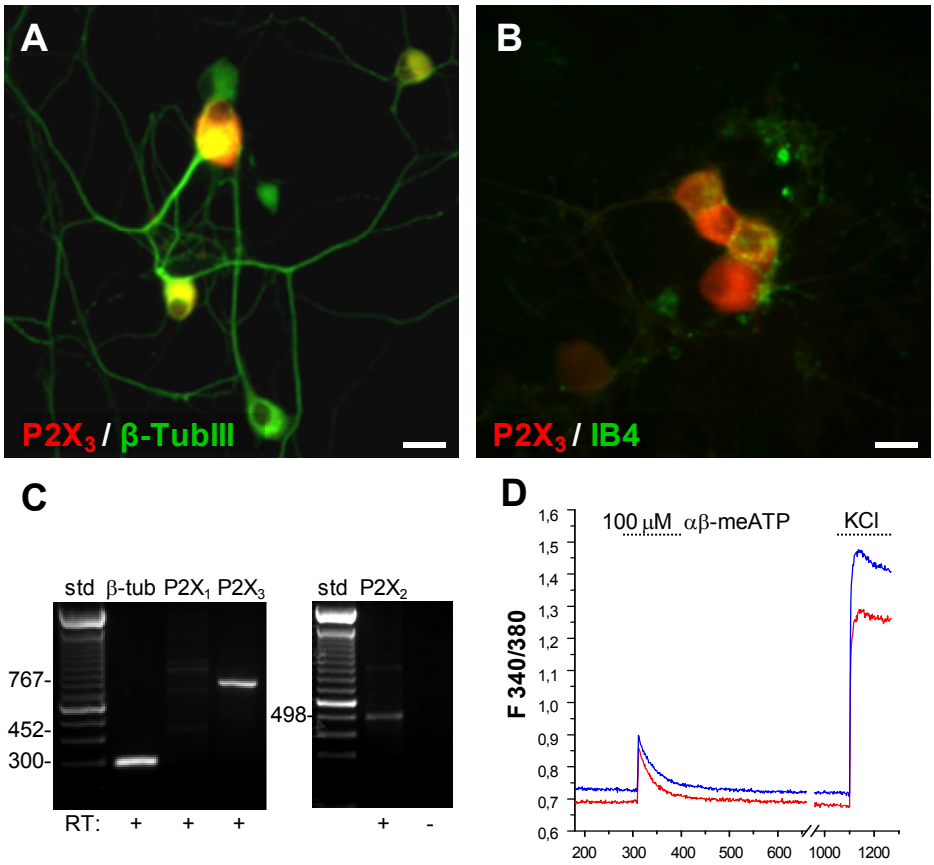


Figure 4.2

$P2X_3$ receptors are expressed and functional in trigeminal ganglion sensory neurons.

A, *Double immunostaining of cultures with anti-β-tubIII (red) and anti-$P2X_3$ receptor antibodies (green), showing that most of β-tubIII-positive cells also express $P2X_3$.* ***B,*** *Representative image showing double staining of cultures with an anti-$P2X_3$ receptor antibody (red) and FITC-IB4 (green). Scale bars: 15 µm.* ***C,*** *RT-PCR analysis showing expression of $P2X_2$ and $P2X_3$, but not of $P2X_1$ receptors, in trigeminal cultures. No amplification products were detected in RNA samples that were not subjected to retrotranscription (indicated as -RT). β-tubIII was used as an internal control for RT-PCR amplification.* ***D,*** *Representative temporal plot of $[Ca^{2+}]_i$ increases recorded from two trigeminal neurons upon stimulation with the $P2X_1/P2X_3$ agonist α,β-meATP (100 µM), followed by application of 50mM KCl.*

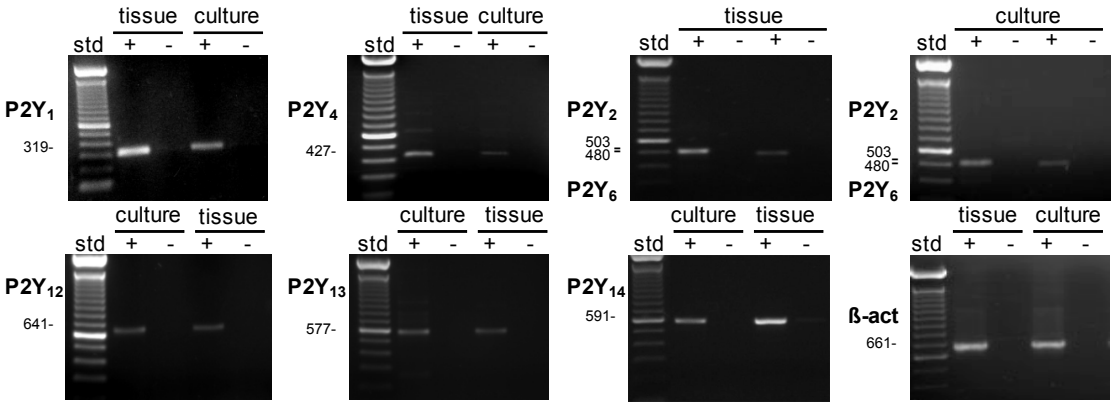


Figure 4.3

Trigeminal ganglia and cultures express all cloned P2Y receptors.

Total RNA isolated from either intact trigeminal ganglia (indicated as "tissue") or from primary trigeminal cultures 48 hours after preparation (indicated as "culture") was subjected to RT and the resulting cDNA was amplified with primers specific for each indicated P2Y receptor (see Table 3.1). No products were detected in the absence of retrotrascription (indicated as -). Parallel expression of the housekeeping gene β-actin (β-act) is shown. No significant differences in P2Y receptor expression were detected between freshly isolated tissue and cultures.

In a similar way, we characterized the functionality of P2Y receptors in TG SGCs. Application of P2Y agonists resulted in $[Ca^{2+}]_i$ increases in most cells, with 78.0±5.2% (n=320) and 83.0±4% (n=331) of SGCs responding to ADP and UTP respectively (Figure 4.5A). The mean calcium increases were 0.73±0.03 and 0.8±0.3 ΔF340/380, for ADP and UTP respectively (Figure 4.5B). A lower percentage of cells (20±6%, n=237) instead responded to UDP, with a mean calcium increase of 0.4±0.06 ΔF340/380 (Figure 4.5A,B). Examples of agonist-induced calcium transients are reported in Figure 4.5C; interestingly, 100μM UTP-evoked calcium responses were more sustained and prolonged compare to the same concentration of ADP and UDP. Experiments performed with MRS2179 and Cangrelor showed that, in glial cells, response to ADP mainly involved the $P2Y_1$ receptor with a smaller contribution of $P2Y_{12}/P2Y_{13}$ receptors (Figure 4.5D). Suramin and RB2 ($P2Y_2/P2Y_2$ antagonists) inhibited UTP-induced $[Ca^{2+}]_i$ transients by 76.6% and 76.7%, respectively, thus suggesting the involvement of $P2Y_2/P2Y_4$ receptors (Figure 4.5E).

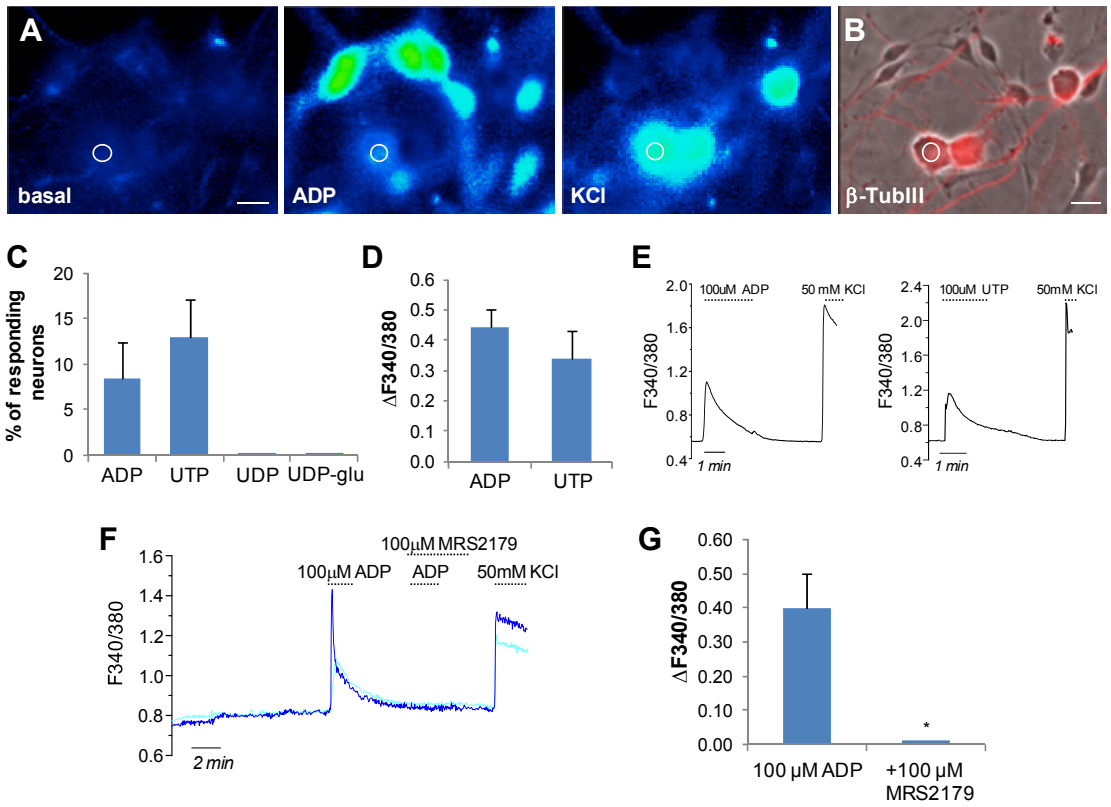


Figure 4.4

Trigeminal ganglion sensory neurons bear some functional P2Y receptors.

A, *Series of pseudocolor images of Fura-2 loaded cells before (basal) and at peak* $[Ca^{2+}]_i$ *responses after the application of 100 μM ADP, followed by 50mM KCl to indentify neuronal phenotype.* ***B****, Identification of neurons was confirmed by immunopositivity to β-tubIII (red). Scale bars: 15 μm.* ***C,*** *Histograms showing the percentage of neurons responding to the indicated P2Y agonists.* ***D,*** *Histograms showing the mean* $[Ca^{2+}]_i$ *increases, evaluated as increase in 340/380 fluorescence ratio (ΔF340/380), induced in neuronal cells by the P2Y agonists ADP and UTP.* ***E,*** *Representative temporal plots of* $[Ca^{2+}]_i$ *increases recorded from neurons upon stimulation with either ADP or UTP (100μM), as indicated, followed by 50 mM KCl. In the case of ADP, the neuron is the one marked with a white circle in* ***A*** *and* ***B****.* ***F,*** *Representative temporal plot of* $[Ca^{2+}]_i$ *changes recorded from two neurons stimulated with 100 μM ADP before and after application of the* $P2Y_1$ *antagonist MRS2179 (100 μM), followed by exposure to 50 mM KCl.* ***G,*** *Quantification of data reported in* ***F*** *expressed as mean calcium responses±s.e.m. *p<0.05 with respect to ADP alone; one-way ANOVA followed by Scheffe's analysis.*

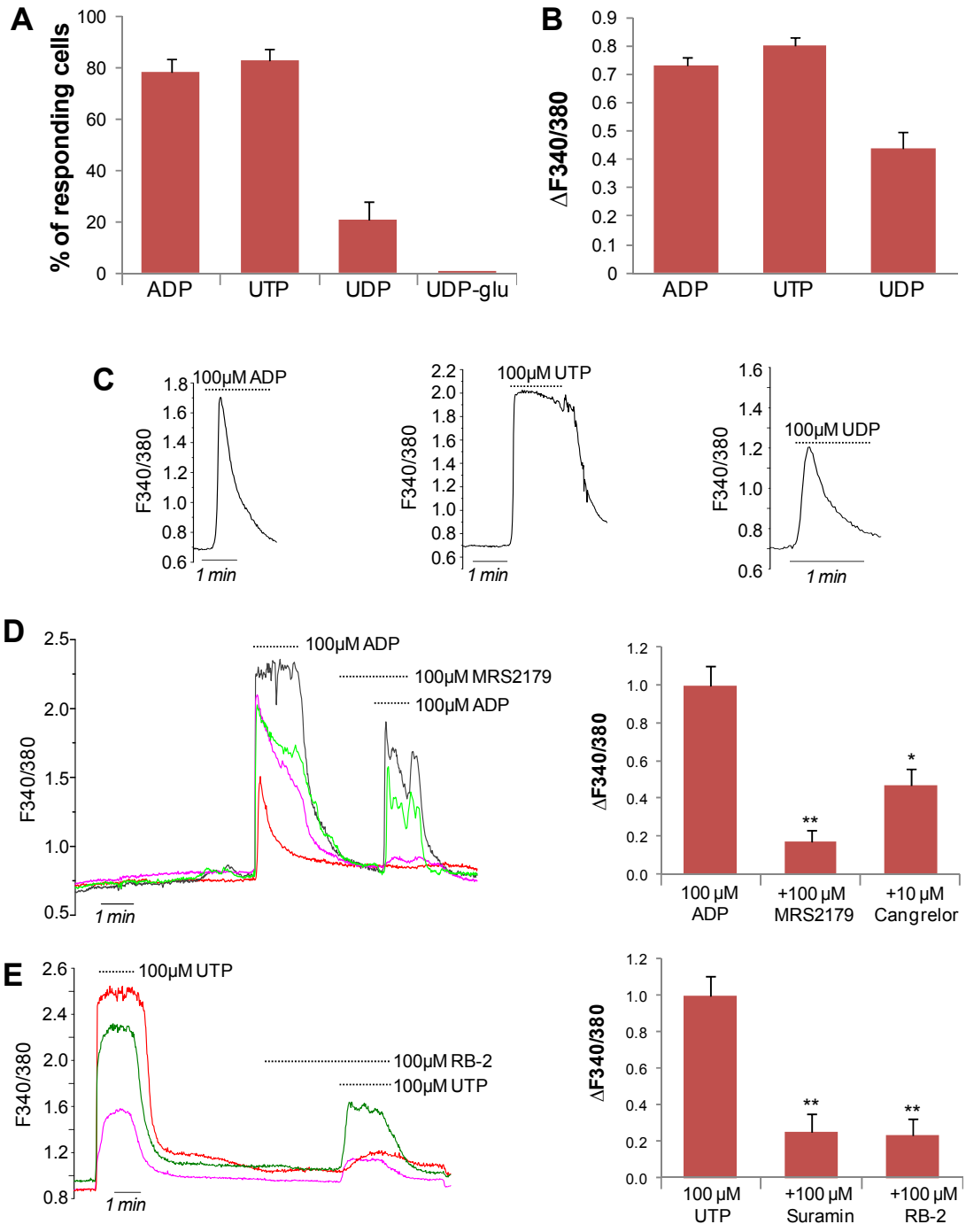


Figure 4.5

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Figure 4.5

Trigeminal ganglion SGCs express functional P2Y receptors.

A, B, *Histograms showing the percentage of responding SGCs (**A**) or the mean $[Ca^{2+}]_i$ increases (**B**) induced by the indicated P2Y receptor agonists.* ***C,*** *Examples of $[Ca^{2+}]_i$ changes recorded from SGCs upon stimulation with various P2Y agonists.* ***D,*** *Left: representative temporal plots of $[Ca^{2+}]_i$ changes recorded from four SGCs stimulated with 100 μM ADP before and after application of the $P2Y_1$ selective antagonist MRS2179 (100 μM), as indicated. Right: Quantification of the mean calcium increases±s.e.m. after stimulation with 100 μM ADP before and after exposure to 100 μM MRS2179 (n=25 cells) or 10 μM Cangrelor (n=20 cells). Values were normalized to results obtained with ADP alone set to 1.00.* ***E,*** *Left: representative temporal plots of $[Ca^{2+}]_i$ changes recorded from three satellite cells stimulated with 100 μM UTP before and after application of the relatively $P2Y_2/P2Y_4$-selective antagonist Reactive Blue-2 (RB-2; 100 μM), as indicated. Right: Quantification of mean calcium increases±s.e.m. induced by 100 μM UTP before and after exposure to 100 μM Suramin (n=24 cells) or 100 μM RB-2 (n=30 cells). Values were normalized to results obtained with UTP alone, set to 1.00. *p<0.05 and **p<0.01 with respect to corresponding agonist alone, by one-way ANOVA followed by Scheffe's analysis.*

4.3 CHRONIC APPLICATION OF THE PRO-INFLAMMATORY MEDIATOR BRADYKININ DIFFERENTIALLY AFFECTS NEURONAL $P2X_3$ AND GLIAL P2Y RECEPTOR FUNCTIONALITY

The pro-inflammatory mediator bradykinin (BK) is a known activator of sensory neurons (Heblich et al., 2001) that was shown to increase the neuronal firing rate in the trigeminal subnucleus caudalis of the medulla oblongata and to enhance the release of CGRP from cultured dorsal horn and TG neurons (Jenkins et al., 2003). Moreover SGCs are also sensitive to BK (England et al., 2001). On this basis, we deemed it interesting to study the effect of either an acute (up to 5 min) or a prolonged (24-hour) exposure to BK on purinergic signalling in both neurons and SGCs in our culture model.

As a first step, we investigated the expression, functionality, and cellular localization of BK receptors (i.e. the B1 and B2 subtypes) in our cultures. RT-PCR analysis demonstrated expression of the constitutive B2 receptor subtype in both the intact tissue and dissociated cultures (Figure 4.6A). Conversely, the inducible B1 receptor subtype was barely present in the intact tissue and clearly expressed in primary cultures, suggesting its upregulation with time in culture (Figure 4.6A). An acute exposure to 100 nM BK evoked changes in the $[Ca^{2+}]_i$ in the 35.11±4.72% (ΔF340/380) of neurons (n=188; 11 independent experiments; Figure 4.6B), with respect to the

3.76±2.01% of SGCs (n=319; 11 independent experiments) suggesting that, in TG, BK receptors are almost exclusively functional on neuronal cells only.

We then studied the effect of BK treatment on the $P2X_3$ and P2Y receptors functionality. An acute application of BK (2-3 min) significantly increased the calcium response induced by the $P2X_3$ agonist α,β-meATP (normalized mean calcium increases: 1.00±0.1 for control cultures, n=24 vs. 1.41±0.3 for BK treated cultures, n=48; Figure 4.6C). No changes were observed in P2Y receptor-mediated modulation of $[Ca^{2+}]_i$ (not shown). This result is in line with previous evidence indicating that acute (50 sec) exposure to BK rapidly sensitised $P2X_3$ receptor functionality in *Xenopus Laevis* oocytes (Paukert et al., 2001).

On the contrary, a 24-hour exposure of trigeminal cultures to 100 nM BK did not modify the amplitude of calcium responses induced by 100 μM α,β-meATP (Figure 4.6D), but resulted in a highly significant reduction of the percentage of responsive neurons which was decreased to 28.5±5.7% (n=125) with respect to 62.1±5.8% in control untreated cultures (n=118; Figure 4.6E). Interestingly, considerable changes in P2Y receptor-mediated responses were recorded in SGCs. In particular, BK-treated cultures showed a significant increase of the mean calcium amplitude (expressed as ΔF340/380 and normalized to BK-untreated control cells exposed to the same P2 agonist set to 1.00) of 1.36±0.11 (n=60), 1.39±0.07 (n=119) and 1.44±0.11 (n=75) after exposure to 100 μM ATP, 100 μM ADP, and 100 μM UTP, respectively (Figure 4.6F), with no changes in the percentage of responding cells (Figure 4.6G). A trend to increase in the mean calcium amplitudes, which did not reach the statistical significance, was also observed after exposure to 100 μM UDP (Figure 4.6F), whereas the percentage of responding SGCs was dramatically increased (% of UDP-responding cells with respect to BK-untreated cells set to 100%: 253.9±5.9%, n=265; Figure 4.6G).

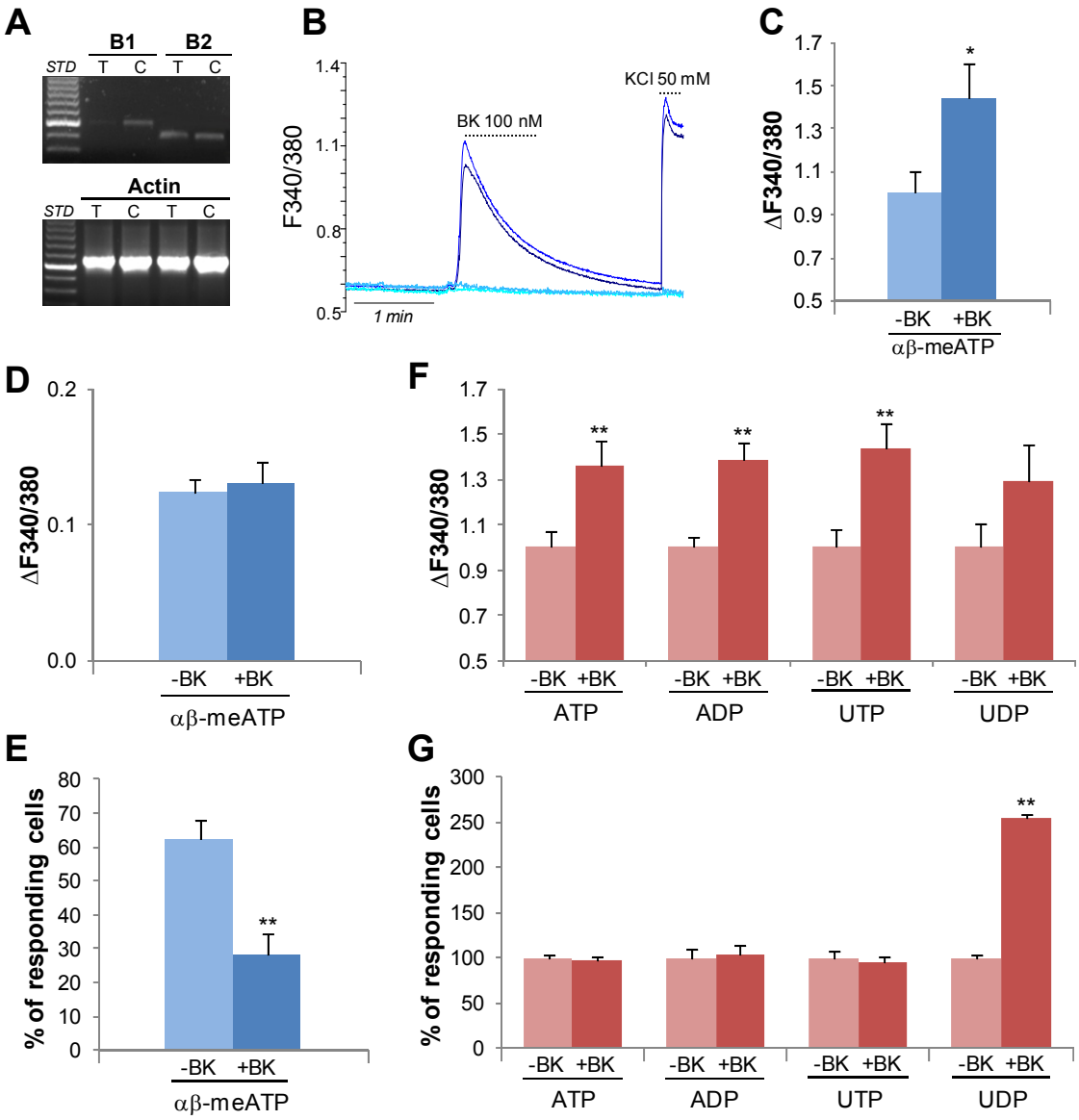

Figure 4.6

Acute and chronic exposure to bradykinin (BK) differentially affects the function of neuronal $P2X_3$ and glial P2Y receptors.

A, RT-PCR experiments demonstrating B1 and B2 BK receptor expression (expected PCR product lengths: 600 and 508 base pairs, respectively) in intact trigeminal ganglia (T) and primary mixed trigeminal cultures (C). The housekeeping gene β-actin (expected PCR product length: 661 bps) was used as an internal positive control. ***B****, Representative temporal plots of BK-evoked $[Ca^{2+}]_i$ increases in two trigeminal neurons (blue lines), and two SGCs (light-blue lines). The depolarizing agent KCl (50 mM) was used to discriminate between neuronal (responding) and non-neuronal (non-responding) cells.* ***C,*** *Quantification of mean calcium increases±s.e.m. induced by 100 µM α,β-meATP in vehicle (-BK) or acutely (2-3 min) BK-treated (100 nM) neurons.* ***D-G,*** *results obtained after a chronic (24 hours) exposure of trigeminal cultures to either vehicle (-BK) or 100 nM BK.* ***D:*** *mean calcium responses to α,β-meATP in neurons.* ***E:*** *mean percentage of neurons responding to α,β-meATP.* ***F:*** *mean calcium responses to various nucleotides (normalized to corresponding vehicle-treated control cells, set to 1.00) recorded in SGCs.* ***G:*** *mean percentage of glial cells responding to ATP, ADP, UTP and UDP (100 µM; normalized to corresponding vehicle-treated control cells, set to 100%). *p<0.05 and **p<0.01 with respect to vehicle-treated cells, one-way ANOVA followed by Scheffe's analysis.*

4.4 FOLLOWING BK APPLICATION, CGRP RELEASED FROM TG NEURONS IS RESPONSIBLE FOR P2Y RECEPTORS UPREGULATION IN SGCs

Our data clearly indicate that exposure of primary mixed neuron-SGCs TG cultures to the pro-algogenic mediator BK significantly enhances the responsiveness of glial P2Y receptors to ADP and UTP. We also demonstrated that BK receptors are almost exclusively functional in TG neurons. For this reason we speculated that the BK-induced upregulation of P2Y receptors in SGCs could be mediated by the release of a neuronal mediator. Since previous studies indicate that stimulation with pro-inflammatory mediators, like BK, is associated to the release of the calcitonin gene-related peptide (CGRP; Ebersberger et al., 1999; Meng et al., 2007; Eberhardt et al., 2008), we chronically (i.e., 24 hours) exposed primary mixed TG cultures to BK alone or in the presence of the CGRP receptor antagonist, $CGRP_{8-37}$, and subsequently recorded ADP- and UTP-induced increases in $[Ca^{2+}]_i$ in SGCs (Figure 4.7A). As expected, BK alone significantly increased the mean peak amplitudes (expressed as ΔF340/380, and normalized to values obtained in CTR cells exposed to the same P2 agonist set to 1.00) exerted by 1 μM ADP (1.52±0.172, n=90 cells), and by 10 μM UTP (1.53±0.182, n=71 cells). Pre-treatment with 2 μM $CGRP_{8-37}$ almost completely abolished the BK-induced effect (normalized ΔF340/380: 1.10±0.08, n=172 cells, and 1.11±0.115, n=95 cells for ADP and UTP, respectively; Figure 4.7A). Similarly to BK, a 24-hour exposure to 1 μM CGRP also induced an increase in P2Y receptor-mediated responses in SGCs (normalized ΔF340/380: 1.56±0.106, n=146 cells and 1.58±0.119, n=121 cells for ADP and UTP, respectively), which is in line with the BK-induced effects (normalized ΔF340/380: 1.46±0.157, n=108 cells, and 1.53±0.182, n=71 cells for ADP and UTP, respectively; Figure 4.7B). No changes in the percentages of SGCs responding cells were observed upon either treatments (data not shown). These results clearly suggest a role for CGRP as the key mediator of neuron-to-SGC communication upon BK exposure.

To further demonstrate this hypothesis, we measured extracellular CGRP concentrations after application of BK. As shown in Figure 4.7C, a 1-hour treatment of primary mixed TG cultures with 100 nM BK almost doubled extracellular CGRP levels, from 19.10±2.28 pg/ml (n=22 coverslips) in CTR cultures, to 36.33±4.07 pg/ml (n=23 coverslips) in BK-treated cultures. The maximal CGRP release was assessed by

exposing cultures to the depolarizing agent KCl (50 mM) for 15 min, and corresponded to 152.5±27.51 pg/ml (n=21 coverslips).

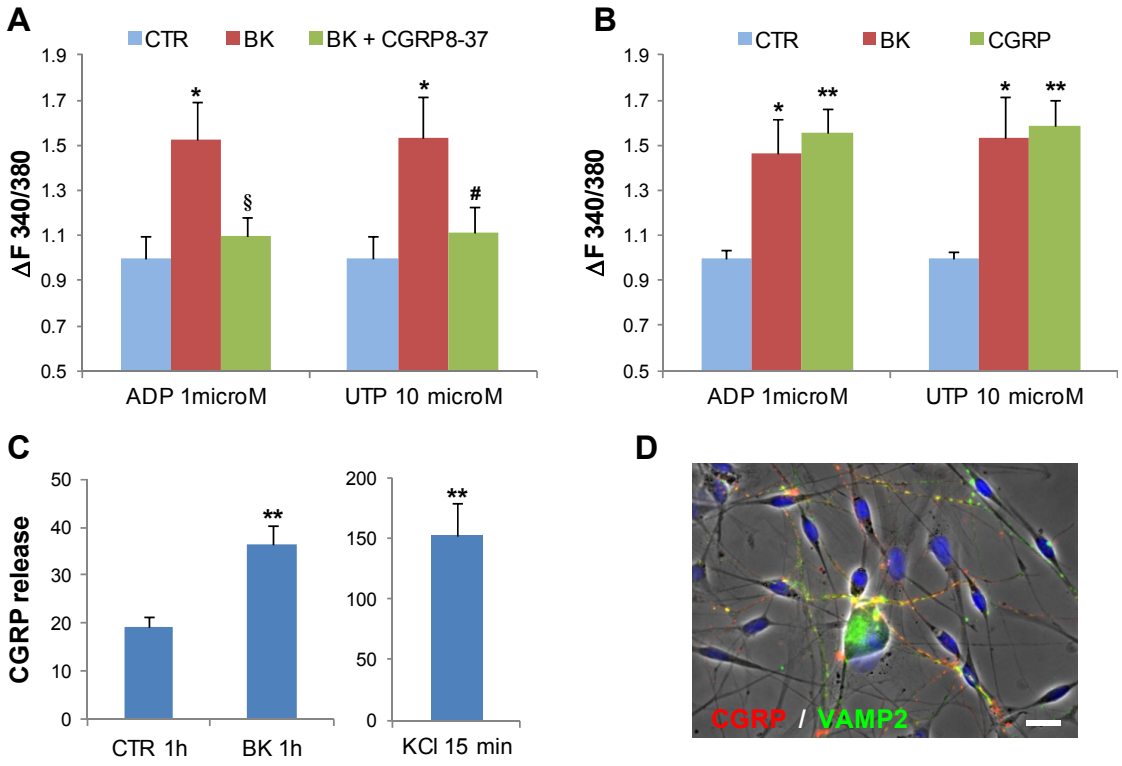

Figure 4.7

BK-induced enhancement of P2Y receptor function in trigeminal SGCs is mediated by the neuronal release of CGRP.

A, *Primary mixed trigeminal cultures were sub-chronically (24 h) treated with vehicle (CTR), or 100 nM BK alone or in the presence of the CGRP receptor antagonist $CGRP_{8-37}$ (2 μM). Cultures were then challenged with 1 μM ADP or 10 μM UTP, and the increases in $[Ca^{2+}]_i$ recorded from SGCs.* ***B,*** *Primary mixed trigeminal cultures were treated for 24 h with vehicle (CTR), 100 nM BK, or 1 μM CGRP, then exposed to ADP or UTP, and changes in $[Ca^{2+}]_i$ analyzed in SGCs (see above). In both* ***A*** *and* ***B****, histograms show the mean $[Ca^{2+}]_i$ normalized to CTR cells set to 1.0 from at least 3 independent experiments. *p<0.05, and **p<0.01 with respect to CTR; §p<0.05, and #p=0.057 with respect to BK alone; one-way ANOVA followed by Scheffé's test.* ***C,*** *Histograms show the mean extracellular CGRP concentrations after 1 h application to primary mixed trigeminal cultures of either vehicle (CTR) or 100 nM BK. A 15-min exposure to 50 mM KCl was utilized as a positive control of maximal neuronal CGRP release. **p<0.01 with respect to CTR, one-way ANOVA followed by Scheffé's test.* ***D,*** *Double immunofluorescence staining showing the specific localization of CGRP (red) to vesicle-associated membrane protein 2 (VAMP2)-positive axons terminals (green) in primary trigeminal cultures. Nuclei were labeled with the Hoechst 33258 dye (blue). Scale bar: 20 μm.*

Data from literature (Xiao et al., 2008) and our data on maximal CGRP release obtained with the depolarizing agent KCl already suggested a neuronal localization and release of this neuropeptide. To unequivocally confirm this, we performed a double immunofluorescence experiment for CGRP and the vesicle-associated membrane protein 2 (VAMP2), demonstrating the selective expression of the neuropeptide in $VAMP2^+$ axons terminals (Figure 4.7D), and therefore the exclusive neuronal localization of CGRP.

4.5 CGRP, BUT NOT BK, RETAINS ITS ABILITY TO INDUCE P2Y RECEPTOR POTENTIATION IN PURIFIED SGCs CULTURES

To get further insights in the molecular pathways linking glial CGRP receptor activation to the upregulation of P2Y receptor function, we generated an *in vitro* model of purified SGCs cultures. As described in more details in *Paragraph 3.1* and in Figure 3.1, five days after preparation of primary mixed neuron-SGCs cultures, SGCs approached confluence and were replated on uncoated wells. This procedure allowed completely removing all neurons, as demonstrated by the lack of staining of the neuronal marker NeuN (Fig. 3.1C). We have also previously demonstrated that there was no difference in P2Y receptor expression and function between purified SGCs cultures and primary mixed neuron-glia cultures (Villa et al., 2010).

RT-PCR analysis for the two subunits composing the functional CGRP receptor (namely, the receptor activity modifying protein 1, RAMP1, and the calcitonin receptor-like receptor, CLR; Lennerz et al., 2008) showed that the expression of CGRP receptor complex was retained in purified SGCs cultures (Figure 4.8A).

Next, we exposed purified SGCs cultures to 100 nM BK and 1 μM CGRP for 24 h, and analyzed the mean $[Ca^{2+}]_i$ responses after ADP and UTP application. Differently from mixed neuron-SGCs cultures (see above), P2Y receptor functionality in purified glial cultures was not affected by BK treatment (normalized ΔF340/380: 0.98±0.151, n=174 cells, and 0.92±0.180, n=99 cells, after exposure to 1 μM ADP and 100 μM UTP, respectively; Figure 4.8B). Conversely, CGRP retained its ability to potentiate glial P2Y receptors even in the absence of neurons (normalized ΔF340/380: 1.42±0.112, n=366 cells, and 1.48±0.144, n=150, in the case of ADP and UTP, respectively; Figure

4.8B-D). Globally, these data further confirm that BK does not directly target SGCs, but acts through its neuronal receptors, and that the mediator of this neuronal-to-glial cell communication is indeed CGRP.

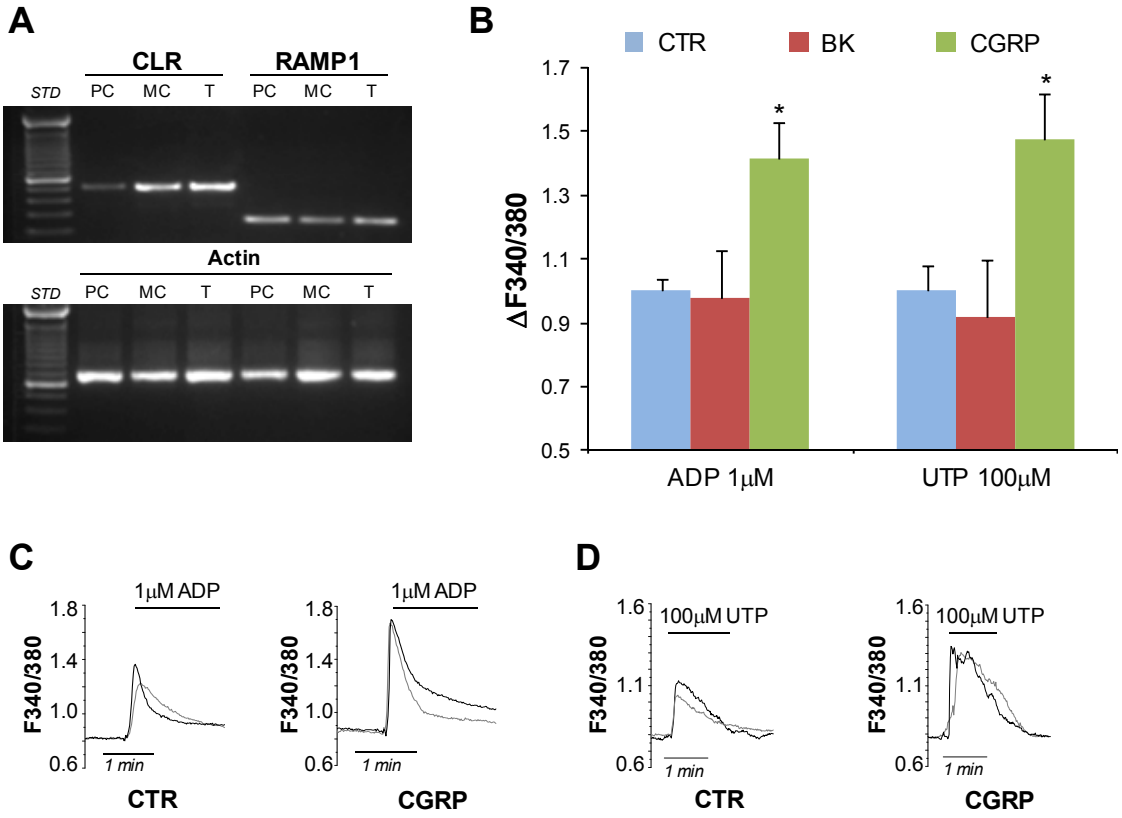

Figure 4.8

CGRP, but not BK, maintains its ability to potentiate P2Y receptors responsiveness in purified SGCs cultures.

A, *RT-PCR analysis from purified SGC cultures (PC), neuron-SGCs mixed cultures (MC), and intact trigeminal ganglia (T). Specific primers for the receptor activity modifying protein 1 (RAMP1; expected PCR product length: 249 bps), the calcitonin receptor-like receptor (CLR; expected PCR product length: 483 bps), and the housekeeping gene β-actin (expected PCR product length: 661 bps) were used.* ***B,*** *Histograms showing the ADP- and UTP-evoked mean $[Ca^{2+}]_i$ increases recorded from purified SGCs cultures after a 24-h treatment with either vehicle (CTR), 100 nM BK, or 1 μM CGRP. Mean $[Ca^{2+}]_i$ values have been normalized to CTR set to 1.0.* ***C, D,*** *Representative plots showing the increased ADP- and UTP-induced $[Ca^{2+}]_i$ peaks in SGCs after chronic exposure to CGRP compared to CTR. *p<0.05 with respect to CTR; one-way ANOVA followed by Scheffé's test.*

4.6 THE ERK1/2 MAP KINASE PATHWAY HAS A PRIMARY ROLE IN CGRP-INDUCED POTENTIATION OF GLIAL P2Y RECEPTORS

Since it is well known that CGRP activates the MAP kinase pathway in trigeminal SGCs (Vause and Durham, 2009; 2010), we exposed purified SGCs cultures to 1 μM CGRP and analyzed the phosphorylation/activation of the ERK1/2 MAP kinase pathway by western blotting. As shown in Figure 4.9A,B, 1 μM CGRP induced a biphasic wave of ERK1/2 activation (measured as the ratio between the intensities of the phosphorylated vs total ERK1/2 protein bands) peaking at 10 and 40 min of incubation, which was completely inhibited by the CGRP receptor antagonist $CGRP_{8-37}$ (Figure 4.9C). Noteworthy, the activation of ERK1/2 pathway is directly correlated with the potentiation of P2Y receptors function, since the inhibitor PD98059 fully prevented the increase in UTP-mediated $[Ca^{2+}]_i$ mobilization induced by CGRP (normalized ΔF340/380: 1.46±0.078, n=63 cells for CGRP alone, and 0.99±0.158, n=40 for CGRP + PD98059; Figure 4.9D).

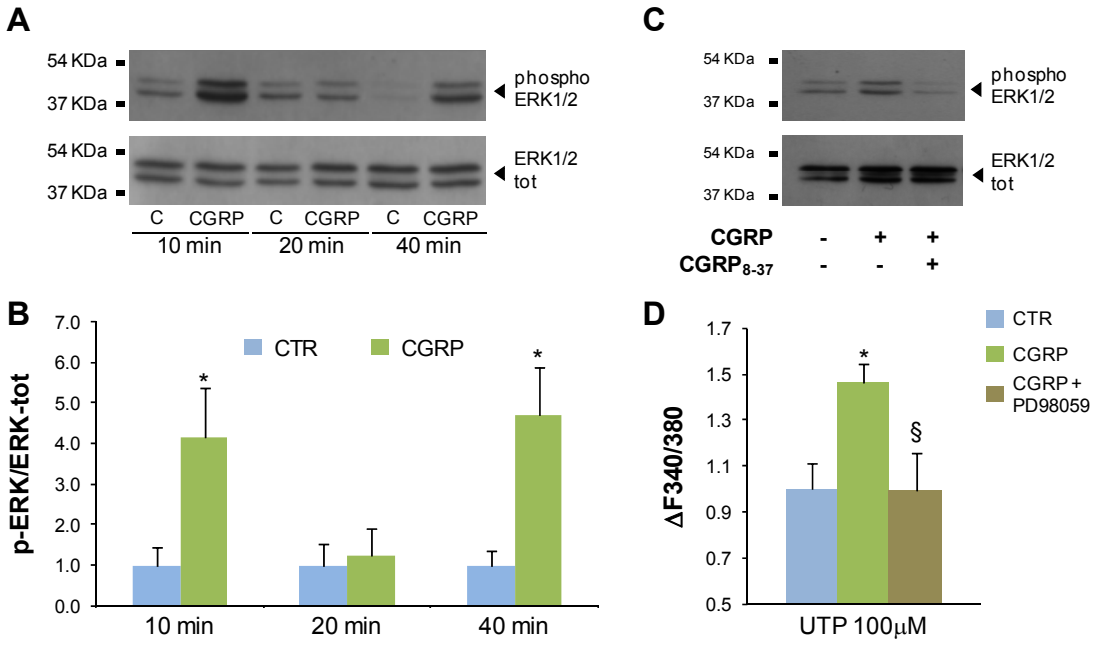

Figure 4.9

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Figure 4.9

The ERK1/2 MAP kinase pathway is involved in CGRP-induced P2Y receptor upregulation.

A, Representative western blotting experiment showing the biphasic activation of extracellular signal-regulated kinase 1/2 (ERK1/2) induced by application of 1 μM CGRP to purified SGCs cultures for 10, 20 and 40 min. ***B,*** *Densitometric analysis of western blotting experiments showing the biphasic activation of ERK1/2 after exposure of purified SGCs to 1 μM CGRP. For each sample, the ratio between the intensities of the phosphorylated and total ERK1/2 (pERK/ERKtot) protein bands has been calculated, and normalized to the corresponding mean CTR value (set to 1.0).* ***C,*** *The CGRP receptor antagonist $CGRP_{8-37}$ (2 μM) inhibits CGRP-induced ERK1/2 activation (10 min).* ***D,*** *The ERK1/2 inhibitor PD98059 prevents the potentiation of UTP-evoked $[Ca^{2+}]_i$ increases induced by CGRP. Histograms show the mean calcium increases in SGCs after chronic (24 h) treatment with vehicle (CTR), 1 μM CGRP alone, or CGRP + 50 μM PD98059. In* ***A*** *and* ***D*** *data are the mean±s.e.m. of 3 independent experiments. *p<0.05 with respect to CTR, §p<0.05 with respect to cells treated with CGRP alone; one-way ANOVA followed by Scheffé's test.*

4.7 CGRP RELEASE IS SIGNIFICANTLY ENHANCED IN TG CULTURES FROM $Ca_V2.1$ α1 R192Q MUTANT KNOCK-IN MICE

Our data point to a key role of CGRP in neuron-to-glia communication between TGs and SGCs and on its contribution to the molecular and signalling network controlling the transmission and integration of painful signals. Therefore, we decided to evaluate both basal and stimulated CGRP release in primary TG cultures obtained from a transgenic mouse model of migraine, the $Ca_V2.1$ α1 R192Q mutant knock-in (KI) mouse (van den Maagdenberg et al., 2004). Figure 4.10A shows that, under basal conditions, a significantly higher CGRP release was detected in primary TG cultures from R192Q KI mice (50.49±7.33 pg/ml, n=29 coverslips) with respect to cultures from R192Q wild type (WT) animals (33.75±2.80 pg/ml, n=31 coverslips from 3 independent experiments). Following exposure to 100nM BK (1h), the increase in CGRP extracellular levels was higher in R192Q KI cultures, with CGRP levels being 61.49±6.73 pg/ml in R192Q WT cultures (n=14 coverslips), and 110.80±19.70 pg/ml (n=10 coverslips) in R192Q KI cultures (Figure 4.10B). Moreover, an over twofold (238%) higher CGRP release was detected after maximal neuronal depolarization by 50 mM KCl (15 min) in R192Q KI cultures compared to R192Q WT cultures (from 194.1±28.25 pg/ml, n=9 coverslips from 3 independent experiments, to 461.1±119.2 pg/ml, n=8 coverslips in WT and KI cultures, respectively; Figure 4.10B).

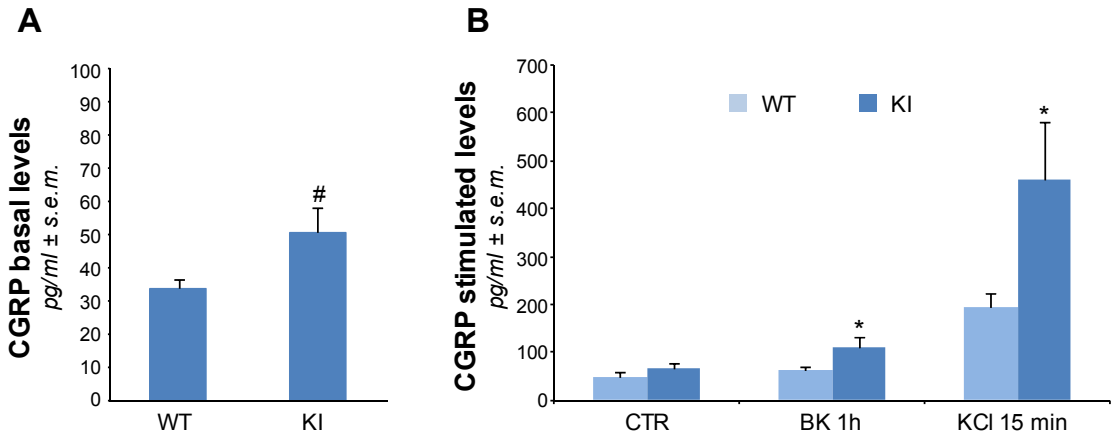

Figure 4.10

Both basal and stimulated extracellular CGRP concentrations are higher in trigeminal cultures from $Ca_V2.1$ α1 R192Q mutant KI mice, compared to WT animals.

A, B, *histograms show the mean extracellular CGRP levels in primary mixed trigeminal cultures from R192Q wild type (WT) and $Ca_V2.1$ α1 R192Q mutant KI mice, after 48 h in vitro. Basal CGRP concentrations were measured after a 15-min incubation with fresh medium alone (**A**), whereas stimulated CGRP levels were measured after a subsequent incubation with vehicle (CTR; 1 h), 100 nM BK (1 h), or 50 mM KCl (15 min) (**B**). #p<0.05 with respect to the basal CGRP levels in WT cultures, *p<0.05 with respect to the corresponding stimulated CGRP levels in WT cultures; one-way ANOVA.*

4.8 APPLICATION OF BK INCREASES THE NUMBER OF ADP- AND UTP-RESPONDING SGCs IN TG CULTURES FROM $Ca_V2.1$ R192Q KI MICE

Having established that the R192Q mutation in $Ca_V2.1$ channels is associated to increases of both basal and stimulated CGRP release, we next evaluated P2Y receptors functionality in TG cultures from KI mice. Since the ability of BK to upregulate ADP and UTP mean calcium amplitudes in SGCs is related to the neuronal release of CGRP (Figure 4.7), we anticipated the BK dependent P2Y receptor upregulation to be even stronger in KI cultures. Surprisingly, no substantial differences in BK-evoked upregulation of ADP and UTP mean calcium amplitudes were observed between SGCs of R192Q WT and KI cultures. In fact, ADP-elicited ΔF340/380 values were 0.95±0.07 (n=132) in control- and 1.18±0.08 (n=116) in BK-treated R192Q WT cultures, whereas values were 0.98±0.06 (n=136) and 1.28±0.08 (n=165) in control and BK-treated R192Q KI cultures, respectively (Figure 4.11A). UTP-elicited ΔF340/380 values were 0.83±0.08 (n=57) in control- and 1.05±0.11 (n=40) in BK-treated R192Q WT cultures, and 0.82±0.07 (n=51) and 1.15±0.08 (n=95) in control- and BK-treated R192Q KI

cultures (Figure 4.11B). However, following chronic treatment with BK the percentage of UTP-responding SGCs was significantly increased in R192Q KI cultures with respect to CTR cultures (Figure 4.11D). A trend to increase was also observed in the case of ADP-responding cells (Figure 4.11C). Following BK exposure, the percentage of nucleotide-responding SGCs was therefore significantly higher in R192Q cultures when compared to BK-treated R192Q WT cultures (% of ADP responding SGCs: 55.6±7.42 in WT cultures vs. 75.7±3.74 in KI cultures; % of UTP responding SGCs: 20.1±2.71% in WT cultures vs. 54.9±5.91% in KI cultures). No changes in both mean calcium increases, and in the percentages of responding cells were observed between R192Q WT and KI neurons (not shown). These data suggests that, in the presence of the gain-of-function R192Q mutation of $Ca_V2.1$ channels, application of nociceptive mediators (i.e., BK), by stimulating a higher release of neurotransmitters (i.e., CGRP) from neurons, can induce an increase in P2Y receptor responsiveness.

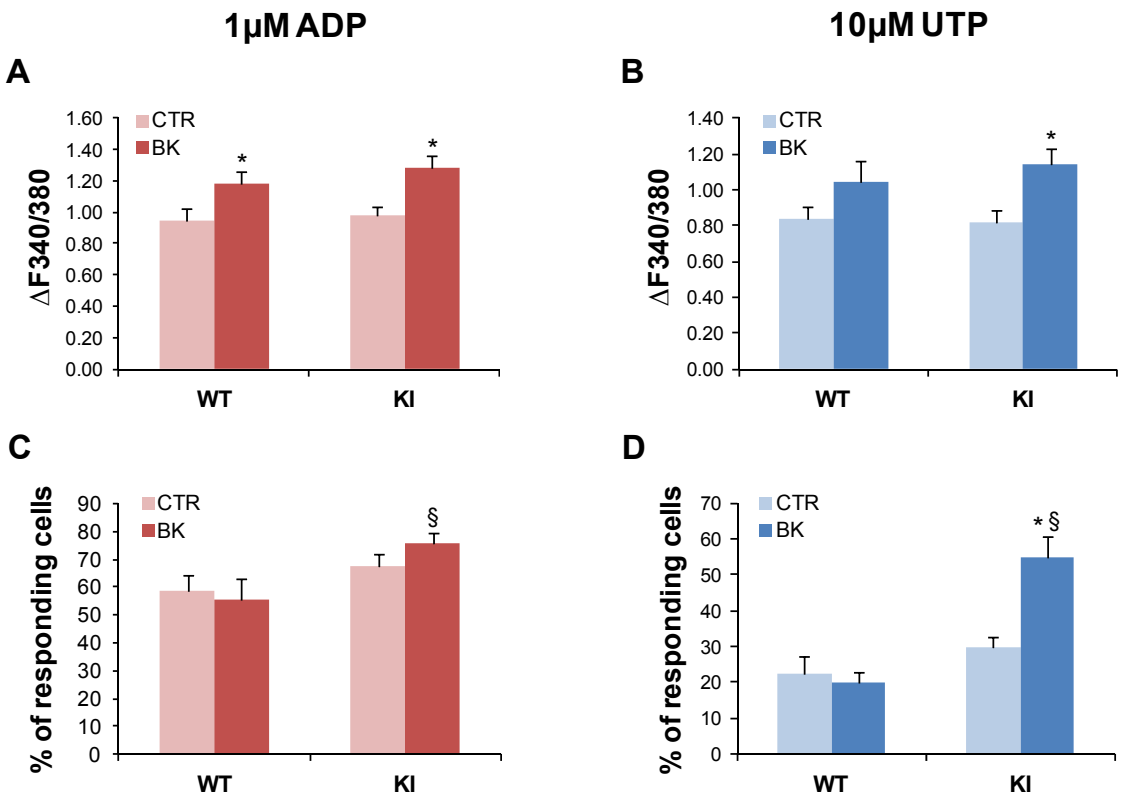

Figure 4.11

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Figure 4.11

The chronic treatment with BK significantly increases the percentage of ADP- and UTP-responding SGCs in KI cultures.

A, B, *histograms show the 1 μM ADP- (**A**) or 10 μM UTP-elicited (**B**) mean $[Ca^{2+}]_i$ increases in WT and KI cultures, after a 24 hours treatment with vehicle alone (CTR) or with 100nM BK.* ***C, D,*** *mean percentage of SGCs responding to ADP (**C**) and UTP (**D**) in R192Q WT and KI cultures, after a 24 hours treatment with vehicle alone (CTR) or with 100nM BK. *p<0.05 with respect to the corresponding WT or KI control cultures; §p<0.05 with respect to WT cultures exposed to BK; one-way ANOVA followed by Scheffe's analysis.*

4. RESULTS

Section II: in vivo studies

4.9. SET UP OF IN VIVO MODELS OF TRIGEMINAL PAIN FOR STUDYING THE ROLE OF THE PURINERGIC SYSTEM IN PAIN TRANSMISSION

The results obtained during the first part of my PhD experimental work demonstrate the presence of functional P2Y receptors in glial cells from TG *in vitro*, and their complex modulation under exposure to pro-inflammatory agents. Taken together these data suggest that glial purinergic receptors act as important players in nociceptive transmission. For this reason, we deemed interesting to set up *in vivo* pain models of TG sensitization in order to study the involvement of the purinergic system in pain transmission, and to validate the role of specific P2Y receptor subtypes through their specific knock down *in vivo*.

To test the role of selected P2Y receptors in the development of pain behaviour, we initially set up a mouse model of acute pain involving trigeminal sensitisation: the oro-facial pain model. A solution of formalin was injected into the upper lip of mice, and their nociceptive behaviour was monitored with a video camera for 30 min. A nociceptive score was then determined by measuring the time (in seconds) that the animal spent in grooming the injected area (Luccarini et al., 2006; see *Methods*). We initially focused our attention on the $P2Y_4$ receptor, due to its expression by trigeminal SGCs, and to previous data implicating this receptor in pain signalling (Weick et al., 2003; Vit et al., 2006). The silencing strategy was based on the injection in the TG of long double-stranded RNAs (dsRNAs) probes designed to knock down the expression of the specific receptors. Sequences directed against the bacterial gene MalE have been also designed and injected in parallel as non-specific dsRNA control. Western Blotting analysis on explanted trigeminal ganglia showed a strong reduction (about 60%) of $P2Y_4$ protein levels in the ipsilateral ganglion of mice injected with $P2Y_4$-dsRNAs, with no significant difference after MalE-dsRNA injection (Figure 4.12A). As expected, behavioural studies showed that the face rubbing time was significantly higher in formalin-injected mice compared to saline-treated animals (Figure 4.12B), but no significant differences were detected when comparing formalin-treated mice that also received $P2Y_4$-dsRNA with respect to formalin-treated animals receiving MalE-dsRNA (Figure 4.12B). These data apparently rule out a role for $P2Y_4$ receptor in acute trigeminal pain transmission.

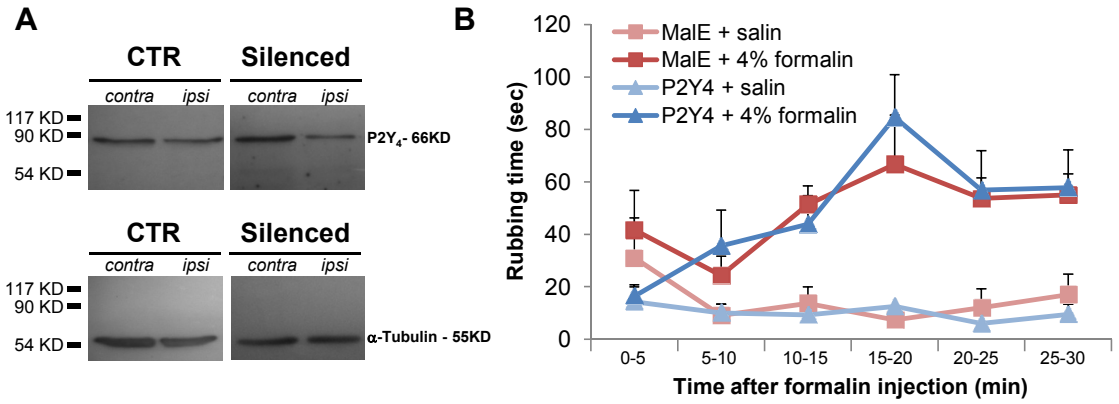

Figure 4.12

$P2Y_4$ receptor knock-down did not produce any change in acute pain behavior following orofacial formalin injection.

A, *Western blot autoradiograms showing the $P2Y_4$ protein levels in contralateral (contra) and ipsilateral (ipsi) trigeminal ganglia from mice injected with the MalE- (CTR) or the $P2Y_4$-dsRNA (Silenced). The housekeeping gene α-Tubulin was used as an internal loading control.* ***B,*** *Time course of the face-rubbing activity observed after subcutaneous injection of either saline or 4% formalin into the upper lip of mice injected with the MalE- or the $P2Y_4$-dsRNA. The mean time±s.e.m spent rubbing within each 5-min block intervals over the 30-min post-injection observation period is shown. At least 4 animals were analyzed in each group.*

We next decided to set up and characterize a model of chronic trigeminal inflammation based on the injection of the Complete Freund's Adjuvant (CFA) into the temporomandibular joint (TMJ) of adult rats. This is a well-established model of inflammatory pain, which shares several characteristics with migraine-associated TG sensitization (Ballegaard et al., 2008; Taub et al., 2008). Since glial cells participate in the genesis and maintenance of chronic pain (See *Paragraph 1.1.3*) and strongly express P2Y receptors (See *Paragraphs 1.3 and 1.4*), but no studies have explored the reaction of glial cells following the induction of TMJ inflammation, we have first characterized the activation of PNS and CNS glial cells to the injection of CFA into the TMJ. The baseline values for the mechanical threshold in non-inflamed rats were determined by probing their orofacial regions with von Frey filaments (see *Methods*). Mean head withdrawal thresholds were measured from the left and right orofacial regions (16.11±2.06 g for the right side and 15.50±1.73 g for the left side; n=12 animals; Table 4.1 and Figure 4.13A). Rats were then injected with either saline or CFA into the left TMJ and tested for their pain behavior. A significantly lower mechanical threshold was measured in the ipsilateral side of CFA injected rats, starting from 24h post injection (p.i.), thus demonstrating the development of mechanical allodynia (see

Table 4.1 and Figure 4.13A). No significant changes between the ipsi- and the contralateral mechanical thresholds were observed in control saline-injected animals (see Figure 4.13A and Table 4.1). To further confirm the establishment of inflammation following CFA injection, we measured the TMJ extravasation of the Evans' blue dye injected into the tail vein. While there was no difference in the low dye concentration in the TMJs of rats injected with saline (0.07±0.07 µg/ml for the ipsilateral side vs. 0.07±0.06 µg/ml for the contralateral side; n=7 animals; Figure 4.13B), a significantly greater amount of dye was extracted from the ipsilateral TMJ of CFA-injected rats, both at 24h p.i. (0.76±0.17 µg/ml vs. 0.13±0.05 µg/ml for ipsi- and contralateral tissue, respectively, n=6 animals), and, to a lesser extent, at 72h p.i. (0.43±0.07 µg/ml vs. 0.07±0.03 µg/ml for ipsi- and contralateral tissue, respectively; n=7 animals; (Figure 4.13B). These results confirm that the injection of CFA into the TMJ induces a persistent inflammation associated with the development of mechanical allodynia.

Table 4.1: Mean head withdrawal threshold values (in grams, g) after saline or CFA injection in the TMJ. Ipsi: ipsilateral side; contra: contralateral side.

Stimulus	**Time post injection**	**Ipsilateral** (g)	**Contralateral** (g)	**n° of rats**	**p value** *(ipsi vs. contra)*	**p value** *(ipsi vs. baseline)*
baseline	-	15.5 ± 1.73	16.1 ± 2.06	12	-	-
saline	4h	15.0 ± 0.00	20.5 ± 5.50	4	0.423	0.813
	24h	12.7 ± 2.33	17.0 ± 4.73	4	0.457	0.395
	48h	17.0 ± 4.73	17.0 ± 4.73	4	1.000	0.845
	72h	16.3 ± 5.24	17.0 ± 4.73	4	0.929	0.962
CFA	4h	10.1 ± 2.59	15.1 ± 3.07	8	0.231	0.088
	24h	6.94 ± 1.45	14.0 ± 2.47	8	0.05	0.01
	48h	5.33 ± 0.67	10.5 ± 0.96	8	0.01	0.01
	72h	5.67 ± 1.20	12.7 ± 1.48	8	0.01	0.01

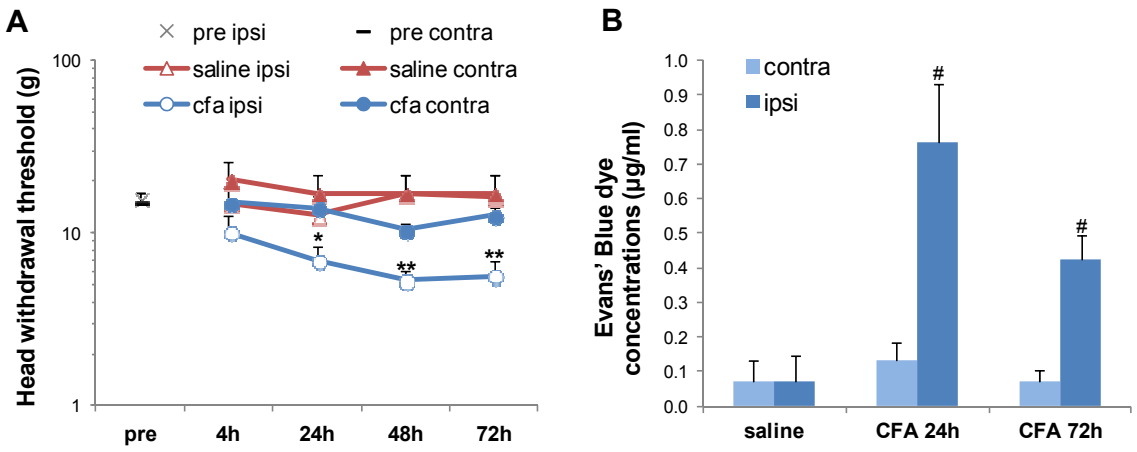

Figure 4.13

Injection of CFA into the TMJ produces mechanical allodynia and plasma extravasation.

A, *Rats were tested for the development of mechanical allodynia by probing the contralateral (contra) and ipsilateral (ipsi) orofacial regions with von Frey filaments before (pre) and post injection of saline or CFA into the TMJ. The head withdrawal threshold force, in grams (g), was measured. Y-axis = log10 scale.* ***B,*** *The development of inflammation was analyzed by injecting the Evans' blue dye through the tail vein. A significantly higher amount of dye was extracted from the ipsilateral TMJs of CFA-injected rats compared to the contralateral side at both 24h and 72h p.i. ** p<0.01, and * p<0.05 compared to the contralateral side of CFA-injected rats, #p<0.01 compared to the contralateral tissue; one-way ANOVA.*

4.10 SGCs AND MACROPHAGES ARE SELECTIVELY ACTIVATED IN TG FOLLOWING TMJ INFLAMMATION

We next evaluated the morphological and biochemical consequences of the induction of TMJ inflammation in the TG, with particular focus on SGCs. Previous studies have indeed reported that either tooth pulp injury or TG inflammation induces SGCs hypertrophy, with increased expression of GFAP (Stephenson et al., 1996; Jimenez-Andrade et al., 2006; Takeda et al., 2007), which is considered to be a marker of reactivity and activation for this particular type of glia. In the contralateral side of CFA injected rats, very low levels of GFAP immunoreactivity were observed, as measured by counting the number of TG neurons encircled by GFAP-positive ($GFAP^+$) SGCs. A large increase in the number of GFAP-encircled neurons was observed in the ipsilateral TG at both 24h (Figure 4.14A',B) and 72h p.i. (Figure 4.14B), as expected (Takeda et al., 2007). From 24h p.i. the number of GFAP-encircled neurons was higher in the ipsilateral V3-mandibular division of the trigeminal nerve (29.38±2.20 in the

ipsilateral side vs. 5.99±0.94 in the contralateral side; n = 7 animals; Figure 4.14B). Similar results were observed in the V2-maxillary division of the trigeminal nerve (28.79±2.30 vs. 4.92±1.00; n=7 animals), and in the V1-ophthalmic division, although to a lesser extent (12.83±1.24 vs. 3.88±0.69; n=7 animals). At 72h p.i. a reduction in GFAP staining compared to the 24 hour values was observed, although it was still significantly higher than the contralateral side or controls (n=7 animals; Figure 4.14B). No differences in the number of GFAP-encircled neurons were found between the ipsi- and contralateral TG in saline-injected rats (n=7 animals; Figure 4.14B).

Macrophages have been reported to infiltrate the DRG following sciatic nerve damage or hindpaw inflammation (Inglis et al., 2005; Hu et al., 2007). We therefore evaluated whether a similar effect also takes place in the TG after the development of TMJ inflammation. Surprisingly, no changes in the number of $Iba1^+$ resident macrophages and no difference in the mean cell size of $Iba1^+$ cells were observed following CFA injection in the ipsilateral TG (n=7 animals; not shown). Nevertheless, clear signs of macrophagic activation were detected by using an antibody directed against the lysosomal antigen ED1, a marker of activated macrophages (Damoiseaux et al., 1994; Mueller et al., 2007; Moxon-Emre et al., 2010). In fact, 24h after CFA injection, the densitometric analysis of ED1 immunostaining yielded to a mean value of 111.39±16.71 pixels for the ipsilateral side vs. 36.44±10.73 pixels for the contralateral side (V3 division; n=6 animals; Figure 4.14C-D). This effect persisted at 72h p.i., although to a lesser and non-significant extent (82.47±27.86 pixels for the ipsilateral side vs. 37.76±15.94 pixels for the contralateral side, V3 division; n=7 animals; Figure 4.14D). These results suggest that, early after induction of TMJ inflammation, there is no recruitment of new inflammatory cells from the bloodstream, but rather a strong activation of local resident macrophages.

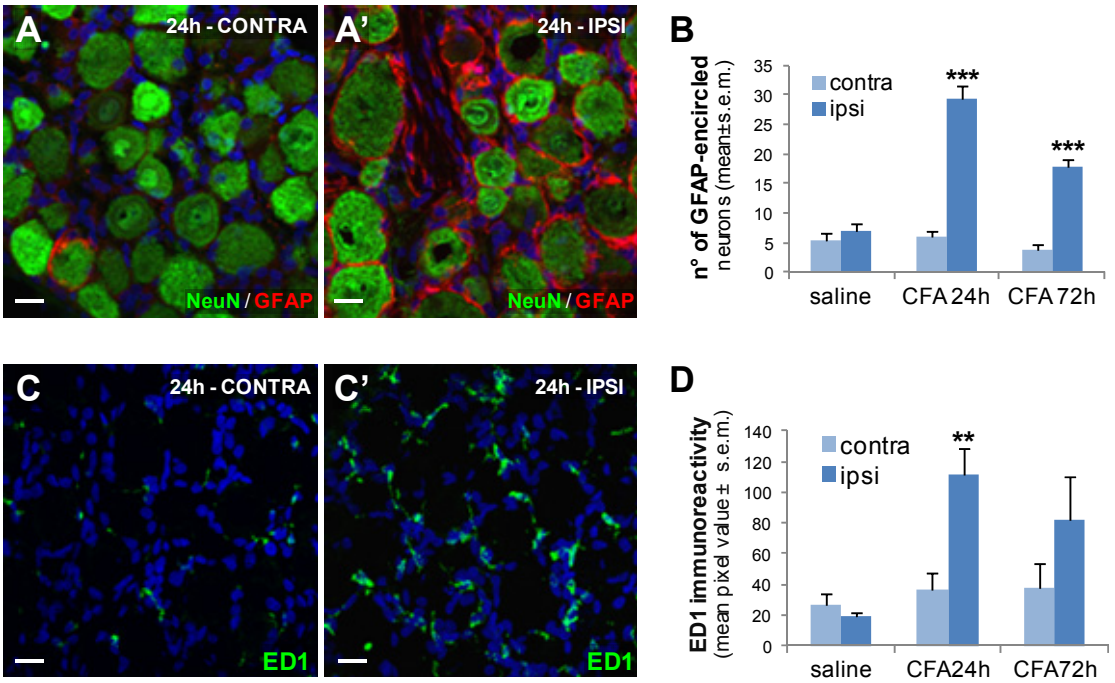

Figure 4.14

SGCs and resident TG macrophages are activated following induction of TMJ inflammation.

*__A,__ Few GFAP$^+$ SGCs (red) encircling NeuN$^+$ neurons (green) were detected in the contralateral (CONTRA) TG of CFA-injected rats, or in TGs from saline-injected rats (not shown). __A',__ A significant increase in the number of GFAP-encircled neurons was instead observed in the ipsilateral (IPSI) TG starting at 24h post CFA injection. __B,__ Histograms showing the increase in the number of GFAP-encircled neurons in the V3-mandibular division of the ipsilateral (ipsi) TG at both 24h and 72h post CFA injection. Similar changes were observed in the V1-ophthalmic and V2-maxillary divisions (see text). Data are expressed as number of GFAP-encircled neurons per counting field at 40x magnification. __C, C',__ ED1 immunostaining (green) of activated macrophages, showing a significant upregulation 24h after the injection of CFA. __D,__ Densitometric analysis, showing that a lower, although not statistically significant, effect was also present at 72h p.i. Nuclei were labeled with the Hoechst 33258 dye (blue). Scale bars: 20 µm. *** $p<0.001$ and ** $p<0.01$ compared to the contralateral side; one-way ANOVA.*

4.11 MICROGLIAL CELLS, BUT NOT ASTROCYTES, ARE ACTIVATED IN THE SPINAL TRIGEMINAL NUCLEUS FOLLOWING TMJ INFLAMMATION

Although previous studies reported that CNS glial cells (astrocyte and microglial cells) become activated following CFA-induced sciatic nerve inflammation (Raghavendra et al., 2004; Sun et al., 2007; Hernstadt et al., 2009), no studies on the reaction of these cells to inflammatory TMJ sensitization are available. Immunoreactivity levels for Iba1 (a marker for microglial cells) and GFAP (a marker for astrocytes) in the different regions of the spinal trigeminal nucleus (see *Paragraph 1.1.2*) were thus evaluated in saline and CFA-injected rats. As shown in Figure 4.15, no changes in Iba1 immunoreactivity were observed between the contra- and ipsi-lateral sides of saline-injected rats in either the trigeminal subnucleus caudalis of the medulla oblongata (n=8 animals; Figure 4.15A) or in the dorsal horn of the cervical spinal cord (n=8 animals; Figure 4.15C). However, 72 hours after CFA administration Iba1 immunoreactivity was significantly up-regulated in the dorsal laminae of the trigeminal subnucleus caudalis (normalized values: 1.51±0.13 pixels for the ipsilateral side vs. 0.90±0.06 pixels for the contralateral side; n=8 animals; Figure 4.15A). In the ipsilateral side, microglial cells displayed shorter and thicker ramifications, a typical characteristic of activated microglia, when compared to the fine processes of microglia in the contralateral side (Figure 4.15B',B''). Similar changes were also observed in the dorsal horn of the cervical spinal cord (normalized values: 1.80±0.21 pixels for the ipsilateral side vs. 1.00±0.12 pixels for the contralateral side; n=7 animals; Figure 4.15C,D).

Conversely, no reactive astrogliosis was detected in the spinal trigeminal nucleus, both in terms of GFAP immunoreactivity (n = 7 animals; Figure 4.16A,C), and of the morphology of $GFAP^+$ astrocytes (Figure 4.16B,D). We conclude that, during the sub-acute phase of CFA-induced TMJ inflammation, microglial cells, but not astrocytes, are selectively activated in the CNS regions relaying nociceptive information.

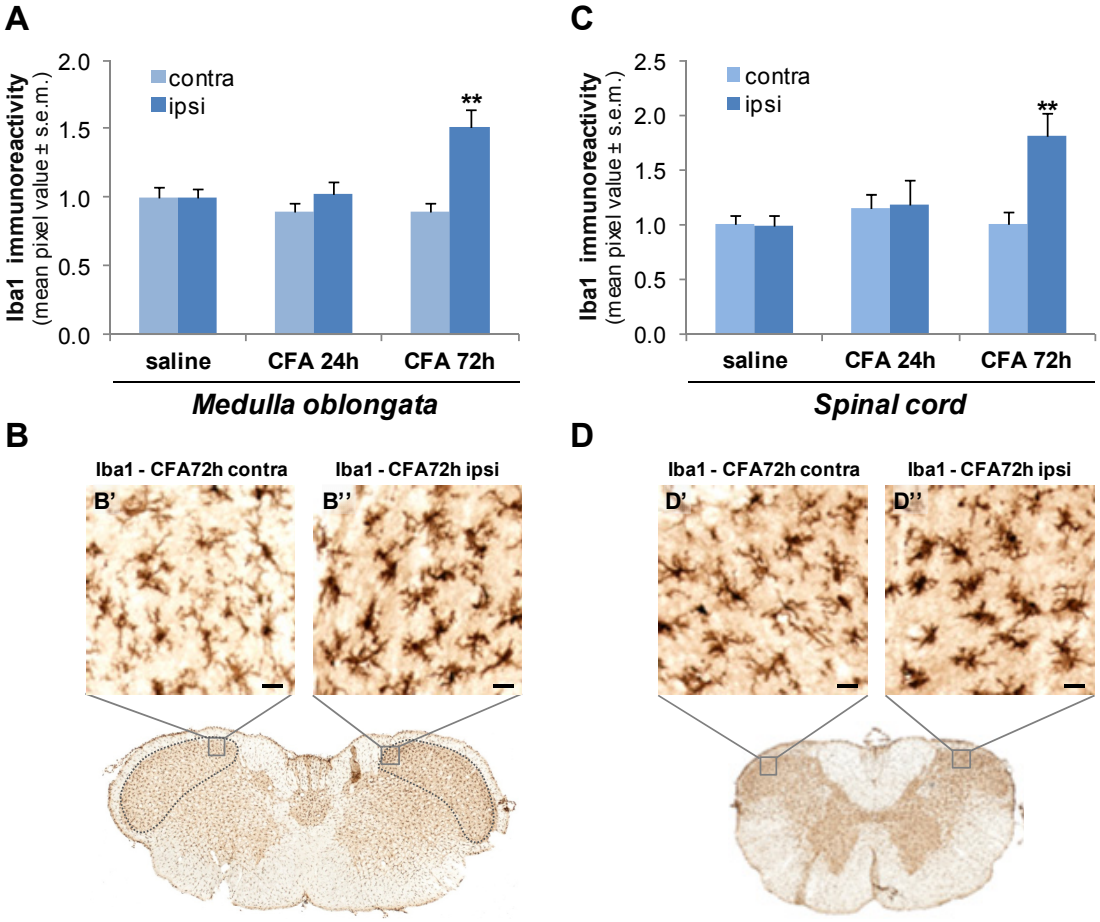

Figure 4.15

TMJ inflammation induces microglial activation in the spinal trigeminal nucleus.

A, C, *Densitometric analysis of Iba1 immunoreactivity (number of immuno-positive pixels, see Methods) showing a significant increase in Iba1 immunoreactivity in the ipsilateral side of both the trigeminal subnucleus caudalis in the medulla oblongata (delimited by the dotted line;* ***A****) and the dorsal horn of the cervical spinal cord (****C****) 72h after CFA injection. The mean values of pixel intensity have been normalized to the values obtained from the contralateral side of saline injected rats, set to 1.0. ** p<0.01 compared to the contralateral side; one-way ANOVA.* ***B-B'', D-D'',*** *Resting microglial cells (i.e., ramified cells with fine processes) were detected in the contralateral side of the trigeminal subnucleus caudalis (****B'****) and of the cervical dorsal horn (****D'****), whereas activated microglial cells (i.e. cells with thicker ramifications) were observed ipsilaterally to the site of injection (****B'', D''****). Scale bars: 20 µm.*

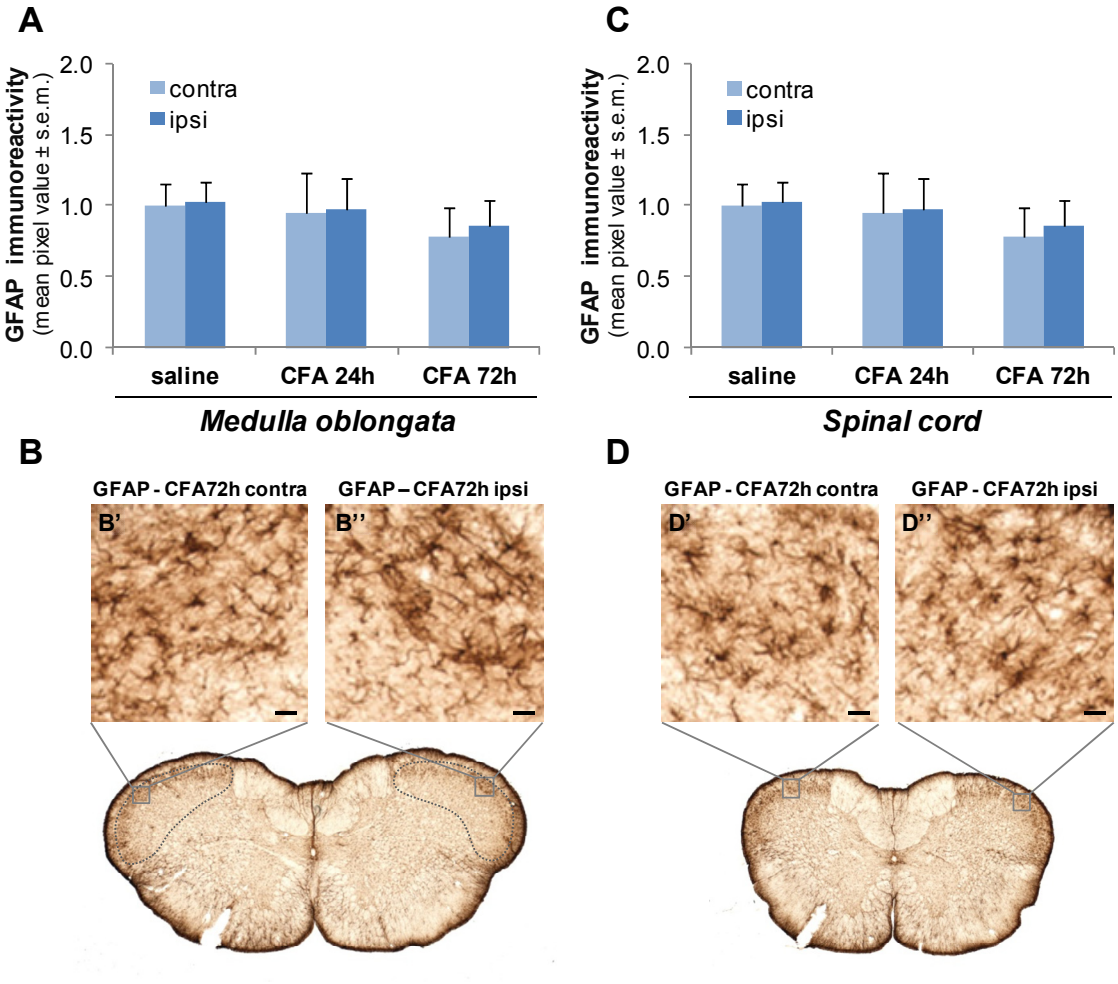

Figure 4.16

TMJ inflammation does not affect the activation state of GFAP$^+$ astroglial cells in the CNS.

A, C, *Densitometric quantification of GFAP immunoreactivity in the trigeminal subnucleus caudalis (delimited by the dotted line;* ***A****) and the cervical dorsal horn (****C****) revealed no changes between the contralateral (contra) and the ipsilateral (ipsi) sides of CFA inflamed rats. The mean values of pixel intensity have been normalized to the contralateral side of saline injected rats, set to 1.0.* ***B-B'', D-D'',*** *No changes in astroglial cell morphology were detected. Scale bars: 20 µm.*

4.12 THE PURINERGIC $P2Y_{12}$ RECEPTOR IS SELECTIVELY EXPRESSED BY MICROGLIAL CELLS IN THE CNS, BUT IT IS NOT UPREGULATED BY TMJ INFLAMMATION

In microglial cells, the purinergic $P2Y_{12}$ receptor subtype was shown to be up-regulated following nerve injury, and its pharmacological or biotechnological inhibition prevented the development of mechanical allodynia (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008). On this basis, we analyzed the possible changes of $P2Y_{12}$ receptor expression in our inflammatory model. Using a specific antibody directed against the C-terminal domain of the rodent $P2Y_{12}$ receptor (Haynes et al., 2006), we were not able to detect any staining within the TG of control animals (Figure 4.17A). This finding is in contrast with a previous report indicating the presence of $P2Y_{12}$ receptor mRNAs in rat DRGs (Kobayashi et al., 2006), and to our RT-PCR analysis on both intact ganglia and primary TG cultures (Figure 4.3). Interestingly, in the same tissue sections $P2Y_{12}$ receptor immunoreactivity was detected at the boundary between the trigeminal nerve root (i.e., the PNS) and the CNS (Figure 4.17B), thus indicating that the expression of this receptor subtype is probably restricted to cells of the CNS. Furthermore, while $P2Y_{12}$ receptor and Iba1 immunostaining colocalized in CNS microglial cells (Figure 4.17B, yellow arrows), $Iba1^+$ macrophages resident in the trigeminal nerve root were $P2Y_{12}$-negative (Figure 4.17B). In the brainstem, the $P2Y_{12}$ receptor was only expressed by $Iba1^+$ microglial cells (Figure 4.17C,C''), confirming previous reports indicating this receptor expressed by CNS microglia (Haynes et al., 2006).

We next evaluated whether or not $P2Y_{12}$ receptor levels in the spinal trigeminal nucleus were affected by CFA injection into the TMJ. Despite the observed upregulation of Iba1 immunoreactivity and the morphological changes observed in microglial cells (see above), no increase in $P2Y_{12}$ receptor immunoreactivity in the ipsilateral side of CFA-injected rats was observed in either the trigeminal subnucleus caudalis (n=7 animals; Figure 4.17D,E) or in the cervical dorsal horn (not shown; n=7 animals). Taken together, these results suggest that the purinergic $P2Y_{12}$ receptor, which has been implicated in some forms of pain sensations (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008), might not be directly involved in the subacute reaction of CNS glial cells to TMJ inflammation.

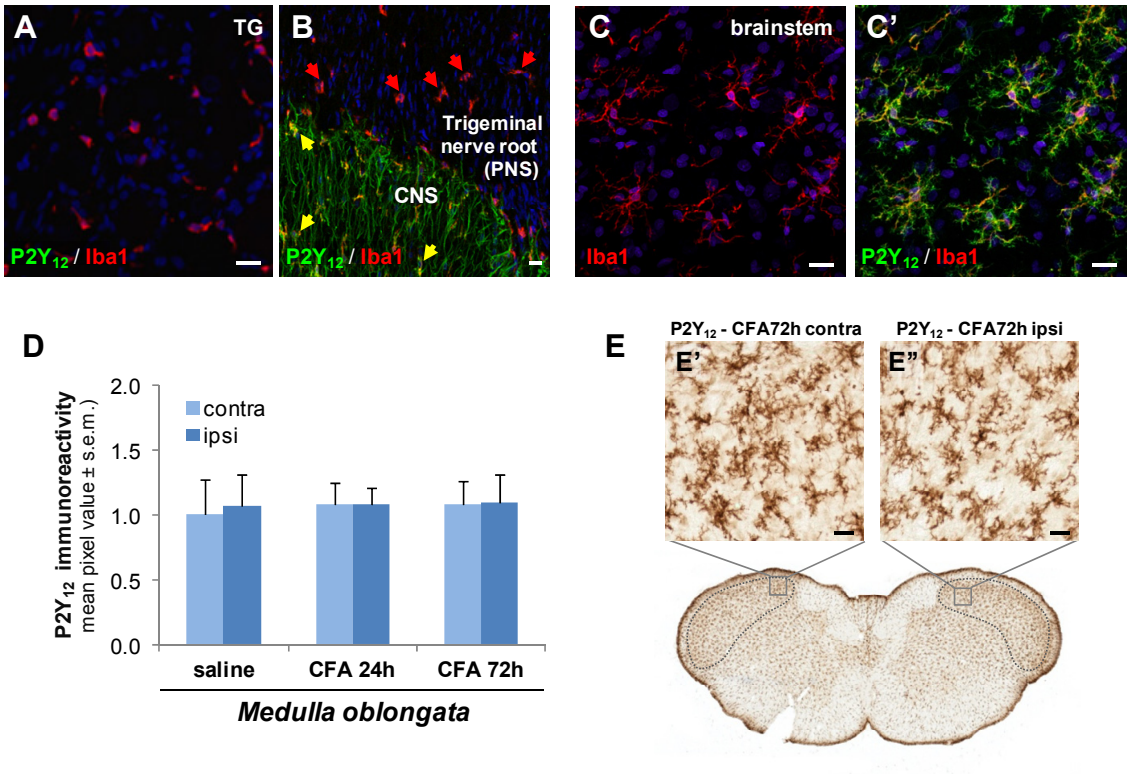

Figure 4.17

$P2Y_{12}$ receptor is selectively expressed by microglial cells of the brainstem, but its expression levels are not affected by TMJ inflammation.

A, *In the TG, no staining for the $P2Y_{12}$ receptor subtype (green) was detected in Iba1-expressing macrophages (red).* ***B,*** *At the trigeminal nerve root (i.e., the PNS/CNS boundary), $Iba1^{+}$/ $P2Y_{12}^{+}$ microglial cells were observed (yellow arrows).* ***C, C',*** *In the brainstem, $P2Y_{12}$ receptor immunoreactivity was specifically found on $Iba1^{+}$ microglial cells (red). Nuclei were labeled with the Hoechst 33258 dye (blue). Scale bars: 20 µm.* ***D,*** *Densitometric quantification of $P2Y_{12}$ receptor immunoreactivity in the trigeminal subnucleus caudalis revealed no changes in the receptor expression between the contralateral (contra) and the ipsilateral (ipsi) sides of inflamed rats. Similar results were detected in the cervical dorsal horn (not shown; see text). The mean values of pixel intensity have been normalized to the contralateral side of saline injected rats, set to 1.0.* ***E-E'',*** *Immunostaining of the $P2Y_{12}$ receptor subtype in the trigeminal subnucleus caudalis 72h after CFA injection. Scale bars: 20 µm.*

5. DISCUSSION

Migraine is a highly disabling neurovascular disorder, affecting about 15% of adults in the Western World, with considerable socio-economic implications on both productivity and quality of life (Goadsby, 2007). Great progress has been made in migraine research during the past few decades: (i) migraine has been better classified and defined (Silberstein, 2004); (ii) imaging studies have shown that cortical spreading depression is most probably the phenomenon that underlies the migraine aura (see *Paragraph 1.2.3*; Pietrobon, 2007); (iii) the neurobiology of the trigeminal vascular system has been better delineated, and several messenger molecules have been strongly implicated in the initiation and triggering mechanisms of migraine (see *Paragraph 1.2.2*; Pietrobon, 2005); and (iv) and novel classes of selective antimigraine drugs, such as triptans and CGRP antagonists, have been developed (see *Paragraph 1.2.4*; Goadsby & Sprenger, 2010).

Despite these progresses, there is little or no evidence that shows with certainty which and how pain fibers are activated during migraine attacks. Most investigators currently believe that a migraine attack starts in the brain, as suggested by premonitory symptoms. However, a debate about migraine origin is still ongoing: some scientists argue for a central origin of the headache pain (Goadsby et al., 2009), whereas others, including our research group, believe that the headache is triggered by activation of peripheral nociceptors. Indeed, the trigeminal nerve conveys sensory information from most extracranial and intracranial structures to the spinal trigeminal nucleus, and it has been therefore hypothesized that migraine could arise from a primary dysfunction leading to activation and sensitization of the trigemino-vascular system (Olesen et al., 2009). On this basis, the final aim of my study have been setting up both *in vitro* and *in vivo* models, as tools for evaluating the molecular and cellular role of the purinergic system in trigeminal pain transmission, in order to identify new potential targets for migraine pain therapy.

5.1 PRIMARY MIXED TG CULTURES AS AN IN VITRO MODEL FOR EVALUATING P2 RECEPTOR EXPRESSION AND FUNCTIONALITY

To characterize calcium signaling via P2 receptors in TG, we have taken advantage of *in vitro* trigeminal cultures that have been previously reported as a reliable

experimental model to study purinergic transmission in trigeminal neurons (Fabbretti et al., 2008; Simonetti et al., 2006). However, these previous studies were only focused on the neuronal $P2X_3$ receptor, and no evidence was provided on the contribution of glial cells to purinergic signaling in TG. As a first step, we have characterized the dissociated TG cultures in terms of presence and structural organization of the different cell populations, demonstrating that primary mixed neuron-glia TG cultures are a reliable *in vitro* model of sensory ganglia. Indeed, cultured neurons survived for several days *in vitro* and displayed a good viability, as indicated by their responsiveness to depolarization and by the MTT cytotoxicity assay (data not shown). An analysis of the distribution of neurons according to their somatic size showed that small and medium nociceptive neurons represent almost the totality of the neuronal population (Figure 4.1H), in full agreement with data obtained in cell cultures from DRG (Gu et al., 2006) and TG (Simonetti et al., 2006). Moreover, most of cultured neurons maintained the typical morphological features of sensory neurons with a single axon forming a T-shape bifurcation, and retained most of the phenotypical characteristics observed *in vivo* (Figure 4.1D-G'). Finally, cultured neurons retained, at least in part, their physical relationships with satellite glial cells (SGCs; Figure 4.1D,E), suggesting that also their functional and modulatory activities are preserved.

We next analyzed single cell calcium changes induced by culture exposure to different subtype-selective P2 agonists in both neurons and glial cells 48 hours after TG dissociation. As a first step, we focused our attention on the $P2X_3$ receptor subtype, the most important purinoceptor involved in pain transmission. Expression levels of $P2X_3$ receptor, and of the $P2X_2/P2X_3$ heterodimer, increases in different inflammatory (Xu & Huang, 2002; Shinoda et al., 2005), and neuropathic (Novakovic et al., 1999) models of pain, and the pharmacological or biotechnological inhibition of $P2X_2/P2X_3$ activation reduces or abolished pain sensation (Honore et al., 2002; McGaraughty et al., 2003; Dorn et al., 2004). Recent studies reported that $P2X_3$ receptor-mediated responses are also increased in trigeminal primary cultures after exposure to pro-algogenic CGRP (Fabbretti et al., 2006), suggesting the possible importance of this receptor subtype also in migraine pain. Confirming to literature data (Bradbury et al., 1998; Guo et al., 1999), we found $P2X_3$ receptors expressed in approximately 70% of neurons (Figure 4.2A), and, relevant to pain transmission, in almost the totality of IB4-positive sensory neurons (Figure 4.2B). In approximately 60% of neurons, $P2X_3$ was coupled to $[Ca^{2+}]_i$ increases

as demonstrated by the application of its relatively selective agonist αβ-meATP (Figure 4.2D). Primary trigeminal cultures also expressed $P2X_2$, which likely dimerizes with $P2X_3$ contributing to pain transmission, but not the $P2X_1$ receptor subtype (Figure 4.2C). Taken together, these results suggest that cultured TG sensory neurons maintain their physiological responses to ATP, and express functional algogenic $P2X_3$ and $P2X_{2/3}$ receptors, thus confirming the suitability of this *in vitro* model to study the modulation of pain transmission by the purinergic system.

As mentioned above, the present study has been principally aimed at characterising P2Y receptors in both neurons and SGCs in the TG cultures. Regarding the presence of these receptors in sensory neurons, immunohistochemical analysis on intact DRG, TG, and nodose ganglia has demonstrated that $P2Y_1$ and $P2Y_4$ receptors are expressed by two distinct neuronal populations (Ruan & Burnstock, 2003), but so far no functional studies have been performed. Interestingly, our RT-PCR analysis showed that all cloned murine P2Y receptors (i.e., $P2Y_{1,2,4,6,12,13,14}$) are expressed in both intact TG and in trigeminal cultures 48 hours after plating (Figure 4.3). Results obtained by single cell calcium recordings from TG neurons indicated that only some P2Y receptors are functional in these cell populations. In particular we showed that TG neurons express metabotropic ADP-sensitive $P2Y_1$ and UTP-responding $P2Y_2$/$P2Y_4$ receptors (Figure 4.4). We have also evaluated the functionality of calcium-linked P2Y receptors on SGCs, for which very few data have been available so far. In fact, in one single study on intact TG ganglion, $P2Y_1$ and $P2Y_2$/$P2Y_4$ receptors were demonstrated to couple to increases of intracellular calcium concentrations in SGCs (Weick et al., 2003). As shown in Figure 4.5, in dissociated TG cultures SGCs expressed functional metabotropic $P2Y_1$ and $P2Y_{12}$/$P2Y_{13}$ receptors responsive to ADP, as well as $P2Y_2$/$P2Y_4$ receptors responsive to UTP, as further indicated by the use of selective antagonists. A smaller response to UDP was also found, indicating that $P2Y_6$ receptors only marginally contribute to P2-mediated calcium increases in these cells, at least under control conditions (see also below). Taken together, our results represent the first systematic evaluation of P2Y receptor expression and function in both neuronal and non-neuronal TG cells.

5.2 EXPOSURE TO ALGOGENS EXERT A COMPLEX MODULATION OF P2 RECEPTOR FUNCTIONALITY IN NEURONS AND GLIAL CELLS

Prolonged exposure to algogenic mediators modulates both neuronal and glial cell activity, thus contributing to the development of chronic pain (Wang et al., 2007; McMahon & Malcangio, 2009). Among known algogens, BK is indeed a known activator of sensory neurons and has been demonstrated to increase the neuronal firing rate in trigeminal nucleus caudalis and to enhance the release of CGRP from cultured dorsal horn and TG neurons (Jenkins et al., 2003). Moreover, literature data indicates that an acute (50 sec) exposure to BK rapidly sensitizes the function of the $P2X_3$ receptor subtype (Paukert et al., 2001). Since subpopulations of SGCs are also sensitive to BK (Heblich et al., 2001), we deemed it interesting to study the effect of an acute (up to 3 min) or a prolonged (24 h) BK exposure on purinergic signaling in both neurons and SGCs. Acute application of BK induced responses in a significant percentage of neurons (see also below), and significantly enhanced the amplitude of neuronal $P2X_3$ receptor-mediated peak calcium response (Figure 4.6C), thus confirming literature data in an expression system (Paukert et al., 2001). Instead, chronic treatment with BK induced a strong and highly significant reduction of the number of αβ-meATP-responding neurons (Figure 4.6E). These data represent the first demonstration of a $P2X_3$ receptor modulation by BK in a native system. Thus, a biphasic response of $P2X_3$ receptor to painful situations, with increased or decreased firing depending on the length of the exposure to the noxious and pro-inflammatory stimulus, might exist. This evidence suggests that different pharmacological manipulations of this receptor subtype might be utilized in acute or chronic pain. Interestingly, in TG cultures a 1 hour exposure to CGRP also led to the upregulation of neuronal $P2X_3$ receptors (Fabbretti et al., 2008).

Upon chronic exposure to BK, a significant upregulation of P2Y receptors responding to either ADP or UTP was also observed in SGCs (Figure 4.6F,G). Of interest, very few SGCs (about 3%) responded to BK, compared to the 35% of responding neurons (data not shown). This allowed us to hypothesize that the observed changes of P2Y receptors functionality in SGCs might be due to a soluble mediator released by neurons upon BK stimulation. We identified this mediator as the neuropeptide CGRP based on the following evidence: (i) BK-mediated effects on P2Y

receptors were inhibited by the CGRP antagonist, $CGRP_{8-37}$ and mimicked by CGRP itself (Figure 4.7A,B); (ii) a significant increase in extracellular CGRP concentrations was detected after exposure of cultures to 100 nM BK (Figure 4.7C); (iii) in purified SGCs cultures (i.e., in the absence of neurons), BK lost its ability to enhance P2Y receptor responses, whereas the effects of CGRP were fully preserved (Figure 4.7B), due to direct activation of CGRP receptors on these cells and subsequent activation of ERK1/2 MAP kinase signalling pathway. In this scenario, it can be envisaged that BK- (and consequently CGRP-) induced upregulation of glial P2Y receptors contributes to the long-term maintenance of the TG pro-algogenic state, possibly by promoting the synthesis and release of additional glial pro-inflammatory mediators (e.g., chemokines, cytokines, or arachidonic acid metabolites), as already demonstrated for the $P2Y_2$ receptor subtypes in astrocytes (Weisman et al., 2005). Nevertheless, at this stage we cannot exclude that the observed plasticity of P2Y receptors represent an attempt to counteract the pro-nociceptive effects of BK and CGRP or that the different receptor subtypes involved (namely, the $P2Y_{1,12,13}$ and the $P2Y_{2,4}$ subtypes for ADP and UTP, respectively) might play opposite roles in the modulation of TG sensitivity.

Our observation that BK-induced effects on SGCs are mediated by the neuronal release of CGRP suggests that this whole molecular network could be involved in migraine pathology. As discussed in *Paragraph 1.2.2,* CGRP play a central role in migraine. For instance, during the acute migraine attack, CGRP is released by TG afferent at the meninges, where it contributes to central neurogenic inflammation and induces pain-associated vasodilatation (Tepper & Stillman, 2008). Accordingly, CGRP plasma levels are increased during a migraine attack (Goadsby et al., 1990), and return to baseline after administration of migraine abortive drugs (Lassen et al., 2002), thus demonstrating its causative correlation with headache pain. No wonder if CGRP antagonists are now evoked as new effective drugs in untreatable migraine (Tepper & Stillman, 2008).

5.3 THE GAIN-OF-FUNCTION MUTATION IN $Ca_V2.1$ CALCIUM CHANNELS AFFECTS P2Y RECEPTORS FUNCTIONALITY IN SGCs

By using TG cultures derived from $Ca_V2.1$ α1 R192Q mutant KI mice we have

demonstrated, for the first time, a significantly increased basal and stimulated extracellular CGRP release compared to wild type cultures. Transgenic mice carrying this mutation were previously shown to exhibit an enhanced Ca^{2+} influx in cortical neurons, which could explain their increased susceptibility to the generation of cortical spreading depression (that is also observed in FHM1 patients, see *Introduction*; van den Maagdenberg et al., 2004; van den Maagdenberg et al., 2010). Our results suggest that, in R192Q KI cultures, mutated $Ca_V2.1$ calcium channels lead to an increased neuronal firing, which in turn promotes CGRP release in TG and may augment their sensitivity to migraine triggers. More specifically, we hypothesize that the presence of a basal pro-inflammatory milieu within the TG of these mice might lower the threshold for neuronal firing. The fact that the already elevated extracellular CGRP levels can be further enhanced by neuronal stimulation stimuli (with BK or KCl) suggests that this pathway can contribute to an amplification and worsening of this basal pro-inflammatory state under conditions that occur during a migraine attack. Accordingly, in the presence of high levels of CGRP, neuronal $P2X_3$ receptors show an enhanced synthesis and a reduced desensitization potential (Fabbretti et al., 2006), contributing to the increased transmission of pain. Moreover, an increase in basal neuronal $P2X_3$ receptor activity has been recently found in TG cultures from $Ca_V2.1$ α1 R192Q KI mice, as a consequence of calcium-dependent alterations of the receptor phosphorylation state (Nair et al., 2010).

We have also evaluated if the augmented CGRP release in R192Q KI cultures was associated to modulation of P2Y receptor functionality in SGCs. We observed no differences in basal ADP- and UTP-related mean calcium increases between R192Q WT or KI SGCs (compare "CTR" histograms in Figure 4.11), thus indicating that the higher basal release of CGRP in KI cultures does not affect P2Y receptor functionality in glial cells, or that the chronic exposure to CGRP might have triggered some compensatory mechanisms. However, following BK application, despite a similar upregulation of P2Y receptors in SGCs from R192Q WT and KI animals (Figure 4.11), a significant increase in the percentage of ADP and UTP responding SGCs was found in R192Q KI cultures (Figure 4.11). This evidence suggests that the larger CGRP release induced by BK in R192Q KI cultures might enhance the transcription of specific P2Y receptor subtypes or that some yet-to-be identified mechanisms of modulation of P2Y receptor function is involved (e.g., receptor phosphorylation or recruitment of scaffold

proteins). It should be noted that the percentage of ADP- and UTP-responding SGCs in R192Q WT cultures is lower to that reported in our previous experiments, as shown in Figure 4.5. This apparent discrepancy could be explained by the use of lower concentration of nucleotides in experiments involving R192Q WT and KI cultures (1μM for ADP and 10 μM for UTP), compared to our previous experiments (100μM for both ADP and UTP). Moreover, the R192Q WT littermates utilized in the last set of experiments were selected during the generation of R192Q KI transgenic mice from C57black/6 mice (van den Maagdenberg et al., 2004), thus some genetic differences with the C57black/6 mice we usually purchase from a commercial source could be present.

In conclusion, we have demonstrated that the gain-of-function R192Q mutation in $Ca_V2.1$ channels is associated to a basal and stimulated increase of CGRP released from TG neurons, which in turn modulates P2Y receptor expression and functionality in SGCs. Thus, the complex interplay between TG neurons and SGCs has implications for migraine pathophysiology and could become even more important in FHM1 patients. To date, the pro- or anti-algogenic role of glial P2Y receptors in pain transmission is still unknown; future *in vivo* studies are therefore needed to clarify this issue.

5.4 SET UP OF IN VIVO MODELS OF TRIGEMINAL PAIN AS TOOLS FOR EVALUATING THE ROLE OF GLIAL P2Y RECEPTORS IN PAIN TRANSMISSION

The results obtained from our TG cultures *in vitro* demonstrate that glial P2Y receptors act as important players in nociception. For this reason, we deemed interesting to set up *in vivo* pain models of TG sensitization, in order to study the involvement of the purinergic system in pain transmission, and to validate the role of specific P2Y receptor subtypes. To this aim, we initially set up a mouse model of acute trigeminal pain, based on the injection of formalin into the upper lip of mice, and we focused our attention on the $P2Y_4$ receptor, due to evidence indicating its expression by trigeminal SGCs (Weick et al., 2003; Vit et al., 2006). Although we successfully reduced $P2Y_4$ protein levels by injecting long double-stranded RNAs (dsRNAs) designed to selectively knock-down P2Y4 receptor expression directly in the TG, no significant

differences in pain behaviour were detected when comparing formalin-treated mice that received $P2Y_4$-dsRNAs to formalin-treated animals receiving control dsRNAs (Figure 4.12). These data apparently rule out a major role for $P2Y_4$ receptors in acute trigeminal pain transmission. Moreover, doubts on the specificity of the anti-$P2Y_4$ commercially available antibody, and on the actual expression of this receptor subtype in SGCs have been raised (Villa et al., 2010). For this reason, we focused our attention on other P2Y receptors subtypes that could be targeted by antibodies whose specificity has been fully validated (see below).

Therefore, we set up and characterized a model of chronic trigeminal inflammation based on the injection of the Complete Freund's Adjuvant (CFA) into the temporomandibular joint (TMJ) of adult rat. This paradigm of pain is a well established model of chronic trigeminal pain, associated with strong neuronal sensitization both in the trigeminal ganglion and in the brainstem (Zhou et al., 1999). Since the trigeminal and CNS nociceptive pathways are sensitized in migraneurs as well (Pietrobon, 2005), this models can be proposed to mimic the molecular mechanisms at the basis of migraine sensitization. It should be also considered that specific *in vivo* models of migraine pain are not available, and even impossible to be achieved. Moreover, relevant links between migraine and inflammatory pain can be found in literature. In fact, clinical studies have demonstrated a strong correlation between migraine and the temporo-mandibular disorders (Taub et al., 2008; Kang et al., 2009), and the pharmacological treatment of various temporo-mandibular symptoms significantly decreases headache pain (Mitrirattanakul & Merrill, 2006). In addition, it has been demonstrated the existence of a prevalence to temporo-mandibular disorders in patients with combined migraine and tension-type headache (Ballegaard et al., 2008).

Due to the limited information on the role glial cells in the spinal-trigeminal system following TG sensitization, we have examined the response of TG and CNS glial cells in our inflammatory pain model. First of all, we have demonstrated that CFA injection into the TMJ produces a significant increase in GFAP expression in SGCs in the TG (Figure 4.14), thus confirming the reaction of this glial cell family to the administration of a pro-algogenic stimulus (Takeda et al., 2009). The key role of SGCs in the development and maintenance of chronic pain has been demonstrated in other pain models by their increased expression and release of IL-1β (Takeda et al., 2007), TNFα (Zhang et al., 2007), as well as augmented gap junction-mediated cell coupling

(Garret et al., 2008; Ledda et al., 2009) following nerve injury. All together, these changes are associated with increased excitability of both primary afferents and CNS neurons, leading to the development of hyperalgesia and allodynia (Takeda et al., 2009).

We also provide new evidence on the behavior of TG macrophages under inflammatory conditions. In particular, following induction of TMJ inflammation a strong upregulation of ED1 (a specific marker for activated macrophages; Hu et al., 2007) in the ipsilateral side of inflamed rats was found (Figure 4.14). Since no increases in either the number or the average cell size of macrophages were detected, we conclude that CFA injection does not trigger macrophages infiltration from the bloodstream into the trigeminal perineuronal regions, but rather modulates the activation state of resident immune cells. These cells can in turn contribute to the development of TG sensitization following TMJ inflammation.

Microglial cells, the resident macrophagic population in the CNS, have been crucially implicated in the initiation and modulation of certain chronic pain states (Smith, 2010). For instance, in various neuropathic pain models, activated microglia was shown to release pro-inflammatory cytokines and other substances that facilitate pain transmission (Inoue & Tsuda, 2009; White et al., 2007). Moreover, several drugs acting as glial cell inhibitors (e.g., propentofylline, pentoxifylline and minocyclin) eventually suppress the development of neuropathic pain by decreasing both microglial activation and cytokine release (Mika, 2008; Sweitzer et al., 2001). Here we report for the first time that injection of CFA into the TMJ induces a significant ipsilateral activation of microglial cells, both in the dorsal laminae of the trigeminal subnucleus caudalis in the medulla oblongata and in the dorsal horn of the cervical spinal cord (Figure 4.15), which represent the territories innervated by the mandibular fibers of the trigeminal nerve (Capra & Dessem, 1992), and represent key stations for the integration of temporomandibular painful sensations. Our data are in agreement with previous papers indicating an increased expression of microglial cell markers in the lumbar spinal cord in CFA-induced ankle or tibio-tarsal joint monoarthritis in rats, associated with the appearance of cells having an activated morphology (Sun et al., 2007; Hernstadt et al., 2009). This confirms that the induction of deep articular inflammation represents a potent trigger for CNS microglial activation.

We also examined astrocyte activation in the brainstem following TMJ inflammation. At variance from previous reports on sciatic nerve inflammation

(Raghavendra et al., 2004; Sun et al., 2007), we did not detect any evidence of astroglial activation (Figure 4.16). This divergence could be due to the fact that the latter studies showed astroglial activation at later time points (11 and 14 days) following the inflammatory insult, thus suggesting that astrocytes are not involved in the first sub-acute phases of tissue sensitization, but might be recruited at later times.

As discussed in *Paragraph 1.4.2*, the purinergic $P2Y_{12}$ receptor is a key player in controlling microglia-associated development of neuropathic pain, and thus represents a possible therapeutic target for treating chronic pain disorders. For these reasons we checked for its possible modulation in our inflammatory pain model. By utilizing a custom-made antibody whose specificity was already successfully validated in $P2Y_{12}$-deficient mice (Haynes et al., 2006), no $P2Y_{12}$ immunostaining was detected on TG sections, although we have detected its mRNA in mouse TG (Figure 4.3). Interestingly, staining for the $P2Y_{12}$ receptor was observed at the boundary between the trigeminal nerve root and the CNS, and in $Iba1^+$ cells of the brainstem (Figure 4.17). This observation correlates with previous reports indicating that, unlike other extracellular nucleotide receptors, such as the $P2X_4$, $P2X_7$, and $P2Y_6$ receptor subtypes, $P2Y_{12}$ receptor expression is restricted to CNS microglia (see *Section 1.4*; Haynes et al., 2006). Quite surprisingly, we detected no changes in microglial $P2Y_{12}$ receptor levels after the injection of CFA in the TMJ, suggesting that this receptor might not participate to the first phases of tissue hypersensitivity following an inflammatory stimulus; however, only pharmacological studies can endorse this evidence. In a different model of inflammatory pain (i.e., the injection of CFA in the hindpaw) the intraperitoneal administration of the $P2Y_{12}$ receptor antagonist 2,2-dimethyl-propionic acid 3-(2-chloro-6-methylaminopurin-9-yl)-2-(2,2-dimethyl-propionyloxymethyl)-propylester (MRS2395) significantly alleviated the mechanical hypersensitivity (Ando et al., 2010), but authors did not evaluated the possible changes in $P2Y_{12}$ receptor expression in their experimental model. Thus, it may well be that, despite the lack of detectable receptor upregulation, $P2Y_{12}$ receptor targeting might be beneficial also for treating other chronic inflammatory pain states involving the sensitization of the spinal-trigeminal system.

In conclusion, this *in vivo* model represents an useful tool for evaluating the role of specific P2Y receptor subtypes, expressed by glial cells, in the development and maintenance of chronic trigeminal pain and migraine-associated pain. We intend now to

look for possible changes in other P2Y receptors expressed by glial cells, and to evaluate the pro- or anti-algogenic effects of their pharmacological or biotechnological inhibition.

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7. ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular calcium concentration
2MeSADP	2-methylthio adenosine 5'-diphosphate
a,ß-meATP	a,ß-methylene-ATP
Ado	adenosine
ADP	adenosine-5'-diphosphate
ADPβS	adenosine 5'-[β-thio]-diphosphate
ASO	antisense oligonucleotides
ATP	adenosine-5'-triphosphate
BDNF	brain derived neurotrophic factor
BK	bradykinin
CaMKII	calcium/calmodulin kinase II
CFA	complete Freund's adjuvant
CGRP	calcitonin-gene related peptide
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CSD	cortical spreading depression
DRG	dorsal root ganglia
dsRNAs	long double-stranded RNAs
EIA	enzyme immunometric assay
FHM1	familial hemiplegic migraine type 1
FHM2	familial hemiplegic migraine type 2
FHM3	familial hemiplegic migraine type 3
GDNF	glial-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GPCR	G protein-coupled receptors
IASP	international association for the study of pain
IB4	isolectin B4
Iba1	ionized calcium-binding adapter molecule
IL	interleukin
ISHH	in situ hybridization histochemistry
KI	knock-in
MA	migraine with aura

MO	migraine without aura
NA	noradrenaline
NGF	nerve growth factor
NO	nitric oxide
NSAIDs	nonsteroidal anti-inflammatory drugs
p.i.	post injection
PET	positron emission tomography
PNS	peripheral nervous system
PPADS	pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid
SCI	spinal cord injury
SGCs	satellite glial cells
SP	substance P
TG	trigeminal ganglia
TMJ	temporomandibular joint
TNFα	tumor necrosis factor α
TNP-ATP	2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate
UDP	uridine-5'-diphosphate
UTP	uridine-5'-triphosphate
VIP	vasoactive intestinal polypeptide
VR1	vanilloid receptor subtype 1
WT	wild type