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The role of TRPA1 and TRPV1 channels in trigeminal pain: data from animal models

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1. INTRODUCTION

1.1. Pain: definition and classifications

The International Association for the Study of Pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. Pain is not classified as a merely sensory phenomenon since it is formed by: a perceptive component (nociception), representing the sensory modality which allows the reception and transport of potential harmful stimuli to the central nervous system (CNS), and an experience component, the psychological condition linked to an unpleasant sensation. The potentially damaging stimuli (of chemical, mechanical or thermal origin) activate some specialized sensory neurons, called nociceptors, which are located on the skin, subcutaneous and internal tissues. The nociceptive afferent fibers, whose cell bodies are located in sensory ganglia, can be divided in two subtypes: the unmyelinated C fibers and the thin myelinated Aδ fibers. A-fiber nociceptors are responsible for the sharp, pricking pain ("first" pain) associated with application of intense heat or sharp objects. C-fiber nociceptors (mostly polymodal) are responsible for the burning pain sensation ("second" pain) from noxious heat stimuli and from prolonged mechanical stimuli.

Based on the origin and neurophysiological characteristics, it is possible to identify a neuropathic and an inflammatory pain; this latter begins following the direct activation of nociceptors produced by a damage of the tissue and it is linked to an inflammatory condition. Neuropathic pain instead is due to a direct lesion of peripheral or central nervous fibers, and it is often referred as a burning sensation.

Pain is defined as acute (or physiological) when it is well localized and transient, and has a stimulus-response relationship. Its fundamental role is to operate a protective system, warning of contact with potentially damaging stimuli, activating spinal and supraspinal responses aimed at preventing or limiting tissue damage. Persistent and intense noxious stimulation leads to chronic pain, giving rise to a sensitization phenomenon, in which peripherally the nociceptive threshold is strongly reduced, and centrally there is an enhanced responsiveness of primary afferent nociceptors. Sensitization is characterized by: allodynia, a pain response from stimuli normally non-painful, and hyperalgesia, an increased sensitivity to pain. Hyperalgesia can be primary hyperalgesia, when it describes

pain sensitivity that occurs directly in the damaged tissues, mostly due to inflammatory mediators that sensitize the nociceptors of the injured site, or secondary, when it occurs in surrounding undamaged tissues and most likely is due to sensitization of neurons in the central nervous system in which mechanisms of synaptic plasticity take place.

Nociceptors sensitization following injury or inflammation may be provoked by chemical substances liberation from damaged cells and surrounding tissues. Some of these molecules are: bradykinin, prostaglandins, histamine, 5-hydroxytryptamine (5-HT) and acetylcholine. These substances activate the nociceptive terminals thus leading to the release of the neuropeptides substance P (SP) and the calcitonin gene-related peptide (CGRP), which contribute to edema diffusion and induce vasodilation (Omoigui, 2007).

1.2. Trigeminal system

The cranial nerve responsible for motor functions and sensation in the face, scalp and mouth is the trigeminal nerve (TN, the 5^{th} cranial nerve). The TN is the largest cranial nerve and it is divided into 3 subdivisions (Figure 1):

- Ophthalmic division (V1), that carries sensory information from the scalp and forehead, the upper eyelid, the conjunctiva and cornea of the eye, the nose, the nasal mucosa, the frontal sinuses and parts of the meninges (the dura and blood vessels).
- Maxillary division (V2), that carries sensory information from the lower eyelid and cheek, the nares and upper lip, the upper teeth and gums, the nasal mucosa, the palate and roof of the pharynx, the maxillary, ethmoid and sphenoid sinuses and parts of the meninges.
- Mandibular division (V3) gives motor supply to the muscles of mastication and carries sensory information from the lower lip, the lower teeth and gums, the chin and jaw, parts of the external ear and parts of the meninges.

The 3 branches of the TN converge in the trigeminal ganglion (TG, also named Gasserian or semilunar) (Figure 1), where the cell bodies of the sensory axons are found. The TG is located in the middle cranial fossa (Meckel's Cave) at the base of the skull, and the cell bodies are organized somatotopically, a feature unique to the TG. The TG is analogous to

the dorsal root ganglia of the spinal cord, which contain the cell bodies of incoming sensory fibers from the rest of the body.

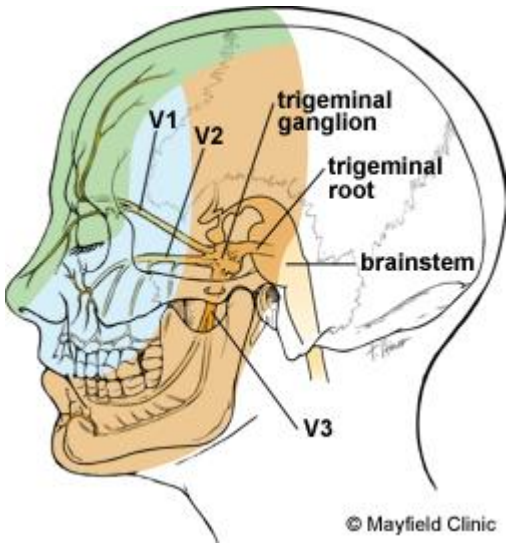

Figure 1. The trigeminal nerve divisions that branch from the trigeminal ganglion: ophthalmic division (V1) provides sensation to the forehead and eye, maxillary division (V2) provides sensation to the cheek, mandibular division (V3) provides sensation to the jaw.

The motor function of TN activates the muscles of mastication, the tensor tympani, tensor veli palatini, mylohyoid and the anterior belly of the digastric. These structures are innervated by the motor nucleus, located in the upper pons ventromedially to the sensory nucleus (Figure 2A). The motor nucleus receives cortical fibres (mostly crossed) for voluntary control of the four muscles of mastication. It also receives input from the mesencephalic and sensory nuclei.

The sensory function of the TN is to provide tactile, proprioceptive, and nociceptive afferents to the face and mouth. Touch-position information is sent to the ventral posteromedial nucleus (VPM) of the thalamus. From there information is projected to the primary somatosensory cortex (S1). Pain-temperature information is sent to the VPM and S1 as well, but, unlike touch-position information, this type of information is also sent to other thalamic nuclei and projected onto additional areas of the cerebral cortex. Some pain-temperature fibers are sent to the medial dorsal thalamic nucleus, which projects to the anterior cingulate cortex. Other fibers are sent to the ventromedial nucleus of the thalamus,

which projects to the insular cortex. Finally, some fibers are sent to the intralaminar nucleus of the thalamus, which projects diffusely to all parts of the cerebral cortex. The insular and cingulate cortices are parts of the brain which represent touch-position and pain-temperature in the context of other simultaneous perceptions (sight, smell, taste, hearing and balance) in the context of memory and emotional state.

There are three sensory nuclei, arranged in a column extending from the midbrain through the pons and medulla and into the upper cervical cord (Figure 2A). The mesencephalic nucleus is mainly involved in proprioception of the face, orbit, muscles of mastication and tongue. This nucleus is unique in that it represents the only structure in the CNS to contain cell bodies of primary afferents. The principal and spinal nuclei of the trigeminal brainstem complex receive most of the sensory inputs from the TN. As the central axons of the trigeminal nerve enter the brainstem from the ganglia through the sensory root, they divide and send short ascending axons up to the principal nucleus and long descending axons down to the spinal nucleus. Axons ending in the principal nucleus carry tactile or light pressure information, while axons that descend to the spinal nucleus carry temperature and pain information. The spinal nucleus, extending from the lower pons to the upper cervical cord, is composed of three subnuclei: oralis, interpolaris and caudalis (Figure 2B). The subnucleus caudalis (or nucleus trigeminalis caudalis, NTC) represents the major nociceptive input for the facial area. The NTC is directly adjacent to the cervical spinal cord, and its organization is comparable to the spinal dorsal horn. Projection neurons from the NTC project to the thalamus and parabrachial nucleus via the posterolateral or anterolateral pathways, similar to the dorsal horn. They also project to the periaqueductal grey (PAG), dorsal reticular nucleus, the amygdaloid complex, the septal nuclei, and the hypothalamus.

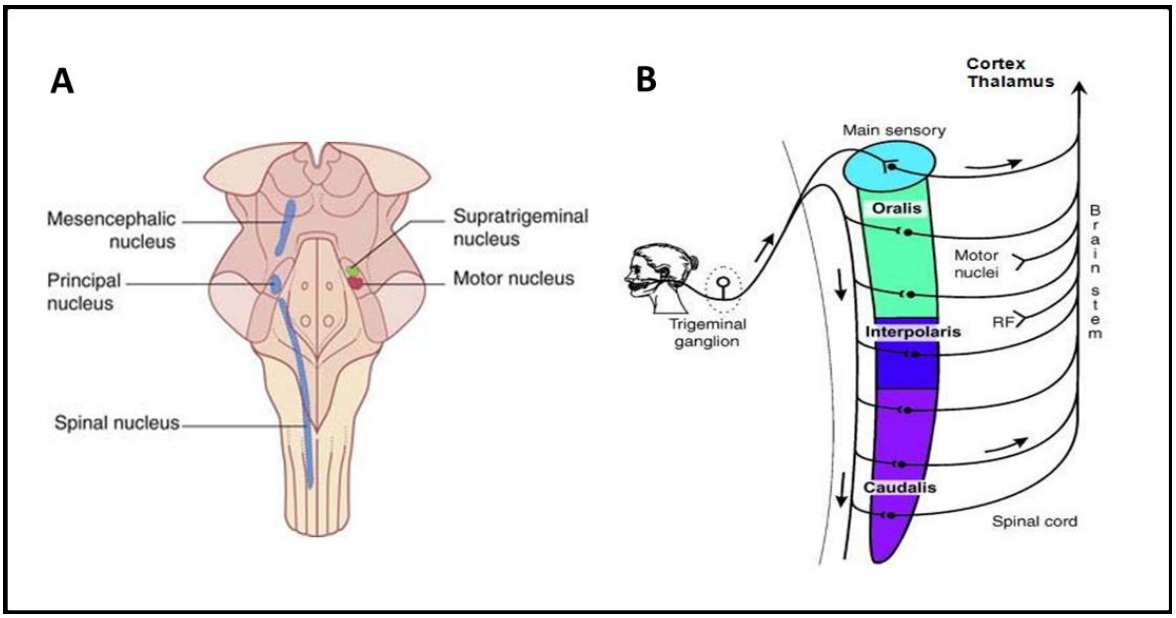

Figure 2. A) The motor and sensory nuclei of the trigeminal nerve (Love and Coakham, 2001); B) The subnuclei of the spinal nucleus: pars oralis, interpolaris and caudalis (adapted from Sessle, 2011).

The trigeminal system displays unique features, indeed the cranial area is made of many particular tissues such as the meninges, cornea, nasal sinuses, oral mucosa and tooth pulp, that require specialized sensory innervations. The orofacial region is one of the most densely innervated areas of the body, with about 50% of the sensory cerebral cortex area dedicated to the processing of orofacial sensation. Therefore, it is clear that the impact of TN injury may be significantly larger than in other areas of the body. Damage to the TN can result in numbness (anaesthesia), tingling altered sensation (paraesthesia), pain or a combination of the three in 50–70% of cases (Renton and Yilmaz, 2011). Pain conditions arising from the structures innervated by the trigeminal system are highly debilitating disorders that impact dramatically the quality of life. Among these pain conditions can be count: the temporomandibular disorders, which include disorders of the temporomandibular joint and disorders of the musculoskeletal structures; neuropathic pains, including episodic (eg, trigeminal neuralgia) and continuous (eg, peripheral/centralized mediated) pains and neurovascular disorders (eg, migraine).

1.3. Migraine

Migraine is a common neurovascular disease, due to environmental factors and genetic predisposition. It is classified as a primary headache disorder (International Classification of Headache Disorders, ICHD 3rd edition-beta version) and it was ranked as the third cause of disability in under 50s by the Global Burden of Disease (GBD) (Steiner et al., 2016). Around 16% of the world population is affected by migraine (GBD 2015), and the prevalence in women is 3-fold higher than men.

Migraine attacks are characterized by mostly unilateral throbbing or pulsating head pain and associated autonomic symptoms such as nausea/vomiting, hypersensitivity to light, sound and smell, and a variety of cognitive, emotional and motor disturbances (Olesen, 2008); often the migraine headache may be exacerbated by movement of the head or by physical activity. The initiation of a migraine attack is frequently associated with a wide variety of internal and external triggers such as stress, hormonal fluctuations, sleep disturbances, skipping meals or sensory overload (Kelman, 2007), and it can last from 4 to 72 hours (Goadsby, 2009).

In about one third of migraine sufferers, the attack is preceded by neurological symptoms collectively known as aura. Generally, it develops and lasts within 20-30 minutes (or even up to 1 hour) and can include visual disturbances with expanding regions of scintillations or light accompanied by some vision loss termed scotomas. However, aura may also present with somatosensory, motor, or language disturbances. These neurological symptoms are associated with a transient cortical malfunction that arises from the phenomenon of cortical spreading depression, which occurs spontaneously in the human cortex before the onset of the headache (Bowyer et al., 2001; Hadjikhani et al., 2001).

Mild to moderate migraine pain may respond to simple analgesics, such as non-steroidal anti-inflammatory drugs (NSAIDs) (Hoffmann and Goadsby, 2014; Belvis et al., 2014), while attacks with moderate or severe pain often require triptans, which account for up to 80% of medications prescribed for migraine (Smitherman et al., 2013; Reddy, 2013). This class of drugs acts as agonists of the 5-HT1B and 5-HT1D serotoninergic receptors, promoting vasoconstriction; for this reason, triptans are contraindicated in patients with risk factors for cardiovascular disease or with hypertension (Talabi et al., 2013). Ergotamine and dihydroergotamine are part of an old generation class of drugs still prescribed for migraines that act like triptans on 5-HT1B/D receptors.

An important clinical concern is that the overuse of these drugs (in particular triptans) can lead to medication overuse headache (ICHD 3rd edition-beta version) (Tepper and Tepper, 2010). When the rate of occurrences of migraine episodes is 15 or more days per month over a period of 3 or more months, then migraine is defined "chronic" (ICHD 3rd edition-beta version). It has been estimated that approximately 14% of patients with episodic migraine will develop chronic migraine (Diener et al., 2012). Some of the possible risk factors for the progression to chronic migraine include female sex, age, head injury, stressful life events, sleep disturbances, obesity, depression and increased caffeine consumption (Diener et al., 2012; Ashina et al., 2010). To manage chronic migraine prophylactic therapies are needed. The most commonly prescribed drugs for migraine prevention are beta-blockers, antidepressants and antiepileptic drugs (such as valproate and topiramate) (Goadsby and Sprenger, 2010); onabotulinumtoxinA has been shown to be effective in migraine prophylaxis as well (Diener et al., 2012; Tassorelli et al., 2017).

Due to the great complexity of migraine disorder, its management is frequently problematic and most abortive and preventive treatments employed give unsatisfactory results in terms of efficacy, safety and tolerability. Current migraine treatment offers pain relief only for a small proportion of migraine patients and might not be tolerable for a relevant portion of patients due to side effects. Thus, research is ongoing to address this unmet need for better prevention with fewer side effects. Advances in migraine pathophysiology have led to new approaches such as neuromodulation and new pharmacologic targets (Tso and Goadsby, 2014). Indeed, in recent years, new compounds targeting either the CGRP peptide or its receptor have been tested with small molecule antagonists or monoclonal antibodies. These drugs proved to be effective in acute migraine treatment in several trials, but were discontinued due to liver toxicity in long-term administration. In particular, several antagonists of the CGRP receptor including olcegepant (BIBN 4096 BS), telcagepant (MK-0974), MK-3207, BI 44370 TA, and BMS-927711 resulted in significantly higher pain-free rates at 2 hours when compared to placebo (Tso and Goadsby, 2017). Unfortunately, development was complicated by pharmacokinetic issues and, in the case of telcagepant and MK-3207, by hepatotoxicity. Monoclonal antibodies against CGRP or its receptor were subsequently developed, and numerous phase 1 and 2 trials and preliminary results of phase 3 trials have been done, showing a good safety/tolerability profile and efficacy in migraine prevention, especially in

high frequent episodic and chronic forms (Giamberardino et al., 2016). Four monoclonal antibodies are currently in development for migraine prevention: galcanezumab (LY2951742), eptinezumab (ALD403), and fremanezumab (TEV-48215) against CGRP; erenumab (AMG-334) against the CGRP receptor.

1.3.1. The trigeminovascular system

Migraine was initially thought to be primarily of vascular origin, since antimigraine therapies had potent vasoconstrictor properties. However, functional imaging studies have shown that migraine headache can occur in the absence of vasodilation, and appears driven from sites within the brain (Schoonman et al., 2008). Although the events that initiate a migraine attack remain unknown, to date, the main mechanism that underlies migraine attacks is thought to be the activation of the trigeminovascular system (Noseda and Burstein, 2013) (Figure 3).

This mechanism provokes the release of multiple excitatory neurotransmitters including CGRP, SP and neurokinin A, from dural afferent terminals, resulting in neurogenic vasodilation of dural blood vessels, release of proinflammatory mediators, degranulation of mast cells and plasma protein extravasation (Goadsby et al., 2009); altogether these neurovascular reactions are known as neurogenic inflammation, a sterile inflammatory reaction brought about by the release of these neuropeptides as a consequence of antidromic or orthodromic stimulation of nociceptive afferent nerves. Neurogenic plasma extravasation is mediated by SP and its role in the pathophysiology of migraine is controversial, although an increase in meningeal blood vessel permeability during migraine aura in humans was demonstrated (Imamura et al., 2008). In contrast the neurogenic vasodilation, mainly mediated by CGRP, seems to play a pivotal role in the pathomechanism of migraine. CGRP has been shown to be released into the jugular blood in humans during migraine headache (Goadsby et al., 1990).

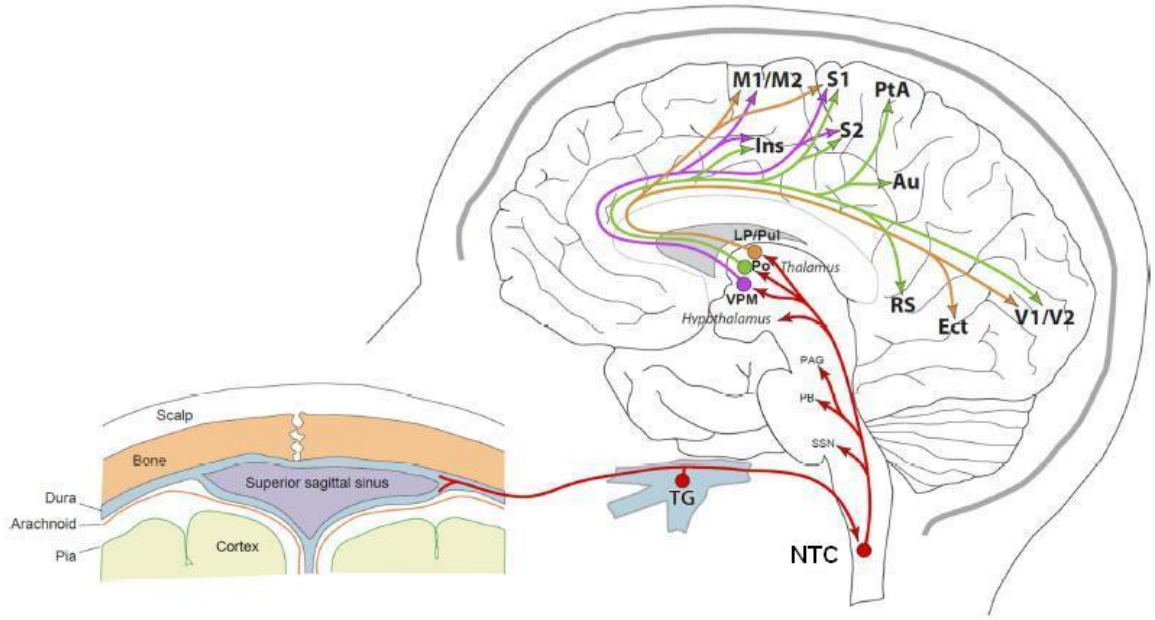

Figure 3. The trigeminovascular system: dural afferent terminals, coming from the trigeminal ganglion (TG), transport nociceptive information to the nucleus trigeminalis caudalis (NTC), where second-order neurons send their projections towards superior structures (thalamus and cortex) (Noseda and Burstein, 2013).

Activation and sensitization of trigeminal nociceptive fibers, contribute to sensitization of the second-order neurons of the NTC resulting in enhanced nociceptive inputs to higher brain centers (thalamus, hypothalamus and cortex) (Figure 3), collectively manifesting as migraine pain (Noseda and Burstein, 2013). The ascending afferents from NTC relay to various brainstem cell stations including PAG, locus coeruleus (LC) and nucleus raphe magnus/rostral ventral medulla. These areas, together with fibers descending from posterior hypothalamus, are involved in descending pain regulation and modulation of NTC activity. Stimulation of PAG, by deep brain stimulation, leads to generation of headache (Raskin et al., 1987) and lesions at or near the PAG may cause migraine-like headache (Afridi and Goadsby, 2003; Goadsby, 2002). Despite these observations, several neuroimaging studies reporting brainstem activation in migraine patients do not include the PAG as an activated region during spontaneous or induced attacks. They do show however, activation in nearby nuclei in the dorsolateral pons that includes the mesencephalic and principal sensory trigeminal nucleus, parabrachial area (PB), vestibular nucleus, inferior colliculus, LC and cuneiform nucleus (Weiller et al., 1995; Bahra et al., 2001; Afridi et al., 2005; Moulton et al., 2008; Stankewitz et al., 2011). It is more likely

that this complex pattern of activation reflects modulation of migraine pain and the associated symptoms (like facial and muscle tenderness, abnormal tactile sensation, nausea, altered auditory perception) rather than to be central migraine generators (Noseda and Burstein, 2013; Schulte and May, 2017).

Several lines of evidence suggest the existence of a condition of trigeminal sensitization in migraineurs, which results in hyperalgesia, allodynia, and cognitive dysfunction during and between episodes (Noseda and Burstein 2013). Thus, migraine patients can be affected by cephalic and extracephalic allodynia, due to the convergence of sensory inputs on sensitized central neuron of the trigeminovascular system (Burnstein et al., 2000, 2010). Moreover, the central sensitization combined with a dysfunction in the descending inhibitory pathway in pain control, could induce hyperalgesia (Fernández-de-las-Peñas et al., 2010; Geppetti et al., 2012). In keeping with this hypothesis, spatial changes in pressure pain hypersensitivity related to central sensitization of spinal tract neurons, have been described in unilateral migraine (Fernández-de-las-Peñas et al., 2010).

1.3.2. Systemic nitroglycerin as animal model of migraine

A central role in the activation of the trigeminovascular system seems to be played by nitric oxide (NO), a potent vasodilator, that activates the perivascular afferent fibers localized at meningeal level, thus contributing to vasoactive neuropeptides release and further vasodilation (Messlinger et al., 2012) (Figure 4). These perivascular trigeminal fibers are immunoreactive for the NO synthase (NOS) (Bredt et al., 1990; Berger et al., 1994; Knyihàr-Csillik and Vécsei 1999), the enzyme catalysing the production of NO.

A relationship between NO and migraine has been proposed since headaches occur as a side-effect of NO donors (Buzzi and Tassorelli, 2010). Nitrovasodilators were originally used in the treatment of ischemic cardiac disease for their vasodilatory effect to produce NO in several body tissues (including the brain). Among the various nitrovasodilators commercially available, nitroglycerin (NTG) is a classic NO donor, that leads to a migraine attack in migraineurs with a latency of 4 to 6 hours (Sances et al., 2004) and causes the facilitation of pain transmission at the spinal level (Perrotta et al., 2011). Given these observations, NTG administration is used as a model in the diagnosis of migraine without aura. Experimental evidences have demonstrated an accumulation of NTG in the

rat brain tissue, since this NO donor is highly lypophilic and easily crosses the blood–brain barrier (Torfgård et al., 1991). Systemic administration of NTG induces neuronal activation in rats by c-Fos protein expression in several brain nuclei belonging to the neurovegetative, neuroendocrine, behavioral and nociceptive systems (as NTC) (Tassorelli and Joseph, 1995; Tassorelli et al., 1997; Greco et al., 2010, 2011). The precise mechanisms involved in NO-triggered migraine and which part of the NO-activated cascade that is involved remain to be determined.

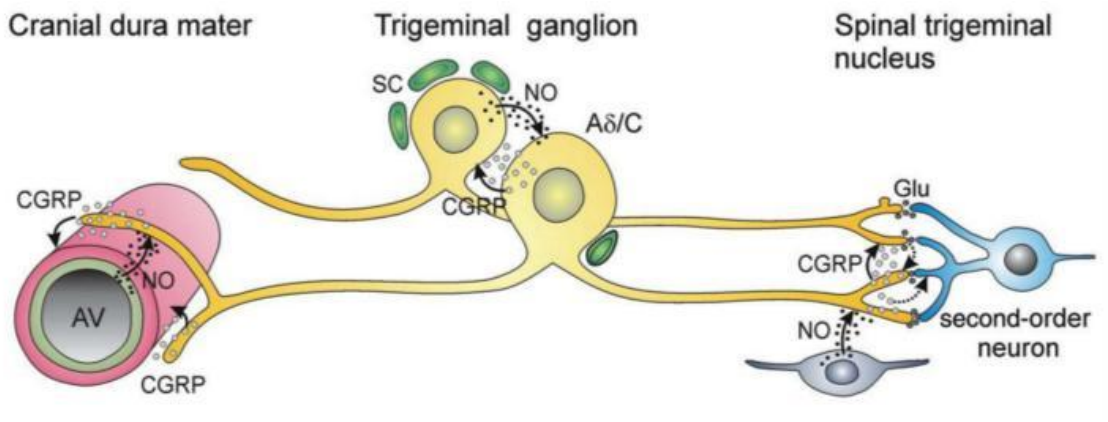

Figure 4. Schematic representation of nitric oxide (NO) activity in the trigeminovascular system. At dural level, neuropeptides release (such as CGRP) from perivascular afferents causes dilation of arterial vessels (AV). NO species, probably mainly derived from vascular endothelium, cause vasorelaxation by a direct action on vascular smooth muscle cells but also by facilitating CGRP release. In the trigeminal ganglion, CGRP may signal to other trigeminal ganglion neurons (Aδ/C) that express CGRP receptors, while NO from neurons and, possibly, satellite glial cells (SC) may signal to other neurons. In the trigeminal nucleus, CGRP facilitates nociceptive transmission to second-order neurons possibly by increasing neurotransmitter (eg, glutamate, Glu) release from adjacent primary afferent terminals, which could also act on presynaptic Glu receptors; alternatively, CGRP could directly signal to second-order neurons. NO-releasing neurons of various types (gray) are assumed to facilitate neurotransmitter and CGRP release (Messlinger et al., 2012).

The temporal profile of neuronal activation following NTG shows that neuronal activation begins as early as 60 minutes post-injection in brain areas that control the cardiovascular function, and c-Fos reaches its maximum expression 4 hours later in nociceptive and integrative structures (Tassorelli et al., 1997). In addition, this modulated temporal course suggested a dual mechanism of action for NTG on the brain; an initial effect on the

vascular compartment followed by the involvement of integrative–nociceptive structures. This activation, which in some areas develops with a latency of hours, contrasts with the very short plasma half-life of the drug, but this may be probably a consequence of NTG accumulation at cerebral level (Torfgård et al., 1991). NTG injection induces an up-regulation of pro-inflammatory genes, with a subsequent delayed inflammatory reaction in the dura mater and central areas of the rat (Reuter et al., 2002; Greco et al., 2016). In particular, intravenous NTG increases NO production within macrophages of dura mater with a delay of hours, via the expression of the inducible isoform of NO synthase (iNOS) (Reuter et al., 2001, 2002). iNOS expression is preceded by significant increase of nuclear factor kappa B (NF-kB) activity (Reuter et al., 2002).

Co-localization studies have shown that NTG-induced neuronal activation takes place in adrenergic, nitrergic and neuropeptidergic structures (Tassorelli et al., 1999), suggesting some of the possible signaling pathways involved in the phenomenon. Neuropharmacological manipulations have suggested that NTG-induced neuronal activation involves probably exogenous (NTG-derived) NO that might directly act at both the vascular and neuronal levels or indirectly activate neurovascular responses via multiple pathways (Tassorelli et al., 2007; Greco et al., 2005, 2017a,b), explaining the hyperalgesic effect of NTG.

1.4. Neuropathic pain

The IASP defines neuropathic pain as "pain caused by a lesion or disease of the somatosensory system". It is a common cause of chronic pain, affecting about 1 in every 10 adults (Yawn et al., 2009) and it negatively impacts the patient's well-being (Hall et al., 2006).

The most common causes of neuropathic pain are diabetic neuropathy, post herpetic neuralgia, trigeminal neuralgia and spinal cord injury, but stroke, multiple sclerosis, cancer and several other conditions may also result in neuropathic pain. Independently of the etiology, the main common feature is an abnormal pain sensation with sensory disturbances without any nociceptive stimuli. Within neuropathic pain conditions may occur: spontaneous and evoked types of pain, paresthesia, dysesthesia, allodynia, hyperpathia and hyperalgesia.

The occurrence of these symptoms is partly due to development of "ectopic discharge", in which there is a large increase in the level of spontaneous firing in the afferent neurons, linked to the injury site (Wall and Gutnick, 1974). Ectopic discharge may be caused by cross-excitation between A- and C-fibers (Amir and Devor, 2000), and can arise in the neuroma, from the dorsal root ganglion or other points along the nerve (Wall and Gutnick, 1974; Wall and Dovor, 1983). The ongoing pain that develops after nerve injury can originate from alterations in membrane stability, characterized by changes in the distribution, expression and biophysical properties of ion channels; indeed, it has been demonstrated that following peripheral nerve injury there is a considerable re-organization of the neuronal afferents at spinal level and changes of expression and nature of various channels, such as voltage-gated Na+ and N-type Ca2+ channels (Matzner and Devor, 1994; Waxman et al., 1994; McCallum et al., 2011). Moreover, the source of ongoing pain may arise from changes in transduction in which can occur: de novo expression of transducers, decreases in inhibitory transducers and/or increases in excitatory transducers, changes in the expression or release of endogenous ligands, emergence of aberrant sources of nociceptor activation (Gold and Gebhart, 2010).

To date, a pivotal role of inflammatory pathways and glial cells activation, in the pain-related mechanisms underlying neuropathic pain, is well established (Thacker et al., 2007; Mika et al., 2013). Peripheral nerve damage is followed by microglial activation in dorsal horns 24 h after the damage, lasting not longer than 3 months; while astrocyte activation was demonstrated to occur on day 3 after the injury. This glial activation is associated with the onset of neuropathic pain symptoms such as allodynia or hyperalgesia (Coyle, 1998; Ledeboer et al., 2005; Zhuang et al., 2005). The recruitment and infiltration of neutrophils and macrophages, followed by macrophage activation, glial cell activation, and cytokine/chemokines release lead to microenvironmental changes that subsequently develop in central sensitization (Liou et al., 2013; Mika et al., 2013). In particular, proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin-1b, and interleukin-6 (IL-6) (Shamash et al., 2002), and chemokines/chemokines receptors have been demonstrated to be involved in acute and chronic hyperalgesia and in allodynia (Watkins et al., 1994; Abbadie et al., 2003; Rittner et al., 2006).

Thus, in neuropathic pain there is an increased activity and decreased inhibition in the somatosensory system. Pharmacotherapy is based on modulation of these phenomena by

decreasing the spontaneous activity and transmitter release, and enhancing the inhibitory mechanisms. The pharmacological therapies for neuropathic pain treatment include: antidepressants (e.g., tricyclic antidepressants, serotonin-norepinephrine reuptake inhibitors), anticonvulsants (e.g., gabapentin pregabalin, carbamazepine and N-methyl-D-aspartate (NMDA) receptor antagonists), opioids, and topical agents (Attal et al., 2010). However, these drugs are not completely effective in attenuating neuropathic pain, because of the complexity of this type of pain, and also have side effects, such as sedation, dizziness, edema, and ataxia (Jensen et al., 2009).

1.4.1. Trigeminal neuropathic pain

Trigeminal neuropathic pain is a disabling pain condition affecting a considerable proportion of the general population (Zakrzewska and Linskey, 2014). Although clinical studies indicate that neuropathic pain is more frequently observed in the trigeminal system than at the spinal level (Sweitzer, 1999), in the last decade most experimental studies were carried on models of spinal nerve injury, probably because of the higher complexity of the trigeminal system. Nevertheless, a number of trigeminal neuropathic pain models have been developed, such as chronic constriction injury (CCI) of the infraorbital (IoN) sensory nerve, that arises from the maxillary division of the TN (IoN-CCI model) (Vos et al., 1994), transection of the inferior alveolar sensory nerve that arises from the mandibular division of the TN (Iwata et al., 2001). Such injuries result in evoked and non-evoked pain-related behaviors in response to stimulation of, or directed at, the vibrissal skin pad, respectively. Another model is represented by the cervical spinal nerve transection model (at C2-C4 level) responsible of extraterritorial facial pain (Kobayashi et al., 2011).

Trigeminal neuropathic pain disorders are consequences of similar etiological factors that are commonly studied in the spinal system, however trigeminal injuries show some differences when compared with those at the spinal level; for instance, a less spontaneous, ectopic discharge of injured primary afferents (Tal and Devor, 1992) has been reported. Moreover, differently from spinal injury (Chung et al., 1996), an absence of sympathetic fiber sprouting into the ganglion was reported (Benoliel et al., 2001), but sprouting of sympathetic fiber does occur in the upper dermis, after bilateral CCI of the mental nerve (innervating the rat lower lip) (Grelik et al., 2005).

Differences in response to nerve injury may be attributed to the particular anatomo-physiological features of the trigeminal system. On the other hand, the trigeminal system and relevant extraterritorial areas may employ mechanisms similar to those observed for central sensitization of the spinal system, including the spatial-temporal activation of glial cells within the medullary dorsal horn. Indeed, an increased activation of microglia and astrocytes after trigeminal injury has been reported by some Authors. In particular, microglia is activated and returns to the basal levels earlier than astrocytes (Kobayashi et al., 2011; Piao et al., 2006; Okada-Ogawa et al., 2009; Xu et al., 2008).

It should be noted, however, that the different animal models of neuropathic pain appear to have distinct features, in terms of onset, severity, duration, and/or a contralateral component (unilateral vs. bilateral). These features are likely related to the type (partial vs. transection vs. non-traumatic) and location of the injury (peripheral vs. central) (Stemkowski and Smith, 2013).

1.4.2. The IoN-CCI model

The unilateral CCI of the rat's IoN described by Vos has been demonstrated to increase nocifensive behaviors in response to mechanical stimulation of the IoN territory and, to develop spontaneous (asymmetric) face grooming directed to the territory of the injured IoN (Vos et al., 1994, 1998; Deseure et al., 2003; Deseure and Hans, 2015b). Depending on the type of IoN ligation (loose, tight or partial tight) the time course of isolated face grooming behavior is different (Deseure and Hans, 2015a), however it seems likely to resemble the spontaneous pain observed in patients.

The behavioral manifestations of increased nociceptive activity, induced by IoN-CCI, are linked to a significant increase in c-Fos protein expression at the medullary dorsal horn ipsilateral to the injury (Vos and Strassman, 1995), reflecting a central activation.

1.5. TRP channels

Transient receptor potential (TRP) channels are a superfamily of non-selective cation channels that currently includes more than 50 members (Vriens et al., 2004). They were firstly discovered in *Drosophila melanogaster*, in which vision is produced by a

mechanism that consists in an initial activation of a transient inward current associated with receptor stimulation (Minke, 1977; Montell, 1999).

TRP proteins consist of six-transmembrane domains (S1-S6) (Figure 5) with both the amino (NH2) and carboxylic acid (COOH) termini localized into the cytosol. The COOH region is highly conserved among TRPs, whereas the NH2 region usually contains different ankyrin repeats, which consist of 33-residue motifs with a conserved backbone and variable residues that mediate specific protein–protein interactions (Sedgwick and Smerdon, 1999). The S1–S6 domains assemble as homo- or hetero-tetramers, with the pore domain formed by loops between S5 and S6 (Figure 5), which permit a non-selective influx of cations.

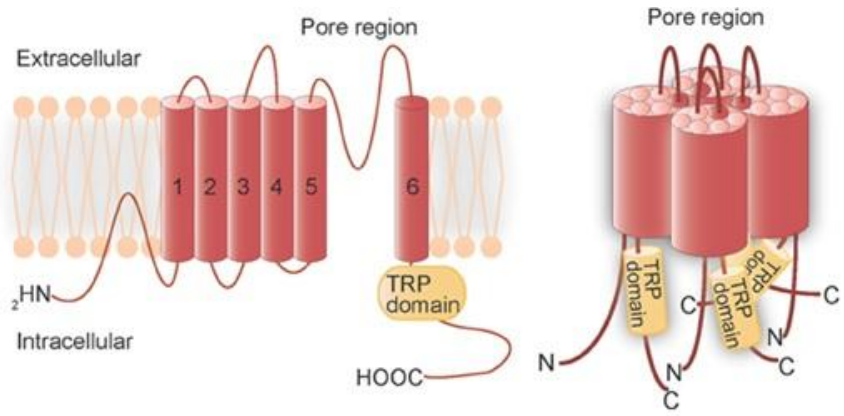

Figure 5. Schematic representation of TRP protein structure (Bessac and Jordt, 2008).

In mammalian systems, TRP family consists of 28 proteins grouped into six subfamilies according to sequence identity: TRP canonical (TRPC), TRP melastatin (TRPM), TRP polycystin (TRPP), TRP mucolipin (TRPML), TRP vanilloid (TRPV) and TRP ankyrin (TRPA) channels (Montell et al., 2002; Clapham, 2003, 2005) (Figure 6).

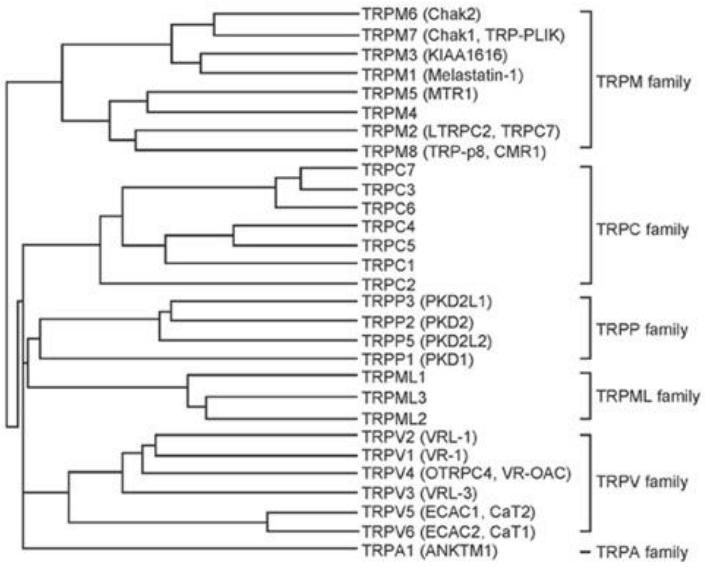

Figure 6. Phylogenetic tree of mammalian TRP channels (Bessac and Jordt, 2008).

The TRPC subfamily comprises seven members (TRPC1–7), whose activation depends on the stimulation of G protein–coupled receptors (GPCRs) and receptor tyrosine kinases (Montell, 1999), although TRPC1 channels seem to be directly activated by membrane stretch (Maroto et al., 2005). The TRPM includes eight different members (TRPM1–8), which differently from TRPA, TRPC and TRPV, do not contain ankyrin repeats within their NH2-terminal domain. Menthol and moderately low temperatures (<25°C) activate the TRPM8 channel. The TRPP is a heterogeneous family, consisting of three members according to structure, and can be divided into PKD1-like (TRPP1-like) and PKD2-like (TRPP2-like) proteins (Hanaoka et al., 2000). Like TRPM channels, TRPP do not contain ankyrin repeats within their NH2-terminal domain. The same is three members (TRPML1–3) of the TRPML family. TRPML1 is widely expressed and appears to reside in late endosomes/lysosomes where it seems to act as a H+-sensitive channel to prevent overacidification (Soyombo et al., 2006). The TRPV subfamily has six members (TRPV1-6), that can be further divided into two groups, TRPV1-4 and TRPV5/TRPV6. TRPV1-4 channels are weakly Ca2+-selective cation channels and are all heat activated; whereas, TRPV5/TRPV6 channels are highly Ca2+ selective but not heat activated (Hellwig et al., 2005; Clapham et al., 2003, 2005). The only member of the TRPA subfamily, TRPA1 (originally designated as ANKTM1), got its name from its 17 predicted ankyrin repeats in the NH2-terminal domain.

Moving away from sequences similarities, TRP channels can be classified on common functional features. For example, TRPA1, TRPM8, TRPV1, TRPV2, TRPV3 and TRPV4 have been collectively labelled as thermo-TRP because they can be activated by a large range of temperatures from noxious cold to noxious heat (Vay et al., 2012). Looking at this kind of classification, it appears clear that members of different subfamilies share similar features. In this sense, it is possible to identify the mechanosensitive TRP channels: TRPA1, TRPC1, TRPC5, TRPC6, TRPM3, TRPM7, TRPP2, TRPV1, TRPV2 and TRPV4. Among those activated by chemical stimuli there are TRPA1, TRPM8 and TRPV1. In addition, it must be noted that, while subunits of the same subfamily do not always coassemble into heteromeric channels, subunits of different subfamilies are found to interact and possibly form heteromeric channel complexes. Examples of cross-subfamily coassembly are between TRPC1/TRPP2 (Bai et al., 2008), TRPV4/TRPP2 (Köttgen et al., 2008), TRPA1/TRPV1 (Salas et al., 2009), and TRPC1/TRPC6/TRPV4 (Alessandri-Haber et al., 2009).

Frequently TRP channels show high Ca2+ permeability (Latorre et al., 2009) however, the TRPs Ca2+/Na+ permeability ratio differs markedly between different members of the superfamily and also among members of each subfamily (Nilius et al., 2007). Thanks to this property, TRP channels play a critical role in many cellular processes via changing cytosolic free Ca2+ concentrations, by acting as Ca2+-permeable channels in the plasma membrane or via modulating the driving force for Ca2+ influx by changing the membrane potential (Nilius et al., 2007). This event is important because among the several cellular processes triggered by free Ca2+ in the cytosol there are gene transcription and release of transmitters (Berridge et al., 2000).

TRP channel gating may depend on direct activation of the channels by a plethora of physicochemical stimuli, including compounds of exogenous origin or endogenous signalling molecules (Nilius et al., 2007). TRP gating may also result from changes in the intracellular machinery as in the case of stimulation of the different isoforms of phospholipase C (PLC; Hardie and Minke, 1992; Niemeyer et al., 1996), following activation of GPCRs or tyrosine kinase receptors (Spehr et al., 2011).

Despite the wide heterogeneity of this family of ion channels, TRPs share a general role serving sensory transduction, because they contribute to pain, vision, taste, olfaction, hearing, touch, and thermo- and osmosensation, making cells able to sense and respond to

environmental changes. This is also possible because of their wide distribution in many tissues and cell types. Part of them appears to be universally expressed, whereas others exhibit more restricted expression patterns. Besides the plasma membrane, TRP channels are also found in intracellular membranes (Dong et al., 2010).

1.5.1. TRPA1 and TRPV1 channels

As already mentioned, TRPA1 and TRPV1 are classified as thermo-TRP and they are sensor of mechanical and chemical stimuli as well. Nevertheless, it should be noted that the ability of TRPA1 channels to function as detectors of mechanical stimuli and noxious cold (<17°C; Story et al., 2003) is still controversial (Jordt et al., 2004; Latorre, 2009). In particular, it was suggested that TRPA1 in the mechanosensation is mostly related to mechanical hypersensitivity of afferents after inflammation (Zygmunt and Hogestatt, 2014).

TRPA1 and TRPV1 activation

A key role played by TRPA1 channels is the detection of pungent or irritant principles, including compounds contained in various spicy foods, such as allyl isothiocyanate (AITC, or mustard oil) contained in horseradish (Jordt et al., 2004), allicin and diallyldisulfide contained in garlic (Bautista et al., 2005), cinnamaldehyde contained in cinnamon (Bandell et al., 2004), and capsiate (Shintaku et al., 2012). Gingerol (in ginger), eugenol (in cloves), methyl salicylate (in wintergreen), carvacrol (in oregano), and thymol (in thyme and oregano; Bandell et al., 2004; Xu et al., 2006; Lee et al., 2008) are also able to gate TRPA1. Additionally, have been recognized as TRPA1 activators some environmental irritants and industry pollutants, such as acetaldehyde, formaldehyde, hydrogen peroxide, hypochlorite, isocyanates, ozone, carbon dioxide, ultraviolet light and acrolein, a highly reactive α,β-unsatured aldehyde present in tear gas, cigarette smoke, smoke from burning vegetation and vehicle exhaust, and hydrogen sulfide (H2S) (Bautista et al., 2006; Bang et al., 2007; McNamara et al., 2007; Andersson et al., 2008; Bessac et al., 2008, 2009; Sawada et al., 2008; Hill and Schaefer, 2009; Taylor-Clark and Undem, 2010; Wang et al., 2010; Miyamoto et al., 2011). Recently, it has been reported the ability of cannabichromene, a non-psychotropic cannabis-derived cannabinoid with anti-inflammatory (Romano et al., 2013) and analgesic properties (Maione et al., 2011) to

activate TRPA1 channels (De Petrocellis et al., 2011). Among the endogenous agonists for TRPA1 channels can be counted: oxidative stress products (e.g. 4-hydroxynonenal; Trevisani et al., 2007) and reactive oxygen species, like hydrogen peroxide and hydroxyl radical (Bessac et al., 2008); lipid peroxidation products (Taylor-Clark et al., 2008); zinc, copper and cadmium (Gu and Lin, 2010).

As regards TRPV1 channels, they were originally identified as receptors for the vanilloid compound capsaicin, the pungent component of chilli peppers (Caterina et al 1997). These channels are also activated by heat >43°C, low pH (< 5.6) (Tominaga et al., 1998) and many other exogenous and endogenous molecules. Among the exogenous agonist, besides capsaicin, can be counted: other vanilloids (e.g. olvanil, resiniferatoxin) (Brand et al., 1987; Szallasi and Blumberg, 1989); capsinoids (e.g. capsiate) (Ohnuki et al., 2001); camphor (Xu et al., 2005); and ethanol (Nicoletti et al., 2008). The endocannabinod anandamide (Zygmunt et al., 1999; Smart et al., 2000), the lipoxygenase products (Hwang et al., 2000; Huang et al., 2002) and N-acyldopamines (Huang et al., 2002) represent the endogenous molecules able to activate TRPV1 channels.

Both TRPV1 and TRPA1 channels are modulated by components of the inflammatory environment and are upregulated during pain and inflammation (Amaya et al., 2003). Indeed, TRPV1 is sensitized following activation of neuronal receptors of proalgesic agents such as bradykinin, nerve growth factor, prostaglandins, histamine and chemokines, (Chuang et al., 2001; Montel, 2005; Shim et al., 2007); likewise, TRPA1 activity can be induced by bradykinin or histamine (Bandell et al., 2004; Bautista et al., 2006; Wang et al., 2008). These proinflammatory mediators promote the activation of PLC and protein kinase A pathways, whose mechanisms contribute to the activation of these channels (Bandell et al., 2004; Bautista et al., 2006; Wang et al., 2008) (Figure 7). Phosphorylation through protein kinase C and other kinases also affects TRP channel activity during inflammation. Heightened Ca2+ levels trigger release of proinflammatory neuropeptides such as SP or CGRP (Figure 7).

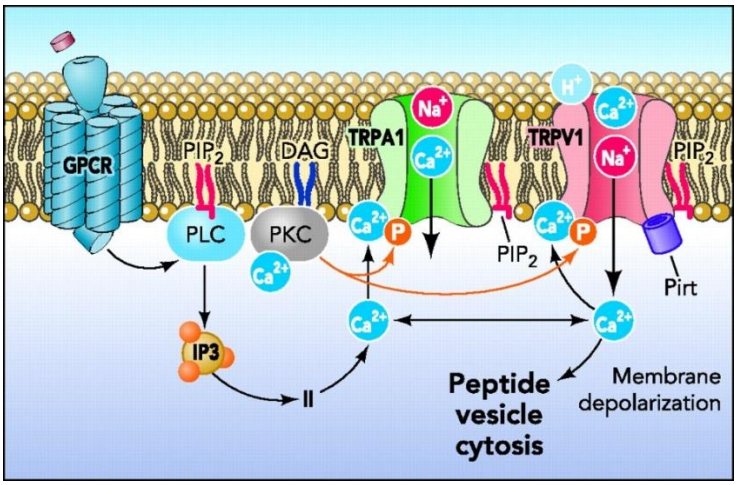

Figure 7. Activation and sensitization of TRPA1 and TRPV1 channels by inflammatory signalling pathways. Activation of proinflammatory agents' receptors (GPCR) lead to the activation of multiple pathways, such as phospholipase C (PLC) and protein kinase C (PKC), that are able to activate and sensitize TRPA1 and TRPV1 channels. The heightened intracellular Ca2+ levels trigger the release of neuropeptides. (Bessac and Jordt, 2008)

TRPA1 and TRPV1 localization

Besides the similarities in functional properties, TRPA1 and TRPV1 seem likely to interact with each other via signaling pathways (Akopian, 2011) and even with physical interaction via the formation of heteromers (Fischer et al., 2014, Salas et al., 2009, Staruschenko et al., 2010). This is also possible since these two channels are often co-expressed (Story et al., 2003). In particular, they are localized preferentially in primary sensory neurons; they can be found both on the peripheral, where they are activated by noxious stimuli, and central terminals, where their activation can lead to the release of transmitters that promote the sensitization of postsynaptic pain transmission pathways (Yeo et al., 2010; Chun et al., 2014; Kim et al., 2014; Koivisto et al., 2014). 97% of TRPA1-expressing sensory neurons express TRPV1, while 30% of TRPV1-expressing neurons express TRPA1 (Story et al., 2003). Importantly, TRPA1 and TRPV1 are often co-expressed with CGRP and SP, whose release can be promoted by TRP channels activation (Story et al., 2003; Nicoletti et al., 2008; Kunkler et al., 2011; Julius, 2013) (Figure 8).

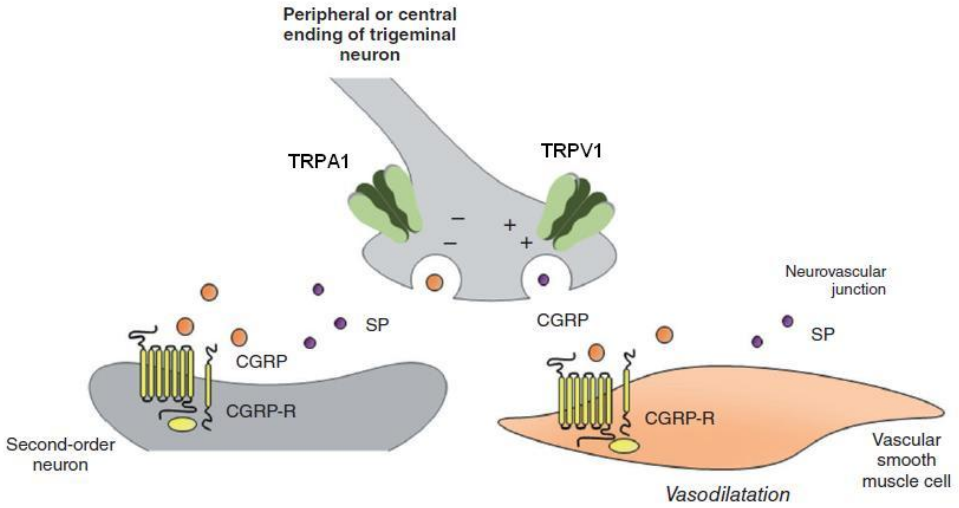

Figure 8. Following the activation of TRPA1 and TRPV1 located at peripheral or central terminals, Ca2+ entry promotes neuron depolarization with the consequent release of SP and CGRP. (Benemei et al., 2014)

Within the nervous system TRPA1 and TRPV1 are widely expressed in the brain (Doihara et al., 2009; Mezey et al., 2000), cerebellum (Doihara et al., 2009; Sasamura et al., 1998), post-ganglionic sympathetic neurons and motor neurons (Anand et al., 2008; Poole et al., 2011).

They can be found also in astrocytes (Shigetomi et al., 2012; Mannari et al., 2013), microglia (Kim et al., 2006), ependymal cells (Jo et al., 2013) and vascular endothelium (Earley et al., 2009; Kark et al., 2008). However, their expression is also extended in non-neuronal cells from many different tissues, such as keratinocytes (Atoyan et al., 2009; Inoue et al., 2002), fibroblast (Atoyan et al., 2009), macrophages and monocytes (Kun et al., 2014; Billeter et al., 2014, 2015).

Thus, it is not surprising that their activities cover a wide spectrum of functions, ranging from pain sensation to vasoconstriction and vasodilation of blood vessels and inflammatory-mediated pathways in different tissues (Fernandes et al., 2012).

1.5.2. TRPA1 and TRPV1 channels in pain

The crucial role of TRPA1 and TRPV1 in pain response is well established; indeed they are able to detect noxious stimuli ranging from plant derivatives and environmental irritants to endogenous products of inflammation or oxidative stress. Many experimental researches, from genetic knockouts to pharmacological manipulation models, reported a critical involvement of these channels in different aspects of pain (Jardín et al., 2017). For these reasons, great efforts have been made in order to develop analgesic TRP-based therapies that are able to desensitize (through agonists) or block (with antagonists) these channels, thus preventing nociceptive signaling.

For instance, the desensitization strategy has been developed in numerous TRPV1 therapeutics including capsaicin cream as well as high concentration patches, used as local analgesic treatment. Similarly, some TRPV1 antagonists have advanced to human trials (Gavva et al., 2008; Quiding et al., 2013), however these studies reported some limitations, including hyperthermia and blockade of thermosensation (Szallasi and Sheta, 2012; Rowbotham et al., 2001). Currently, TRPV1 antagonists that do not affect body temperature in humans are under investigation (Chiche et al., 2016).

Differently from TRPV1, the blockade of TRPA1 channels seems to have no influence on body temperature (Chen et al., 2011; de Oliveira et al., 2014). Recently, it was suggested that the activation of TRPA1 channels, by systemic agonists, would be a possible means of reducing inflammation and generating hypoalgesia (Kistner et al., 2016).

TRPA1 and TRPV1 in migraine pain

In recent years, clinical and experimental data have supported the critical role of TRPA1 and TRPV1 channels in the pathophysiology of primary headaches, in particular, migraine. Among the above cited TRP agonists, there are some that are known to be migraine triggers; for examples, acrolein and nicotine, the active compounds of cigarette smoke (Lima et al., 2011), tear gas (Brone et al., 2008), formalin (McNamara et al., 2007) and Umbellularia californica (the “headache tree”) (Nassini et al., 2012) for TRPA1 channels. As regard TRPV1, ethanol contained in alcoholic beverages is known to be a migraine trigger (Kelman, 2007). Application to the rat nasal mucosa of the TRPA1 agonist acrolein, produces a TRPA1-dependent and CGRP-mediated increase in meningeal blood flow (Kunkler et al., 2011). Similarly, capsaicin can dilate dural vessels in a TRPV1-dependent mechanism (Akerman et al., 2003), leading to extracellular signal–regulated kinase

activation in trigeminal ganglion neurons (Iwashita et al., 2013) and behavioral responses consistent with headache (Yan et al., 2011; Bove and Moskowitz 1997). Interestingly, the TRPA1 agonist AITC, injected onto the dura, was used to induce migraine pain in rats, producing a concentration-dependent depression of wheel running (reflecting the duration and magnitude of pain). This response was reversed by sumatriptan treatment (Kandasamy et al., 2017).

Thus, in the trigeminovascular system, TRPA1 and TRPV1 channels activation induce CGRP release from trigeminal neurons and dural tissue, stimulating meningeal vasodilatation (Kunkler et al., 2011; Nassini et al., 2012; Dux et al., 2016; Marics et al., 2017; Akerman et al., 2004; Nicoletti et al., 2008), and neuronal activation in NTC (Jordt et al., 2004).

The desensitization of TRPV1 channels by means of agonists has proved effective in migraine patients, after intranasal administration of TRPV1 agonists (Fusco et al., 2003; Diamond et al., 2000). However, side effects due to TRPV1 activation within the nasal cavity (Diamond et al., 2000), has limited the clinical potential of these agents. Additionally, many TRPV1 antagonists have been tested as well in several preclinical models related to headache, but the results were inconsistent (Akerman et al., 2003; Lambert et al., 2009; Summ et al., 2011). Accordingly, the TRPV1 antagonist SB-705498 in a clinical phase II study showed no significant effect (Palmer et al., 2009), indicating that further studies on TRPV1-based therapies are needed.

Parthenolide, one of the major constituents of feverfew, commonly used for migraine treatment, was identified as a partial TRPA1 agonist (Materazzi et al., 2013), suggesting that one of its anti-migraine effects may be mediated through TRPA1 channels desensitization. Similarly, the anti-migraine action of butterbur, an herbaceous plant currently recommended for migraine prophylaxis (Holland et al., 2012), evokes TRPA1-dependent desensitization of nociceptors that mediate neurogenic inflammation (Benemei et al., 2017). Other herbal constituents used for the treatment of pain and headaches, such as ligustilide, an active ingredient of umbelliferous plants, act on TRPA1 channels (Zhong et al., 2011). Finally, the pyrazolone derivatives, dipyrone and propyphenazone (old analgesics with a well-established anti-migraine effect) (Bigal et al., 2002) selectively antagonize the TRPA1 channels (Nassini et al., 2015).

TRPA1 in neuropathic pain

Numerous experimental evidences, from both pharmacological and genetics studies, pointed out the involvement of TRPA1 in different patterns of neuropathic pain. Indeed, its contribution has been reported in different models of nerve injury, such as the lumbar spinal nerve ligation (Obata et al., 2005) and sciatic nerve injury by chronic constriction or transection (Katsura et al., 2006; Caspani et al., 2009; Staaf et al., 2009). In these models, it was demonstrated an up-regulation of TRPA1 associated with mechanical and thermal hyperalgesia; this condition was reversed by using TRPA1 antagonists (Eid et al., 2008; Chen et al., 2011). Additionally, in a recent study Trevisan et al., (2016) reported that, in the constriction of the infraorbital nerve model of trigeminal neuropathic pain, pain-like behaviors are mediated by the TRPA1 channel, activated by increased oxidative stress by-products released from monocytes and macrophages clumping at the site of nerve injury.

A role of the TRPA1 channel in neuropathic pain has been proposed also in those conditions which do not result from mechanical trauma, such as the pain secondary to diabetic neuropathy and neuropathy associated to chemotherapy (Ta et al., 2010; Wei et al., 2010). In a rodent model of induced diabetes, the blockade of TRPA1 was shown to reduce mechanical hypersensitivity (Wei et al., 2009). It has been hypothesized that the reactive compounds produced in diabetes induce a sustained TRPA1 channel-mediated activation of nociceptive nerve fibers, causing both pain hypersensitivity and the subsequent loss of cutaneous nerve fiber function, responsible for the detrimental long-term effects (Koivisto et al., 2012). Similarly, TRPA1 genetic deletion or pharmacological inhibition reduced/abolished the mechanical and cold hypersensitivities induced in a rodent model of chemotherapeutic-induced peripheral neuropathy (Joseph and Levine 2009; Nassini et al., 2011; Zhou et al., 2016; Tonello et al., 2017). Treatment with chemotherapeutic agents caused an up-regulation of TRPA1 mRNA both *in vitro* and *in vivo* dorsal root ganglion neurons (Ta et al., 2010). The hypothesis is that the oxidative stress by-products, produced by chemotherapeutic agents, activate/sensitize TRPA1 channels, thus inducing a chronic hypersensitivity state (Nassini et al., 2011; Trevisan et al., 2013; Nakagawa and Kaneko, 2017).

Only one TRPA1 antagonist has reported to have gone through human phase II clinical trials, Glenmark's GRC 17536. Though the full results have not been published, Glenmark reported that in a subset of patients with painful diabetic neuropathy, the TRPA1 antagonist significantly reduced pain scores (Moran and Szallasi, 2017).

Interestingly, in a recent case report study, it was suggested that the attacks of trigeminal neuralgia evoked by sour and spicy foods could be related to direct activation of trigeminal C-fibers, probably through TRPA1 channels (Eisenberg, 2016).

2. AIM

Pain conditions arising from the structures innervated by the trigeminal system are highly debilitating disorders that impact dramatically the quality of life. Migraine pain and trigeminal neuropathic pain are very common diseases which have a high socio-economic and personal impact. These neurological disorders present a clinical treatment challenge and often the medications available give unsatisfactory results. For this reason, there is a strong medical need for development of new therapeutics, together with the clear identification of the underlying pathomechanisms.

Experimental and clinical observations pointed out a critical involvement of TRP channels, in particular TRPA1 and TRPV1, in migraine, trigeminal neuropathic pain and associated symptoms, including hyperalgesia and allodynia. However, researches on the importance of TRP channels in the pathophysiology of these pain disorders and in general, in the trigeminal pain, are still inconclusive.

The aim of this study was to investigate the role of TRP channels in trigeminal pain in two animal models of diseases related to the trigeminal system. In particular, TRPA1 and TRPV1 antagonists were used in the NTG-induced hyperalgesia at the trigeminal level induced by means of the orofacial formalin test, a well validated animal model of migraine. As regards the trigeminal neuropathic pain, the role of TRPA1 channels was investigated by evaluating mechanical allodynia in a model of chronic constriction injury of the infraorbital nerve (IoN-CCI). Additionally, in order to gain new insights on the TRP role upon the trigeminal nociception, the attention was focused specifically on changes in c-Fos, TRPA1, TRPV1, CGRP and SP gene expression in specific cerebral and peripheral areas involved in sensitization, in combination with the changes in CGRP and SP protein expression in the NTC.

3. MATERIALS AND METHODS

3.1. TRP antagonists

The TRPV1 antagonist AMG9810 was diluted, accordingly to the supplier's instructions, in 100% dimethylsulfoxide (DMSO), one of the most common solvents used experimentally to dissolve hydrophobic substances for *in vivo* and *in vitro* purposes (Greco et al., 2015; Rahn et al., 2008; 2011; Gao and Duan, 2010). The high concentration of DMSO was chosen because lower concentrations were unable to completely dissolve the drug. AMG9810 was administered intraperitoneally (i.p.) at the dose of 30 mg/Kg (Gavva et al., 2005); its solubility in DMSO is 16 mg/ml, thus the volume of vehicle used was 1,87 ml/Kg. The analgesic and antihyperalgesic effects of AMG9810 have been already demonstrated in different pain models (Gavva et al., 5005; Tékus et al., 2010; Wu et al., 2013).

The TRPA1 antagonist used is a novel lipoic-based molecule: ADM_12, synthesized in the Laboratory of Prof. Cristina Nativi (University of Florence, Italy). The IC50 values obtained for ADM_12 after activation of TRPA1 by AITC were 8.2 ± 0.8 μM and for the inhibition of 30 μM menthol-induced currents, 7.3 ± 2.1 μM (Gualdani et al., 2015). Gualdani et al. (2015) reported *in vivo* the ability of ADM_12 to reduce mechanical facial allodynia after complete Freund's adjuvant injection, a model used to induce a chronic inflammation. *In vitro* experiments show that ADM_12 is able to block not only the currents elicited by TRPA1 agonists but also the ones elicited by capsaicin, a TRPV1 agonist, thus suggesting that ADM_12 also acts as modulator of a TRPA1/TRPV1 heterodimer (Gualdani et al., 2015).

Since ADM_12 has high affinity for TRPA1 and an elevated solubility in physiological solution, we used this antagonist for experimental studies. ADM_12 was dissolved in saline (1 ml/Kg) and administered i.p. at the dose of 30 mg/Kg (Gualdani et al., 2015).

3.2. Animal model of migraine

Nitroglycerin (NTG) (Bioindustria L.I.M. Novi Ligure (AL), Italy) was prepared from a stock solution of 5.0 mg/1.5 mL dissolved in 27% alcohol and 73% propylene glycol. For the injections, NTG was further diluted in saline (0.9% NaCl) to reach the final concentration of alcohol 6% and propylene glycol 16%. The diluted NTG is injected i.p. at the dose of 10 mg/Kg, (Tassorelli et al., 2003; Greco et al., 2015, 2017b). An equivalent volume of saline (0.9% NaCl), alcohol 6% and propylene glycol 16% was used as vehicle.

3.2.1. Animals

Adult male Sprague-Dawley rats were used (weight 200-250g) following the IASP's guidelines for pain research in animals (Zimmerman, 1983). Rats were housed in plastic boxes in groups of 2 with water and food available *ad libitum* and kept on a 12:12 hours light-dark cycle. All procedures were in accordance with the European Convention for Care and Use of Laboratory Animals and were approved by the Italian Ministry of Health (Document number 1239/2015PR).

3.2.2. Experimental plan

The animals were allocated in 8 groups and randomly assigned to different experimental sets, according to the experimental protocol illustrated in Table 1. Briefly, TRP antagonist/vehicle was administered 3 hours after NTG/vehicle treatment; 4 hours after NTG or vehicle administration - which corresponds to the timing of the maximal expression of NTG-induced hyperalgesia (Greco et al., 2015, 2017b) - the rats underwent the orofacial formalin test. All animals were acclimatized to the test chamber 30 minutes before testing. At the end of the behavioral test, each rat was sacrificed with a lethal dose of anesthetic (Chloral hydrate 800 mg/kg, i.p., and Tiletamine-Zolazepam, 50 mg/Kg, intramuscular) and samples were collected and processed either for the detection of gene expression levels with real time polymerase chain reaction (RT-PCR) or the evaluation of CGRP and SP expression with immunohistochemistry (IHC).

Table 1. Schematic representation of experimental groups, timing (T) of administration and number (N) of animals *per* group that underwent the orofacial formalin test (OFT). The samples of subsets of these animals were processed for real time polymerase chain reaction (RT-PCR) of for immunohistochemistry (IHC).

EXPERIMENTAL GROUPS	**T0**	**T3h**	**T4h**	**OFT**	**RT-PCR**	**IHC**
DMSO	NTG vehicle	DMSO	formalin	N=5	-	-
AMG	NTG vehicle	AMG9810	formalin	N=5	-	-
NTG+DMSO	NTG	DMSO	formalin	N=5	-	-
NTG+AMG	NTG	AMG9810	formalin	N=5	-	-
Saline	NTG vehicle	-	saline	N=5	N=5	-
Control (CT)	NTG vehicle	saline	formalin	N=13	N=7	N=6
ADM	NTG vehicle	ADM_12	formalin	N=13	N=7	N=6
NTG	NTG	saline	formalin	N=13	N=7	N=6
NTG+ADM	NTG	ADM_12	formalin	N=13	N=7	N=6

ADM=ADM_12; AMG=AMG9810; DMSO=dimethylsulfoxide; NTG=nitroglycerin

3.2.3. Orofacial formalin test

The formalin test is a widely used nociceptive pain model in which the subcutaneous injection of diluted formalin causes tissue injuries and generates behavioral as well as electrophysiological responses. Typically, the time course of the response to formalin is biphasic: an early and short-lasting first phase is followed, after a quiescent period, by a second prolonged (tonic) phase. The first phase results from direct stimulation of nociceptors, whereas during the second one inflammatory phenomena take place (Dubisson and Dennis, 1977; Tjølsen et al., 1992).

In this study we performed the orofacial formalin test, in which formalin (1.5%, 50µl), an aqueous solution of 37% formaldehyde, or saline (50µl) was injected subcutaneously into the right upper lip, just lateral to the nose (Raboisson and Dallel, 2004). Immediately after the injection, each animal was placed into the observation box (30x30x30 cm glass chamber with mirrored sides) and rubbing behavior was recorded for 45 minutes with a camera, located at 50 cm from the box, for the off-line analysis. Pain-related behavior, linked to the trigeminal activation, was quantified by measuring the seconds the animal spent grooming the injected area (face rubbing) with the ipsilateral fore- or hindpaw. The observation time was divided into 15 blocks of 3 minutes each for the time course analysis (Raboisson and Dallel, 2004). The test consisted of 2 phases spaced by a latency period of 6-12 min: Phase I (0-6 min) refers to the acute pain, while Phase II (12-45 min) reflects

the combined effects of afferent input and central sensitization (Raboisson and Dallel, 2004). After completion of the orofacial formalin test, a subset of 7 rats per experimental group served for the evaluation of gene expression by means of RT-PCR, while the remaining subset of 6 animals per experimental group underwent evaluation of protein expression by means of IHC (Table 1). To evaluate whether formalin injection *per se* (CT group) induces an increase in mRNA expression of all the investigated genes in all areas under evaluation, we included an additional experimental group (Saline, N=5) injected with saline into the right upper lip.

3.3. Trigeminal neuropathic pain model

3.3.1. Animals

Male Sprague-Dawley rats (Charles River, weighing 225-250 g at arrival) were housed in solid-bottom polycarbonate cages in group of 2 in a colony room with water and food available *ad libitum*. Rats were kept under a reversed 12:12 h dark/light cycle (lights on at 20 h). Animals were treated and cared for according to the guidelines of the Committee for Research and Ethical Issues of IASP (Zimmerman, 1983). The protocol was approved by the institutional Ethical Committee (number 2017-16).

Rats were allowed to acclimate for 8 days to the housing conditions before the surgery, and were habituated to the behavioral test procedure daily for three days before pre-operative testing.

3.3.2. Experimental plan

The animals were randomly allocated in four groups of 12 animals each and assigned to different experimental set as shown in Table 2.

Rats underwent the mechanical stimulation testing **(**MST) one day before the surgery and 5, 12, 18, 26 and 27 days after. On day +27, sham and operated rats (see next paragraphs) were treated with ADM_12 (ADM) or saline 1 hour prior to the MST (Figure 9). At the end of the behavioral test, each rat was sacrificed with an i.p. overdose of pentobarbital (150 mg/Kg). A subset of 6 rats per experimental group served for the detection of gene expression levels by means of RT-PCR; another subset of 6 animals per experimental group underwent the evaluation protein expression by means of IHC (Table 2).

Table 2. Experimental groups and number (N) of animals *per* group that underwent the mechanical stimulation test (MST). The samples of subsets of these animals were processed for real time polymerase chain reaction (RT-PCR) of for immunohistochemistry (IHC).

EXPERIMENTAL GROUPS	**MST**	**RT-PCR**	**IHC**
Sham+saline	N=12	N=6	N=6
Sham+ADM	N=12	N=6	N=6
CCI+saline	N=12	N=6	N=6
CCI+ADM	N=12	N=6	N=6

CCI=IoN-ligated rats; ADM=ADM_12

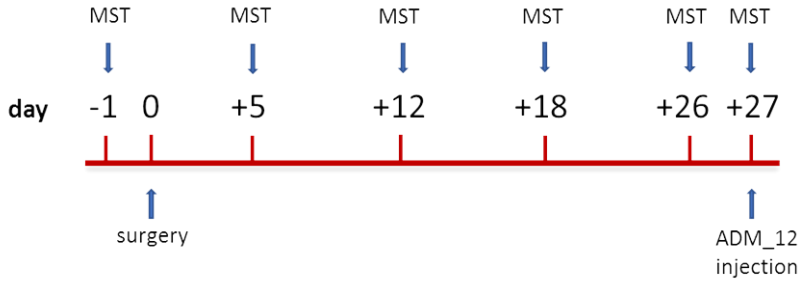

Figure 9. Schematic representation of the experimental design.

3.3.3. Surgery

The chronic constriction injury of the infraorbital nerve (IoN-CCI) was performed following the procedure described by Vos et al., (1994). Rats were anaesthetized with pentobarbital (60 mg/Kg, i.p.) and treated with atropine (0.1 mg/Kg, i.p.). All surgery was performed under direct visual control using a Zeiss operation microscope (x10-25). The rat's head was fixed in a stereotaxic frame and a mid-line scalp incision was made, exposing skull and nasal bone. The edge of the orbit, formed by the maxillary, frontal, lacrimal and zygomatic bones, was dissected free. To give access to the left IoN, the orbital contents were gently deflected with a cotton-tipped wooden rod. The IoN was dissected free at its most rostral extent in the orbital cavity. Two chromic catgut ligatures (5-0) were tied around the IoN (2 mm apart) (Figure 10). The scalp incision was closed using

polyester sutures (4-0). In sham operated rats, the IoN was exposed using the same procedure, but the exposed IoN was not ligated.

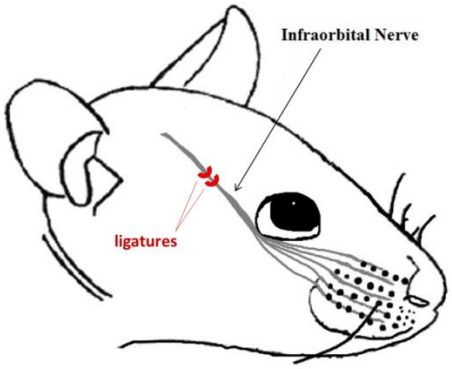

Figure 10. Schematic representation of the rat Infraorbital Nerve and sites of nerve ligation.

3.3.4. Mechanical stimulation testing (MST)

Baseline data were obtained 1 day before surgery. Following surgery, rats were tested on post-operative days +5, +12, +18, +26 and +27 (Figure 9). A graded series of five Von Frey hairs (Pressure Aesthesiometer®, Stoelting Co, Chicago, IL) were applied within the IoN territory, near the center of the vibrissal pad, on the hairy skin surrounding the mystacial vibrissae (Vos et al., 1994; Deseure and hans 2015b). The force required to bend the filaments was 0.015 g, 0.127 g, 0.217 g, 0.745 g and 2.150 g, respectively. Stimuli are applied in an ascending order of intensity either ipsi- or contralaterally. The scoring system described by Vos et al. (1994) was used to evaluate the rats' response to the stimulation: (score 0) no response; (score 1) detection: the rat turns the head toward the stimulating object and the stimulus object is then explored; (score 2) withdrawal reaction: the rat turns the head slowly away or pulls it briskly backward when the stimulation is applied; sometimes a single face wipe ipsilateral to the stimulated area occurs; (score 3) escape/attack: the rat avoids further contact with the stimulus object, either passively by moving its body away from the stimulating object to assume a crouching position against the cage wall, or actively by attacking the stimulus object, making biting and grabbing movements; (score 4) asymmetric face grooming: the rat displays an uninterrupted series

of at least three face-wash strokes directed toward the stimulated facial area. For each rat, and at every designated time, a mean score for the five Von Frey filaments was determined.

3.4. Real time polymerase chain reaction (RT-PCR)

The trigeminal ganglia (TG), cervical spinal cord (CSC, C1-C2 level) and medulla-pons (bregma, −13.30 to −14.60 mm) of each animal were removed and frozen at -80°C. Samples were then processed to evaluate the expression levels of the genes encoding for c-Fos (c-fos), TRPA1 (Trpa1), TRPV1 (Trpv1), CGRP (Calca) and SP (preprotachykinin-A, PPT-A).

Total RNA was extracted from samples with TRIzol® reagent (Invitrogen, USA), according to the method of Chomczynski and Mackey (1994), in combination with tissue homogenization by means of ceramic beads (PRECELLYS, Berthin Pharma). RNA was quantified by measuring the absorbance at 260/280 nm using a nanodrop spectrophotometer (Euroclone); cDNA was generated using the iScript cDNA Synthesis kit (BIO-RAD) following the supplier's instructions. Gene expression was analyzed using the Fast Eva Green supermix (BIO-RAD). Primers sequences, obtained from the AutoPrime software (http://www.autoprime.de/AutoPrimeWeb), are reported in Table 3. The expression of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), remained constant in all the experimental groups considered. The amplification was performed through two-step cycling (95–60°C) for 45 cycles with a light Cycler 480 Instrument RT-PCR Detection System (Roche) following the supplier's instructions. All samples were assayed in triplicate and the ΔΔCq method was used to investigate the differences in the gene expression levels.

Table 3. Sequences of primers used.

Gene	*Forward primer*	*Reverse primer*
GAPDH	AACCTGCCAAGTATGATGAC	GGAGTTGCTGTTGAAGTCA
c-fos	TACGCTCCAAGCGGAGAC	TTTCCTTCTCTTTCAGTAGATTGG
Trpa1	CTCCCCGAGTGCATGAAAGT	TGCATATACGCGGGGATGTC
Trpv1	CTTGCTCCATTTGGGGTGTG	CTGGAGGTGGCTTGCAGTTA
Calca	CAGTCTCAGCTCCAAGTCATC	TTCCAAGGTTGACCTCAAAG
PPT-A	GCTCTTTATGGGCATGGTC	GGGTTTATTTACGCCTTCTTTC

3.5. Immunohistochemical staining

After the behavioral test (orofacial formalin test or MST) the animals were anaesthetized and perfused transcardially with saline and 4% paraformaldehyde. The medullary segment containing the NTC, between +1 and -5 mm from the obex, was removed and post-fixed for 24 h in the same fixative; subsequently, samples were transferred in solutions of sucrose at increasing concentrations (up to 30%) during the following 72 h. All samples were cut transversely at 30 µm on a freezing sliding microtome. CGRP and SP protein expression was evaluated using the free-floating immunohistochemical technique. Following several rinses in a potassium phosphate buffered saline (KPBS) solution, sections were incubated in a blocking solution (4% normal goat serum) for 30 min; subsequently, sections were incubated in primary antibodies in a KPBS solution containing 0.4% Triton X-100 (TX) and 4% normal goat/horse serum for 24 h at room temperature. For CGRP were used an anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:3200. For SP were used an anti-rabbit antibody (Chemicon, Temecula, CA, USA) at a dilution of 1:5000. After several rinses in a KPBS solution containing 0.04% TX, sections were incubated at room temperature with the secondary biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) and then with the avidin-biotin complex (Vectastain, Vector Laboratories). Peroxidase substrate kit DAB (3′3′-diaminobenzidine tetrahydrochloride) (Vector Laboratories, Burlingame, CA, USA) was used for visualization.

Negative control staining was performed by omitting the primary antibodies and, in order to avoid variability in the background staining due to the procedure, each treated animal was simultaneously stained with the corresponding control and processed at the same time. After staining, sections were rinsed in KPBS, mounted onto glass slides, air dried and cover slipped.

3.6. Statistical evaluation

Statistical analysis was performed with GraphPad Prism program (GraphPad Software, San Diego, CA). For the orofacial formalin test, the time spent (in seconds) in face rubbing was counted separately for Phase I and for Phase II. For CGRP and SP protein expression, the area covered by immunoreactive fibres in the NTC ipsilateral to the formalin injection, was expressed as optical density (OD) values (Greco et al., 2008, 2017b). OD was obtained

using an AxioSkop 2 microscope (Zeiss) and a computerized image analysis system (AxioCam, Zeiss), equipped with dedicated software (AxioVision Rel 4.2, Zeiss, Germany). The mean OD was determined by rounding off the stained structure of interest (NTC) and subtracting the OD of the background (slide, mounting medium and coverslip) for each section, considering a total of 12 sections per animal. All sections were averaged and reported as the mean ±SEM of OD values.

For mRNA expression, results were analyzed using the ΔCt method to compare expression of genes of interest with that of GAPDH.

In the MST, for each rat and at every designated time, a mean score for the five Von Frey hairs was determined. To evaluate the drug treatment effect, a comparison between groups within +26 and +27 post-surgery days was made. The IoN-CCI rats were compared to the sham-operated rats.

All data were tested for normality using the Kolmogorov-Smirnov normality test and considered normal. Differences between groups or between ipsilateral and contralateral sides were analyzed by the one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test, or by means of the two-way ANOVA followed by Bonferroni post-hoc test respectively. Differences between two groups were analyzed with the Student's t test. A probability level of less than 5% was regarded as significant.

4. MIGRAINE MODEL: RESULTS and DISCUSSION

4.1. Results

4.1.1. AMG9810 effect on behavioral response

No difference was seen between NTG+DMSO and control (DMSO) group in both Phases of the orofacial formalin test. As regards the TRPV1 antagonist AMG9810, when administered with NTG vehicle, it induced a significant increase of face rubbing behavior compared to DMSO group during Phase II of the test. When administered in combination with NTG, AMG9810 significantly increased the nocifensive behavior in Phase I of the test compared to NTG+DMSO group (Figure 11).

The findings obtained are inconclusive, this could probably due to the high dose of DMSO we had to use that resulted as confounding factor.

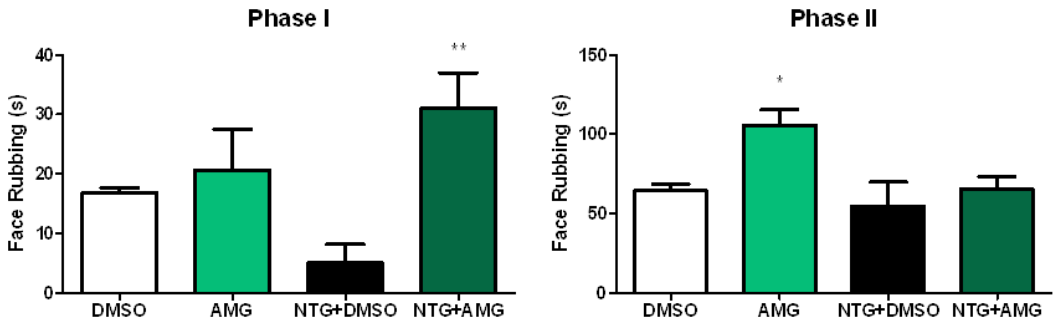

Figure 11. Orofacial formalin test. Total time (seconds) spent in face rubbing in Phases I and II. Data are expressed as mean ± SEM. ANOVA followed by Tukey's Multiple Comparison Test, *p<0.05 vs DMSO and NTG+DMSO; **p<0.01 vs NTG+DMSO.

4.1.2. ADM_12 effect on behavioral response

NTG administration significantly increases nocifensive behavior in Phase II (hyperalgesic phase) of the orofacial formalin test, when compared to control group (CT) (Figure 12A). No difference was found in Phase I. ADM_12 administration induced a slight, non-significant reduction in the nocifensive behavior during Phase I of test when used alone; on the contrary, when administered in association with NTG (NTG+ADM group) it

significantly reduced the face rubbing time during Phase II when compared to NTG group (Figure 12A).

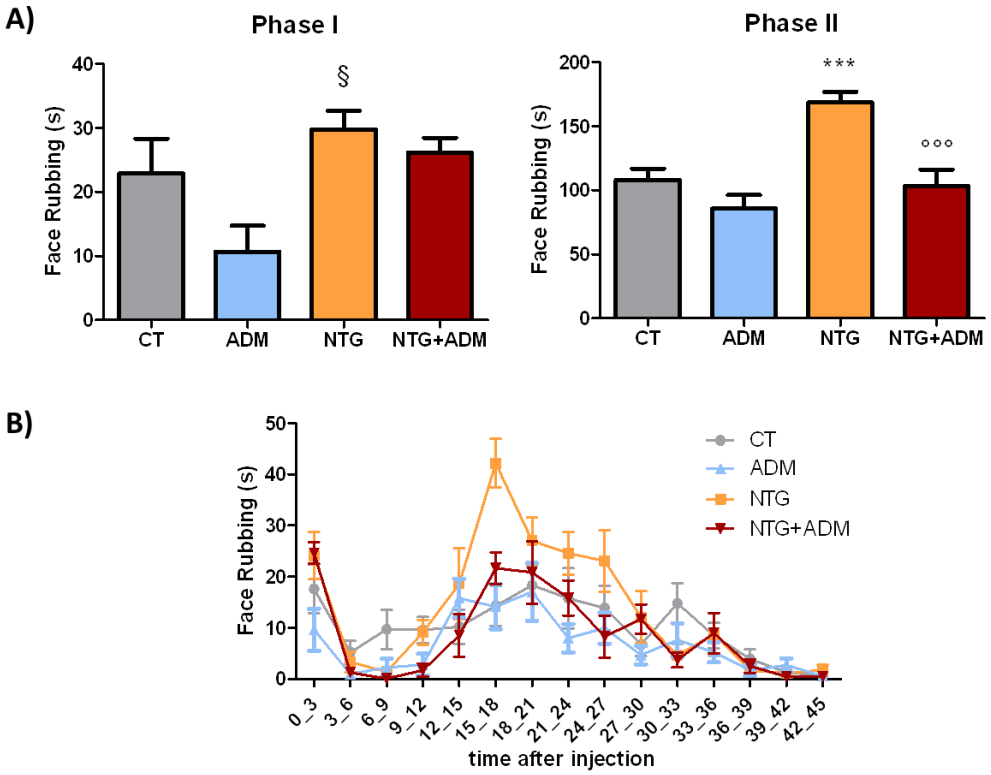

Figure 12. Orofacial formalin test. A) Total time (seconds) spent in face rubbing in Phases I and II. Data are expressed as mean ± SEM. ANOVA followed by Tukey's Multiple Comparison Test, §$p<0.05$ vs ADM; ***$p<0.001$ vs CT and ADM; °°°$p<0.001$ vs NTG. B) Time course of the face rubbing. Data are expressed as mean ± SEM.

4.1.3. ADM_12 effect on gene expression

The expression of c-fos, Calca, PPT-A, Trpa1 and Trpv1 was evaluated in the trigeminal ganglion (TG) and the cervical spinal cord (CSC) ipsilateral to the formalin or saline injection and in the medulla-pons *in toto*.

Following formalin injection (CT group), mRNA expression of all genes was significantly increased compared with saline group in all areas investigated (Figure 13). NTG administration caused a further increase in the expression of all the genes in all the areas under evaluation, compared to CT group (Figure 14). ADM_12 treatment significantly prevented NTG-induced increase in c-fos, Trpa1, Calca and PPT-A expression (Figure 14); as regards Trpv1, a decreasing trend was seen in all areas when ADM_12 was administered in combination with NTG (Figure 14). No change was observed in gene expression levels when ADM_12 was administered with NTG vehicle (Figure 14).

4.1.4. ADM_12 effect on neuropeptides protein expression

CGRP and SP protein expression was evaluated in the NTC ipsilaterally to the formalin injection. No significant difference in the density of immunoreactive fibers for CGRP and SP protein was observed when comparing NTG and CT groups (Figures 15 and 16). ADM_12 administration did not provoke any change in CGRP and SP expression either with NTG vehicle or in combination with NTG (Figures 15 and 16).

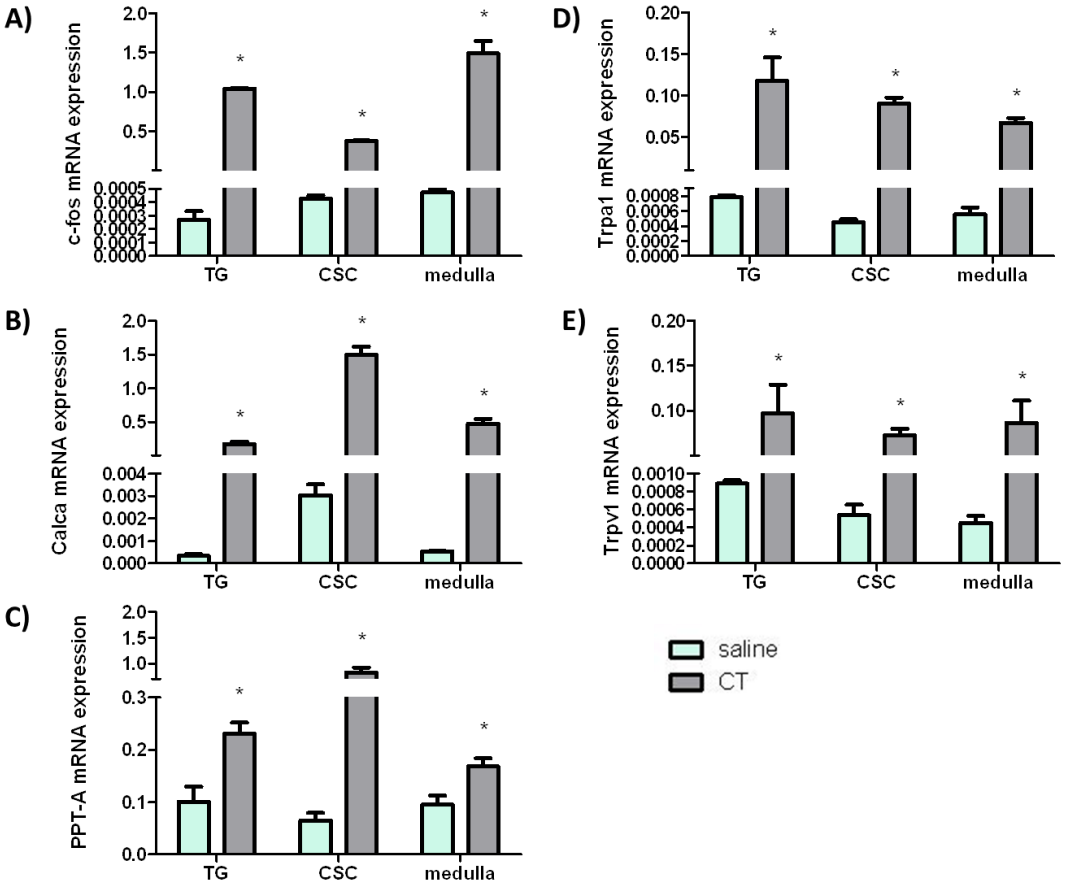

Figure 13. The panels show the mRNA expression of c-fos (A), Calca (B), PPT-A (C), Trpa1 (D) and Trpv1 (E) in trigeminal ganglion (TG) and cervical spinal cord (CSC) ipsilateral to the formalin injection (CT group) or the saline injection (saline group), and in medulla *in toto*. Data are expressed as mean ± SEM. Student's t test, *$p<0.05$ vs saline.

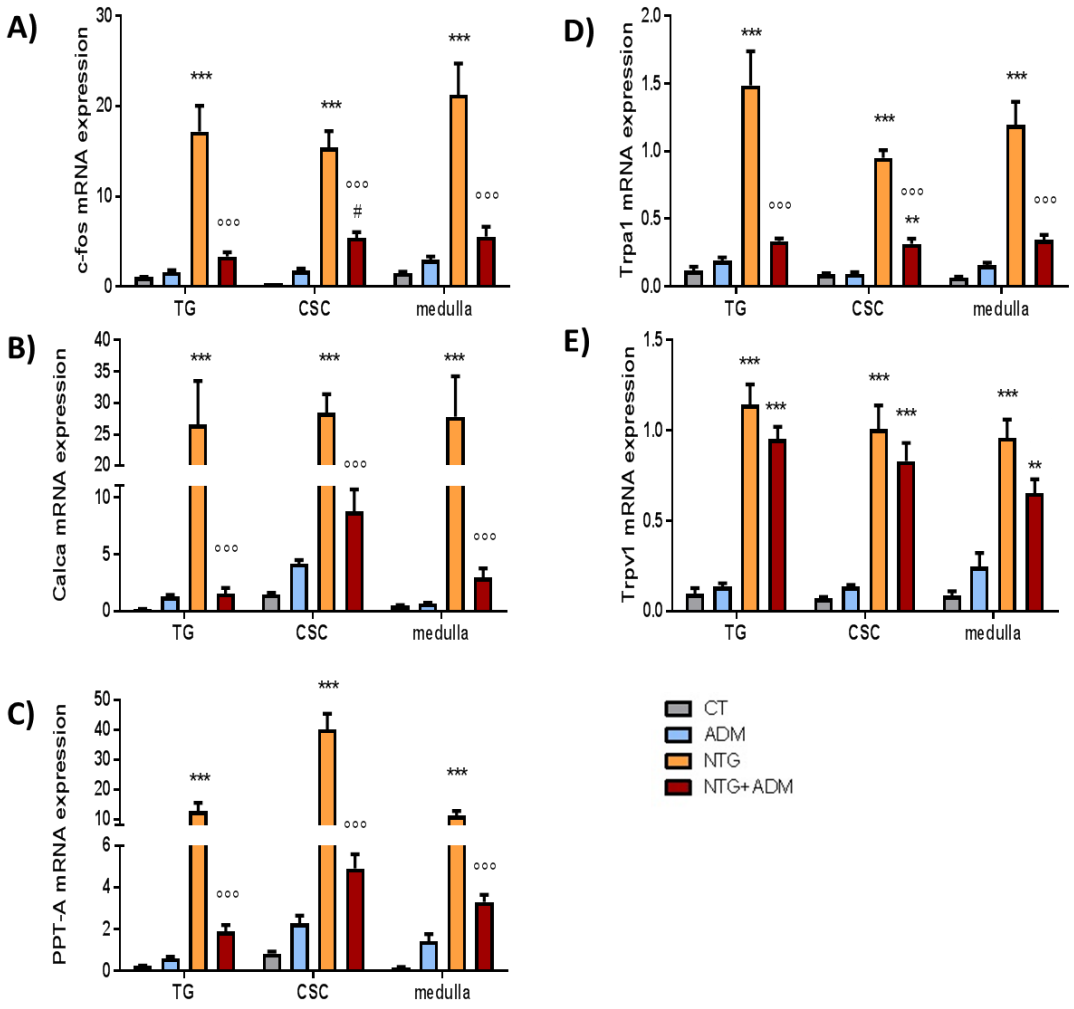

Figure 14. The panels show the mRNA expression of c-fos (A), Calca (B), PPT-A (C), Trpa1 (D) and Trpv1 (E) in trigeminal ganglion (TG) and cervical spinal cord (CSC) ipsilateral to the formalin injection, and in medulla-pons *in toto*. Data are expressed as mean ± SEM. ANOVA followed by Tukey's Multiple Comparison Test, **p<0.01 and ***p<0.001 vs CT and ADM; °°°p<0.001 vs NTG; #p<0.05 vs ADM.

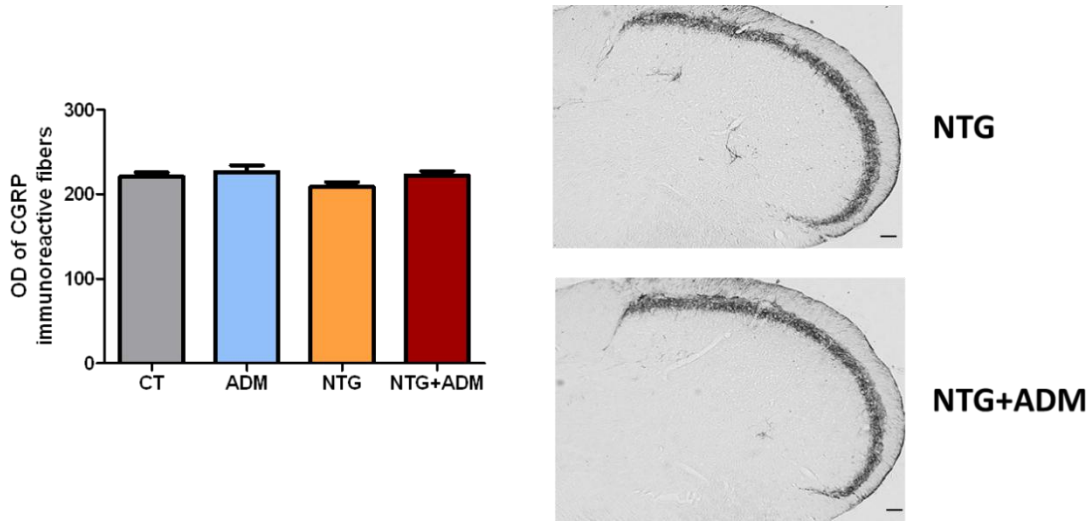

Figure 15. Optical density (OD) values of CGRP immunoreactive fibers in the NTC ipsilateral to the formalin injection. Data are expressed as mean ± SEM. ANOVA followed by Tukey's Multiple Comparison Test. Right: representative photomicrographs of CGRP immunoreactive fibers in NTG and NTG+ADM group. Scale bar: 100 µm.

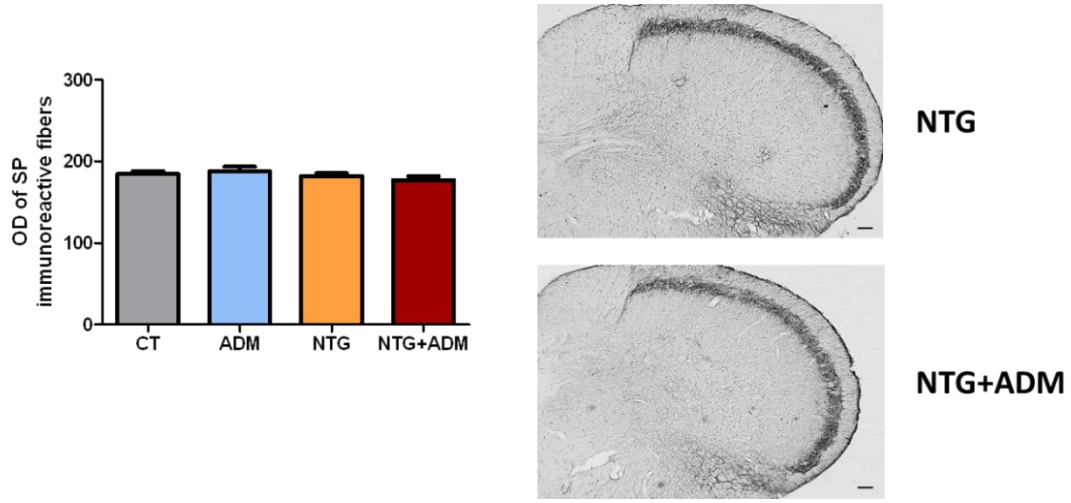

Figure 16. Optical density (OD) values of SP immunoreactive fibers in the NTC ipsilateral to the formalin injection. Data are expressed as mean ± SEM. ANOVA followed by Tukey's Multiple Comparison Test. Right: representative photomicrographs of SP immunoreactive fibers in NTG and NTG+ADM group. Scale bar: 100 µm.

4.2. Discussion

Clinical and experimental studies have pointed to the possible involvement of the TRPA1 and TRPV1 channels in migraine pain (Dussor et al., 2014). The activation of these channels, expressed on primary sensory neurons, leads to the release of SP and CGRP, key neuropeptides implicated in the trigeminovascular system activation (Tajti et al., 2015). In this study, we investigated the effects of AMG9810 and ADM_12, TRPV1 and TRPA1 antagonist respectively, in NTG-induced hyperalgesia at the trigeminal level in rats, using the quantification of nocifensive behavior induced by the orofacial formalin test. Since AMG9810 effects in the animal model of migraine are unclear, we evaluated only biochemical and molecular changes induced by ADM_12. In particular, we evaluated the mRNA expression of the genes coding for c-Fos (c-fos), TRPA1 (Trpa1), TRPV1 (Trpv1), CGRP (Calca) and SP (PPT-A) in specific peripheral and central areas involved in trigeminal nociception. CGRP and SP protein expression was also investigated in the NTC. We focused our attention only at the side ipsilateral to the formalin injection because it was demonstrated that there was no difference between the two sides (Greco et al., 2017b).

4.2.1. AMG9810 effects

The findings obtained using the TRPV1 antagonist AMG9810 in the NTG-induced hyperalgesia associated to the orofacial formalin test are inconclusive. It is possible that the effect of AMG9810 was confounded by DMSO. Indeed, these results are in contrast with previous studies, where we showed that NTG administration, alone or with DMSO (1ml/Kg), significantly increased nocifensive behavior in Phase II, when compared to either NTG vehicle or NTG vehicle+DMSO group, thus showing that NTG induces trigeminal hyperalgesia and confirming the validity of the model even with use of DMSO as vehicle (Greco et al., 2015, 2017a). In the present study, however we had to use a higher dose of DMSO, almost two-fold higher (1,87 ml/Kg), in order to achieve AMG9810 dilution. DMSO is one of the most common solvents used experimentally to dissolve hydrophobic substances for *in vivo* and *in vitro* purposes. However, previous studies have suggested that DMSO may variably interfere with pain perception. Colucci et al. (2008) showed an increase in the nocifensive behavior in both phases of the formalin test when DMSO was applied subcutaneously in the mouse paw, but also an anti-nociceptive activity when it was administered centrally or i.p. At variance, Păunescu et al. (2009) reported that

DMSO administered i.p. did not have an analgesic effect in the mouse writhing test. El-Morsy et al., (2013) showed that low concentrations of DMSO are devoid of significant antinociceptive effect, but may nonetheless mask the antinociceptive activity of paracetamol when used as a vehicle at the formalin test. Thus, to better understand the role of TRPV1 in trigeminal hyperalgesia, further studies will be needed, using a different vehicle or other antagonists.

4.2.2. ADM_12 effects

Our findings show the ability of ADM_12 to reduce NTG-induced hyperalgesia in the second phase of the orofacial formalin test. This effect is associated to a significant inhibition of NTG-induced increase in c-fos, Trpa1, Calca and PPT-A mRNA levels in medulla-pons, in the ipsilateral cervical spinal cord (CSC) and in the ipsilateral trigeminal ganglion (TG). By contrast, ADM_12 did not influence gene expression when used in animals that were not made hyperalgesic by NTG treatment, and induced only a moderate, not significant reduction in the nocifensive behavior during phase I of the orofacial formalin test. Since formalin is able to activate TRPA1 channels (McNamara et al., 2007), this reduction is probably linked to the antagonist action of ADM_12 on the TRPA1 channels localized at the peripheral endings of the primary sensory neurons or on non-neuronal cells (Atoyan et al., 2009).

c-Fos, TRPA1 and TRPV1 mRNA expression

The protein and mRNA expression of c-fos is commonly used as a marker of neuronal activation following painful stimuli (Tassorelli et al., 2007; Greco et al., 2015; Launay et al., 2016). In our model, the orofacial formalin injection induced an increase in c-Fos mRNA levels in medulla-pons, CSC and TG when compared to orofacial saline injection. NTG treatment induced a further amplification of primary and second order neurons activation, as demonstrated by c-Fos mRNA levels in TG and in central areas compared to CT group. NTG is able indeed to activate and sensitize spinal trigeminal neurons (Lambert et al., 2000; Greco et al., 2015, 2017b). Probably, the increase in inflammatory response (e.g. TNF-α, IL-6) (Greco et al., 2016, 2017b) induced by NTG in all areas investigated, contributes to the intensification of c-fos expression (Zhuang et al., 2000; Imbe et al., 2001). The treatment with the TRPA1 antagonist reverted the NTG-induced increase in c-

Fos mRNA expression. Interestingly, the selective TRPA1 antagonist HC-030031 downregulates IL-6 and Prostaglandin E2 (PGE2) production (Nummenmaa et al., 2016), and decreases PGE2-induced hyperalgesia (Dall'Acqua et al., 2014), confirming that TRPA1 play a role in the upregulation/modulation of these inflammatory factors.

TRPA1 and TRPV1 mRNA levels are increased in all areas involved in trigeminal nociception after formalin injection, and they are further increased following NTG administration. The mechanisms underlying NTG-induced hyperalgesia are believed to depend upon an increased availability of nitric oxide (NO), either released directly from the drug (Torfgård et al., 1989) or synthetized *ex novo* in the meninges (Reuter et al., 2001). Increased availability of NO would in turn stimulate trigeminovascular terminals to induce inflammation (Greco et al., 2005, 2015, 2016), and possibly to upregulate TRPA1 and TRPV1 channels (Amaya et al., 2003; Diogenes et al., 2007; Devesa et al., 2011). It is known that the cysteine residues of both channels are target of NO and NO nitrosylation (Yoshida et al., 2006) could contribute to channels sensitization, which in turn would amplify neuropeptides release (Miyamoto et al., 2009). On the other hand, it is also known that pro-inflammatory agents activate and/or sensitize nociceptors by means of TRPA1 and TRPV1 channels (Huang et al., 2006) and their stimulation causes the release of neuropeptides.

The present data confirm the contribution of TRPA1 to NTG-induced hyperalgesia. In agreement, it was shown that antisense mRNA for TRPA1 prevents carrageenan-induced inflammatory hyperalgesia, suggesting that channel activation is necessary for both the development and the maintenance of hyperalgesia (Bonet et al., 2013). TRPA1 transcripts are upregulated in response to CFA in rats, and inflammatory signals sensitize the channel, consistent with TRPA1 being involved in hyperalgesia (Obata et al., 2005; Diogenes et al., 2007; Dai et al., 2007). The understanding of the molecular mechanisms involved in the regulation of these TRP channels expression is limited. However, Hatano et al. (2012) have suggested that TRPA1 gene expression is induced via the nuclear factor-κB (NF-κB) signaling. In this frame, therefore, it is possible that ADM_12 administration caused a reduction of calcium influx through TRPA1 channels, which in turn interfered with the cascade of second-messenger molecules (e.g. via the PLC/Ca^{2+} signaling pathway) and with the Ca^{2+}-interacting proteins (Zurborg et al., 2007; Mandadi et al., 2011), ultimately preventing NTG-induced NF-kB activation (Greco et al., 2005).

The functions of TRPA1 and TRPV1 are strongly linked to each other (Akopian, 2011; Fischer et al., 2014). This is especially evident in relation to pain and neurogenic inflammation also because TRPV1 is co-expressed on the vast majority of the TRPA1-expressing sensory nerves (Story et al., 2003) and both channels integrate a variety of noxious stimuli. The NTG-induced increase in TRPV1 mRNA expression was not significantly modulated by ADM_12 treatment, showing *in vivo* the antagonist's specificity for TRPA1 channel. On the other hand, *in vitro* experiments performed by Gualdani et al, (2015) show that ADM_12 is able to block not only the currents elicited by TRPA1 agonists but also the ones elicited by capsaicin, a TRPV1 agonist, thus suggesting that ADM_12 also acts as modulator of a TRPA1/TRPV1 heterodimer. However, the ability of these two channels to form heteromultimeric complexes (Staruschenko et al., 2010; Fischer et al., 2014) is still under investigation. Furthermore, it was demonstrated *in vitro* that ADM_12 may act as TRPV1 agonist if only this channel (and not TRPA1) is expressed (Gualdani et al., 2015).

Neuropeptides expression

SP and neurokinin A are encoded by the gene PPT-A. Neurokinins exert a variety of biological activities including nociception, synaptic transmission (as excitatory neurotransmitters), and neurogenic inflammation. In particular, SP is the best characterized of these neuropeptides and has been shown to be related to nociceptive (pain) responses and neurogenic inflammation. SP often coexists and is co-released with CGRP (Roza and Reeh, 2001) and glutamate (Juránek and Lembeck, 1997) in the TG and NTC. It is known that in response to prolonged noxious stimuli, SP and CGRP are released from trigeminal sensory nerve fibers around dural blood vessels, leading to endothelium dependent vasodilation, increased microvascular permeability, and plasma and protein extravasation. TRPA1 channels in primary sensory neurons frequently colocalize with CGRP and SP (Story et al., 2003; Huang et al., 2012) and their activation after NTG administration could promote neuron depolarization and the consequent biosynthesis and releasing of neuropeptides. TRPA1 channels are also expressed on a multitude of non-neuronal sites. Formalin injection may indeed induce TRPA1 activation in various cell types including keratinocytes (Fischer et al., 2015), which may release a large variety of different mediators to indirectly activate and/or sensitize primary sensory neurons (Schaible and Schmidt, 1988; Binshtok et al., 2008). Therefore, it is possible to hypothesize that a direct

effect on TG or at central level may occur as well as an indirect activation via exposed keratinocytes or other cells in the nerve endings proximity. TRPA1 channels have also been described in macrophages (Kun et al., 2014; Billeter et al., 2015), thus it is also possible an effect of ADM_12 on these cells by inhibition of NF-κB activation and other inflammatory mediators that may interact with sensory nerves to affect pain and neurogenic inflammation.

Here, a significant increasing in Calca and PPT-A mRNA expression in the evaluated areas after NTG was reported, while no difference in protein expression was found at the same time point in the NTC. This apparently paradoxical finding may be related to compensatory mechanisms aimed at reintegrating CGRP and SP stores after these neuropeptides have been released at the trigeminovascular endings subsequently to the NTG administration (Greco et al., 2008). In a previous study, the group I work with, detected a reduction in CGRP-immunoreactivity (ir) that occurred from the 1st until the 4th hour after NTG administration, while SP-ir increased transiently 1 hour after NTG administration and returned to baselined levels at the 4th hour (Greco et al., 2008). In the present study, CGRP and SP protein expression was evaluated at the end of orofacial formalin test, thus 5 hours after NTG administration, it is therefore possible that the discrepancy observed is related to the different timings required for the different biological processes (synthesis of mRNA on one side and storage of newly synthetized peptides on the other) and to the different time of evaluation (4 hours in the previous study, 5 hours in the present one). Other studies show an increase in CGRP release from the TG neurons during neurogenic inflammation or after NO donor treatments together with an increased CGRP gene transcription (Helyes et al., 1997; Chen et al., 2010). In addition, stimulation of peripheral afferent fibers causes SP release within the trigeminal ganglia and this release is greatly amplified following orofacial inflammation (Neubert et al., 2000). Similarly, a significant increase in the expression of SP was found 2 hours after orofacial formalin test in the NTC (Lu et al., 2009). Altogether, these results reinforce the role of SP and CGRP in persistent pain by acting at both peripheral and central levels. In the experimental condition of NTG-induced hyperalgesia, the increased levels of NO and the release of inflammatory agents can sensitize the TRPA1 channels, thus amplifying neuropeptides release. In this frame, the inhibitory effect of ADM_12 on Calca and PPT-A mRNA expression suggests its capability to reduce NTG-induced formation/release of neuropeptides. In line with these observations, Nakamura et al., (2012) demonstrated that TRPA1 activation evokes SP

release from the primary sensory neurons through phosphorylation of p38 mitogen-activated protein kinase, via an increase in intracellular Ca^{2+}, and inflammatory responses.

5. TRIGEMINAL NEUROPATHIC PAIN MODEL: RESULTS and DISCUSSION

5.1. Results

5.1.1. ADM_12 effect on behavioral response

Operated rats (CCI+saline and CCI+ADM groups) display a lack of responsiveness to ipsilateral MST of the IoN territory 5 days post operation compared to sham-operated rats (Figure 17). During the following days, this hyporesponsiveness (still visible at 12 days post operation) is replaced by a hyperresponsiveness (at day +26) (Figure 17).

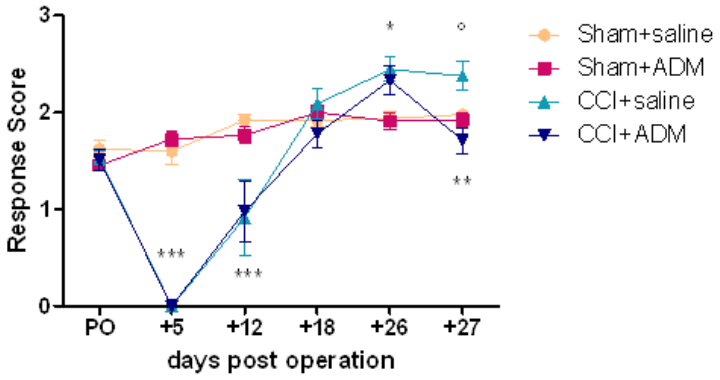

Figure 17. Mechanical stimulation testing (MST). Mean response score to Von Frey hair stimulation of the ligated/sham IoN territory, on pre-operative day (PO) and on +5, +12, +18, +26 and +27 days post operation. Data are expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni post-hoc test, ***p<0.001 CCI+sal and CCI+ADM vs sham+sal and sham+ADM; *p<0.05 CCI+sal and CCI+ADM vs sham+sal and sham+ADM; **p<0.01 CCI+ADM vs CCI+sal; °p<0.05 CCI+sal vs sham+sal and sham+ADM.

TRPA1 antagonist treatment in operated rats (CCI+ADM group) (on day +27) reduced the response score of the mechanical stimulation compared to CCI+saline group (Figure 17 and 18); whereas, ADM_12 treatment in sham-operated rats (Sham+ADM) did not change the mechanical response (Figure 17 and 18). A significant difference in response score of the mechanical stimulation was found between days +26 and +27 in CCI+ADM group (Figure 18).

No difference in the mechanical response score was reported at the level of contralateral side of IoN-CCI rats compared to sham-operated rats (data not shown).

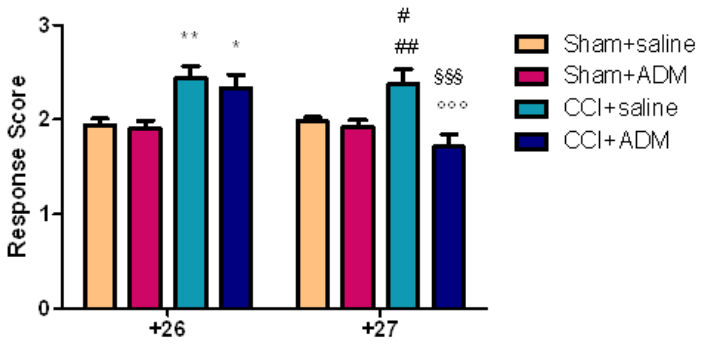

Figure 18. Drug treatment effect on mechanical stimulation testing (MST). Mean response score to von frey hair stimulation of the ligated/sham IoN territory before ADM_12 or saline treatment day (+26) and after treatment (+27). Data are expressed as mean ± SEM. Two-way ANOVA followed by Bonferroni post-hoc test, *p<0.05 and **p<0.01 vs Sham+sal and Sham+ADM (+26); #p<0.05 vs Sham+sal (+27); ##p<0.01 vs Sham+ADM (+27); °°°p<0.001 vs CCI+sal (+27); §§§p<0.001 vs CCI+ADM (+26).

5.1.2. ADM_12 effect on gene expression

The expression of Trpa1, Trpv1, Calca and PPT-A was evaluated in the trigeminal ganglion (TG) and the cervical spinal cord (CSC) ipsilateral (ipsi) and contralateral (contra) to the IoN ligation and in the medulla-pons *in toto*.

Trpa1 mRNA expression

In the ipsilateral TG and CSC and in medulla-pons region, Trpa1 mRNA expression levels were significantly increased in the CCI+saline group compared with Sham+saline and Sham+ADM groups (Figure 19). The increased mRNA levels were significantly reduced after treatment with ADM_12 in CCI rats (CCI+ADM group) in the same regions (Figure 19). ADM_12 administration did not provoke any changes in sham-operated rats.

A significant difference in mRNA levels was seen between the ipsi- and contralateral side (both in TG and CSC) in the CCI+saline group; whereas there was no difference between groups within the contralateral side of TG and CSC (Figure 19A and B).

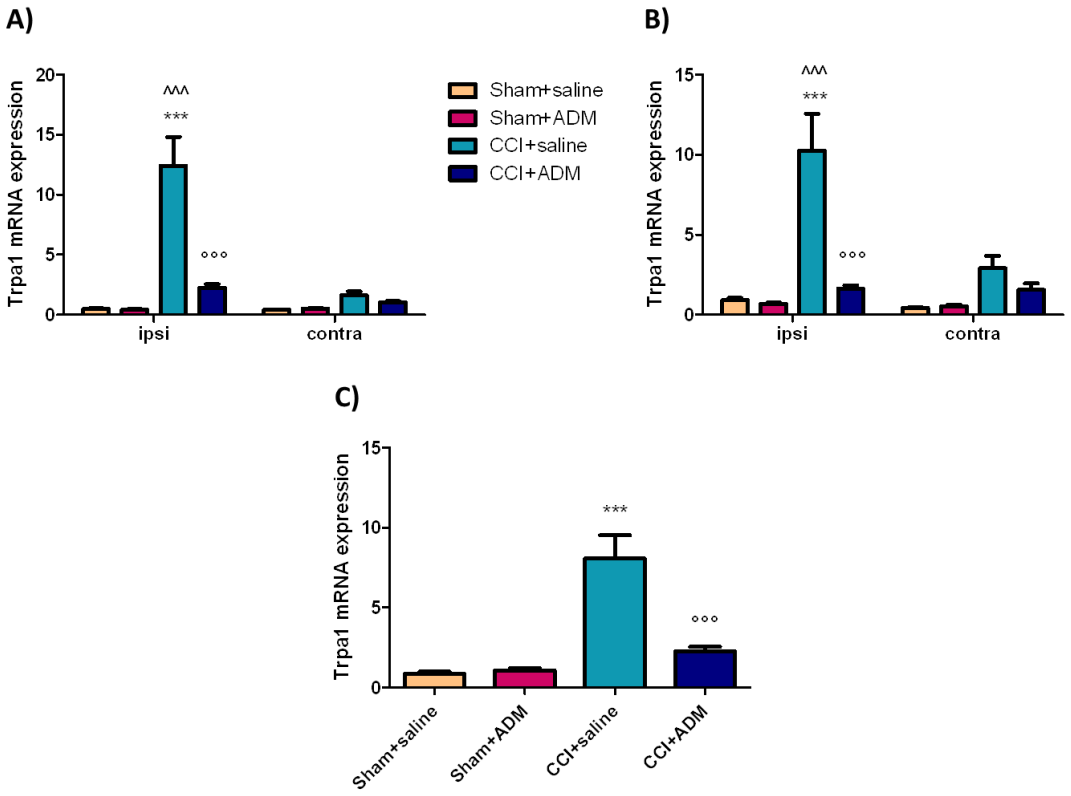

Figure 19. Trpa1 mRNA expression in TGs (A), CSC (B) and medulla (C). Data are expressed as mean ± SEM. One way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test or Two-way ANOVA followed by Bonferroni post-hoc test, ***p<0.001 vs Sham+saline and Sham+ADM (ipsi); °°°p<0.001 vs CCI+saline (ipsi); ^^^p<0.001 vs CCI+saline (contra).

Trpv1 mRNA expression

In the ipsilateral TG and CSC and in medulla-pons region, Trpv1 mRNA expression levels were significantly increased in the CCI+saline group compared with Sham+saline and Sham+ADM groups (Figure 20). Moreover, Trpv1 mRNA levels in CCI+saline group were significantly increased also in the contralateral CSC, compared to sham groups (Figure 20B). The increased mRNA levels were significantly reduced after treatment with ADM_12 in CCI rats (CCI+ADM group) in ipsilateral TG and CSC and in medulla *in toto* (Figure 20). ADM_12 administration did not provoke any changes in sham-operated rats.

A significant difference was seen between the ipsi- and contralateral side (both in TG and CSC) in the CCI+saline group (Figure 20A and B).

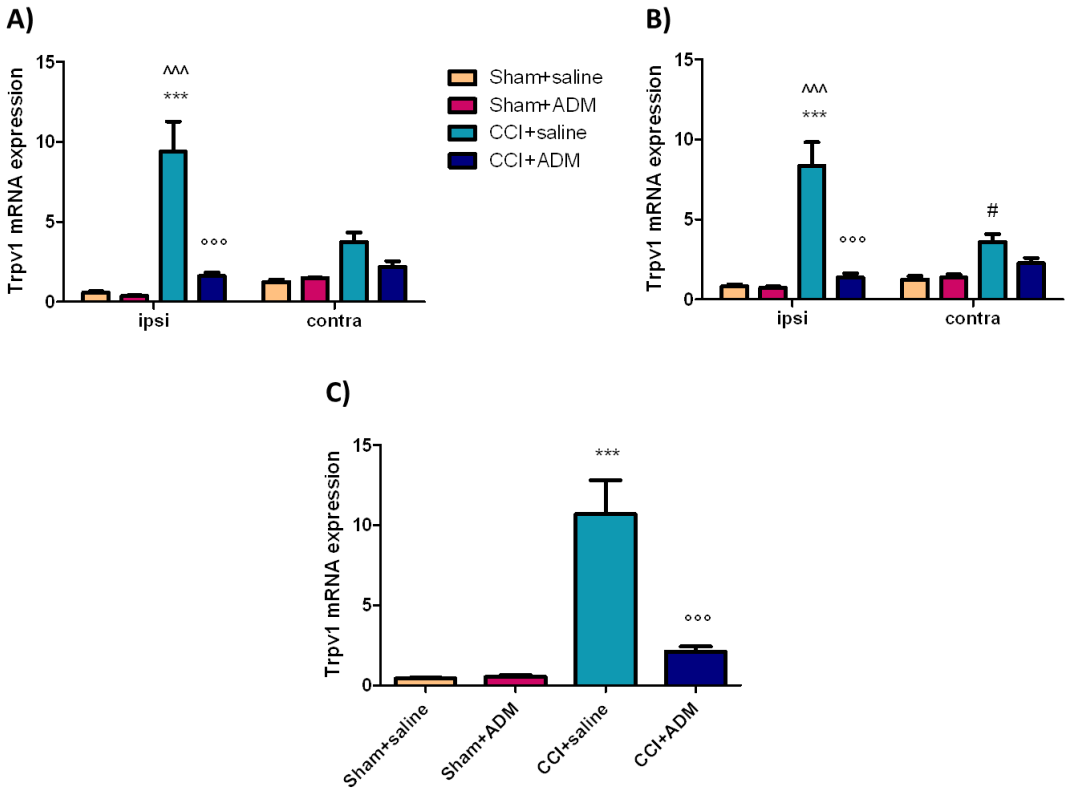

Figure 20. Trpv1 mRNA expression in TGs (A), CSC (B) and medulla (C). Data are expressed as mean ± SEM. One way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test or Two-way ANOVA followed by Bonferroni post-hoc test, ***p<0.001 vs Sham+saline and Sham+ADM (ipsi); °°°p<0.001 vs CCI+saline (ipsi); ^^^p<0.001 vs CCI+saline (contra), #p<0.05 vs Sham+saline and Sham+ADM (contra).

Calca mRNA expression

In the ipsilateral TG and CSC and in medulla-pons region, Calca mRNA expression levels were significantly increased in the CCI+saline group compared with Sham+saline and Sham+ADM groups (Figure 21). Moreover, Calca mRNA levels in CCI+saline and CCI+ADM groups were significantly increased also in the contralateral TG, compared to sham groups (Figure 21A). The increased mRNA levels were significantly reduced after treatment with ADM_12 in CCI rats (CCI+ADM group) in ipsilateral TG and CSC and in medulla *in toto* (Figure 21). ADM_12 administration did not provoke any changes in sham-operated rats (Figure 21).

A significant difference was seen between the ipsi- and contralateral side (both in TG and CSC) in the CCI+saline group (Figure 21A and B).

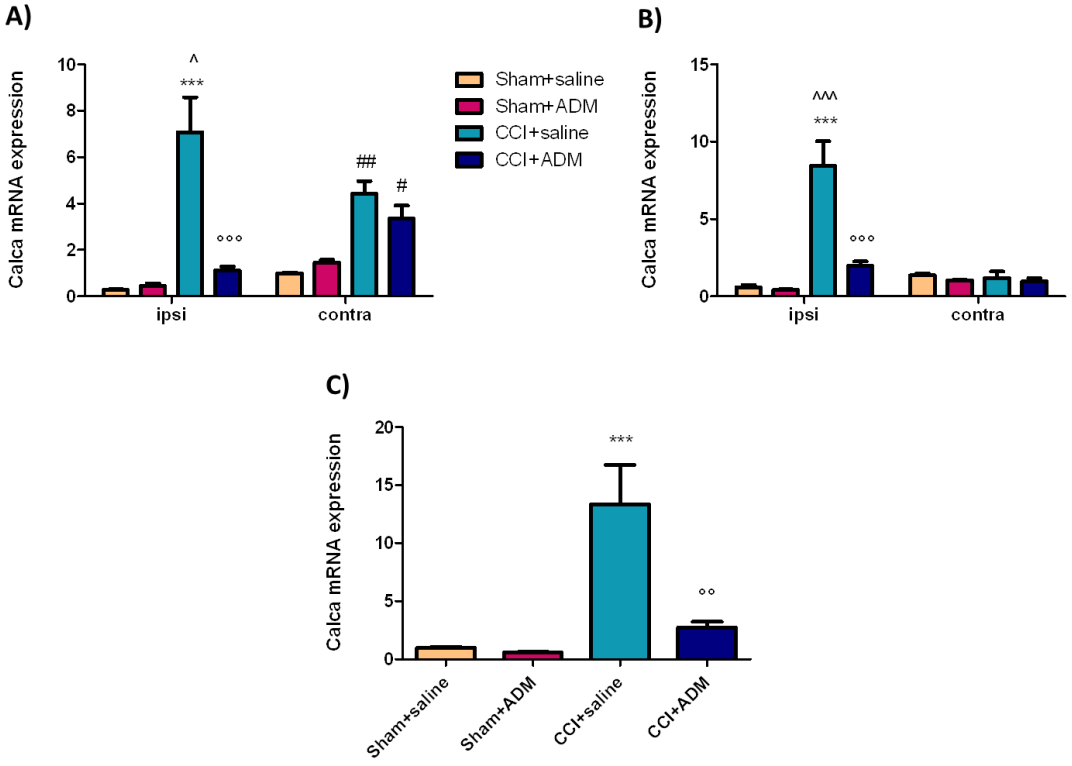

Figure 21. Calca mRNA expression in TGs (A), CSC (B) and medulla (C). Data are expressed as mean ± SEM. One way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test or Two-way ANOVA followed by Bonferroni post-hoc test, ***p<0.001 vs Sham+saline and Sham+ADM (ipsi); °°p<0.01 and °°°p<0.001 vs CCI+saline (ipsi); ^p<0.05 and ^^^p<0.001 vs CCI+saline (contra), #p<0.05 and ##p<0.01 vs Sham+saline and Sham+ADM (contra).

PPT-A mRNA expression

In the ipsilateral TG and CSC and in medulla-pons region, PPT-A mRNA expression levels were significantly increased in the CCI+saline group compared with Sham+saline and Sham+ADM groups (Figure 22). The increased mRNA levels were significantly reduced after treatment with ADM_12 in CCI rats (CCI+ADM group) in the same regions (Figure 22). ADM_12 administration did not cause any changes in sham-operated rats.

A significant difference was seen between the ipsi- and contralateral side (both in TG and CSC) in the CCI+saline group, as well as in the CCI+ADM group at the TG level; whereas

there is no difference between groups within the contralateral side of TG and CSC (Figure 22A and B).

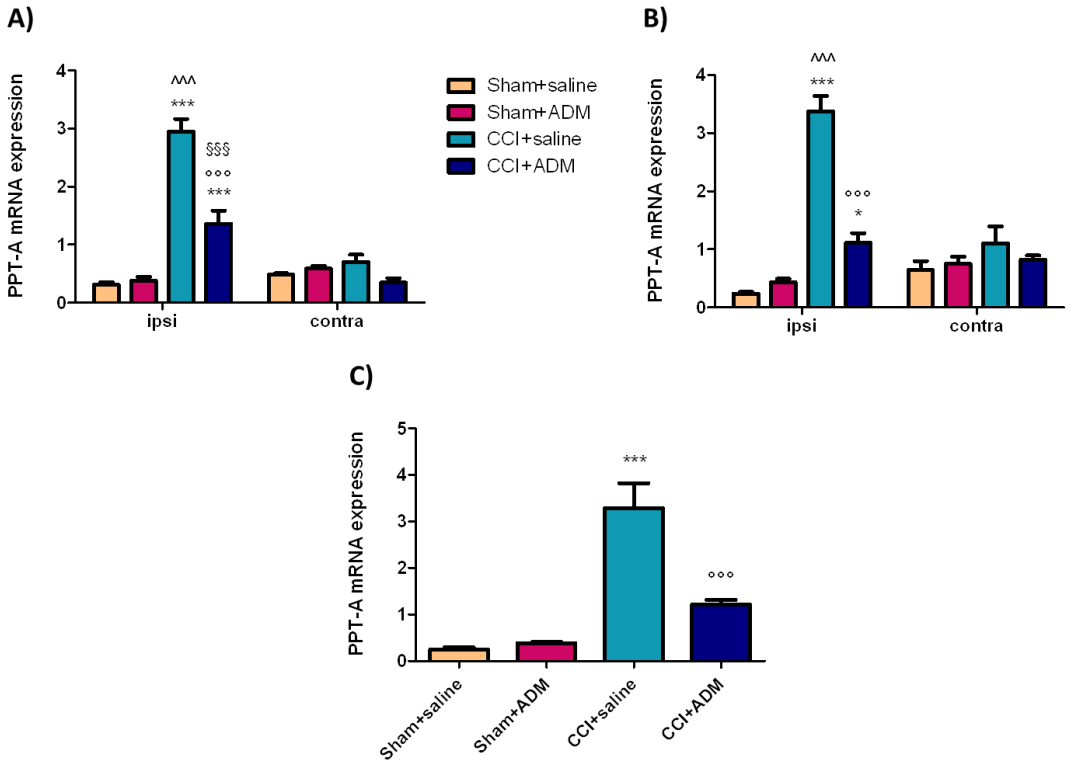

Figure 22. PPT-A mRNA expression in TGs (A), CSC (B) and medulla (C). Data are expressed as mean ± SEM. One way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test or Two-way ANOVA followed by Bonferroni post-hoc test, *p<0.05 and ***p<0.001 vs Sham+saline and Sham+ADM (ipsi); °°°p<0.001 vs CCI+saline (ipsi); ^^^p<0.001 vs CCI+saline (contra); §§§p<0.001 vs CCI+ADM (contra).

5.1.3. ADM_12 effect on neuropeptides protein expression

CGRP and SP protein expression was evaluated in both sides of NTC. A slight, non-significant difference in the density of immunoreactive fibers for CGRP and SP protein was observed between the ipsilateral and contralateral side in the CCI groups (CCI+saline and CCI+ADM) (Figures 23 and 24). No significant change was seen between sham and operated rats (Figure 23). ADM_12 administration did not provoke any change in CGRP and SP expression either in sham or in CCI operated rats (Figure 23).

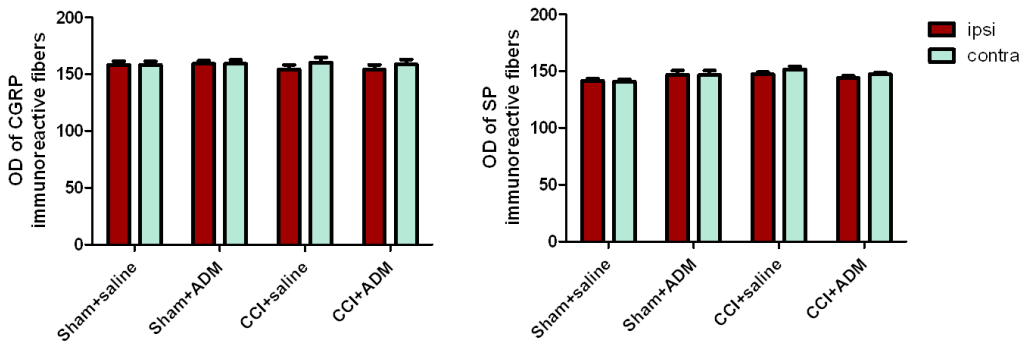

Figure 23. Optical density (OD) values of CGRP and SP immunoreactive fibers in the NTC ipsilateral (ipsi) and contralateral (contra) to the IoN-CCI. Data are expressed as mean ± SEM. Two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test.

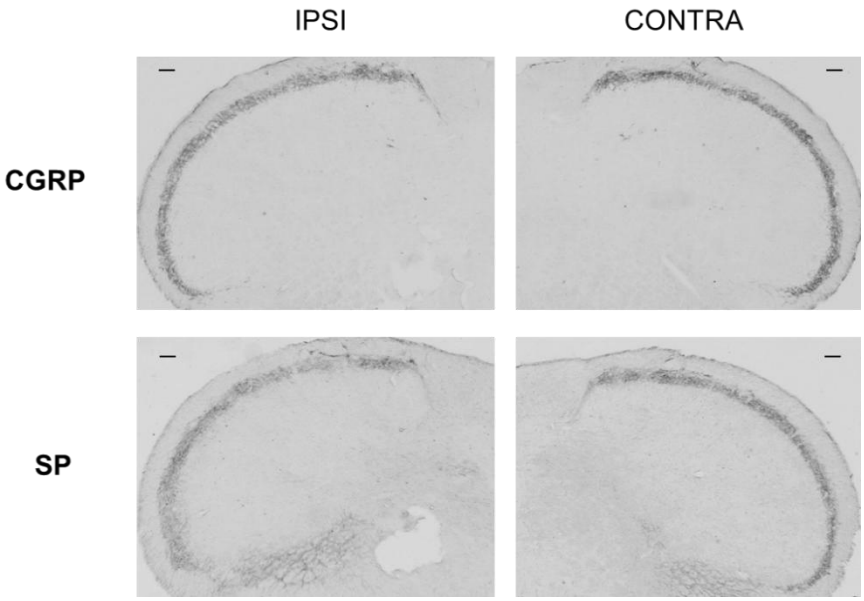

Figure 24. Representative photomicrographs of CGRP and SP immunoreactive fibers in the NTC ipsilateral (ipsi) and contralateral (contra) to the IoN-CCI (CCI+saline group). Scale bar: 100 µm.

5.2. Discussion

Numerous experimental evidences suggest a strong involvement of TRPA1 in different patterns of neuropathic pain, and recently its role was demonstrated in a trigeminal neuropathic pain model as well (Trevisan et al., 2016).

Here we evaluated the role of TRPA1 channels using the antagonist ADM_12 as a probe, in an animal model of trigeminal neuropathic pain in which the infraorbital nerve was loosely ligated (IoN-CCI model). The ADM_12 effects were evaluated both in the behavioral response to mechanical stimulation and neurochemical analysis.

Behavioral response

Infraorbital nerve injury in rats leads to the development, in the ipsilateral side, of a hyporesponsiveness to mechanical stimulation within the first week post operation, followed by a hyperresponsiveness that, according to other studies (Vos et al., 1994; Deseure and Hans, 2015b), reflects a condition of mechanical allodynia. This change in response is probably related to the demyelination process, occurring in the early post-operative period, and remyelination process, that occur in the late post-operative period (Costa et al., 2016). Differently from the above cited papers (Vos et al., 1994; Deseure and Hans, 2015b; Costa et al., 2016), the time needed in this study to develop allodynia is greater, probably because the IoN was tighter ligated; different types of IoN ligation may indeed produce different time course in face grooming behavior (Deseure and Hans, 2015a), and this can be true also for mechanical allodynia.

The allodynic response of operated rats was abolished after treatment with TRPA1 antagonist ADM_12. Probably, the blockade of TRPA1 channels located on the trigeminal afferents prevented the release of neuropeptides thus resulting in a reduced neurogenic inflammation and ultimately the nociceptors sensitization (Gold and Gebhart, 2010). In agreement with this idea, we reported a significant reduction in neuropeptides mRNA expression in IoN-CCI rats after ADM_12 treatment. Moreover, the inhibition of TRPA1 located on glial cells in the nervous system, or on non-neuronal cells, such as keratinocytes and macrophages, in the tissues surrounding the damaged nerve, could contribute in reducing the release of pro-inflammatory factors (Atoyan et al., 2009), which are known to play a crucial role in the pathophysiology of neuropathic pain (Thacker et al., 2007; Mika et al., 2013). Altogether, these processes contribute to the decrease of mechanical allodynia.

The involvement of TRPA1 in mechanosensation has been extensively studied; both genetic deletion of TRPA1 and pharmacological blockade abrogate mechanical pain-like behaviors (Trevisan et al., 2016; Kwan et al., 2009; Kerstein et al., 2009). Recently, Trevisan et al, (2016) confirmed the critical role played by TRPA1 channels in mechanical allodynia induced by trigeminal neuropathic pain; conversely, in a model of sciatic nerve injury, Lehto et al, (2016) reported a not significant involvement of these channels in the mechanical sensitivity. On the other hand, other authors showed that TRPA1 blockade attenuated mechanical hypersensitivity following spinal nerve injury (Wei et al., 2011; Park et al., 2015), or neuropathic pain induced by chemotherapeutic agents (Materazzi et al., 2012; Trevisan et al., 2013). Altogether these observations suggest that mechanical allodynia could be differently mediated by TRPA1 channels depending of type pain or site of damage or to the different distribution profile in TG and DRGs (Vanderwauw et al., 2013). Moreover, the different responses observed in the experimental models could be related also to the different TRPA1 antagonists used, that may inhibit the channel through binding at different sites, with specific regulatory mechanisms (Paulsen et al., 2015).

The present data reinforce the involvement of TRPA1 in mechanical allodynia (Trevisan et al., 2016; Green et al., 2016) at the trigeminal level; mechanical allodynia is one of the major features in clinical presentation of the trigeminal neuropathic pain (Zakrzewska, 2013; Ossipov et al., 2000), and TRPA1 could be a suitable therapeutic target (Garrison and Stucky, 2011; Chen and Hackos, 2015).

TRPA1 and TRPV1 mRNA expression

Chronic constriction injury of the IoN produced a markedly increase in the Trpa1 and Trpv1 mRNA expression in central and peripheral areas ipsilaterally, and a slight increase even at the contralateral side, compared to sham group. This increase is probably due to activation of inflammatory processes occurring after nerve injury, which can affect also the contralateral side (Jancalek, 2011). It is known that TRPA1 and TRPV1 channels can be sensitized by inflammatory agents, causing up-regulation of these channels (Amaya et al., 2003; Diogenes et al., 2007; Devesa et al., 2011). For example, TRPA1 expression has been shown to be up-regulated by TNFα and interleukin-1 α via transcriptional factor hypoxia-inducible factor-1α and NF-kB pathways (Hatano et al. 2012). Similarly, TNFα can up-regulate TRPV1 in DRG and TG neurons (Hensellek et al., 2007; Khan et al., 2008).

Furthermore, an important role in neuropathic pain seems to be played by oxidative stress (Kim et al., 2004; Naik et al., 2006) whose components can directly activate TRPA1 channels (Andersson et al., 2008), thereby contributing to inflammation in a TRPA1-dependent manner. Indeed, it was recently found that the trigeminal neuropathic pain behaviors were mediated by TRPA1 targeted by oxidative stress by-products released from monocytes and macrophages surrounding the site of nerve injury (Trevisan et al., 2016).
In agreement to our study, an up-regulation of TRPA1 and TRPV1 mRNA levels, as well as protein levels, in TG, DRGs and dorsal horns were seen in different models of neuropathic pain, (Urano et al., 2012; Hudson et al., 2001; Obata et al., 2005; Frederick et al., 2007; Park et al., 2015; Fukuoka et al., 2002; Quartu et al., 2014; Kim et al., 2012).

In contrast to the data obtained in the migraine animal model, systemic administration of ADM_12 markedly reduced the mRNA expression levels of both TRPs induced by IoN ligation. Thus, ADM_12 appears to be a specific antagonist for TRPA1 channels in migraine pain, but in trigeminal neuropathic pain it seems to act also on TRPV1 channels. Probably, the damage induced by the nerve injury lead to a re-organization in expression and nature of the channels (Matzner and Devor, 1994; Waxman et al., 1994; McCallum et al., 2011) that made ADM_12 able to block TRPV1 channels. Alternatively, since TRPA1 and TRPV1 are functionally linked, ADM_12 could have a direct effect on TRPA1 and an indirect effect on TRPV1 channels. Indeed, these channels are able to form heteromultimeric complexes (Staruschenko et al., 2010; Fischer et al., 2014), that may result in considerable effects on channel biophysical properties, pharmacology, signalling, regulation, and ultimately function (Akopian, 2011). Therefore, further studies on protein and pattern expression are needed to fully characterize the involvement and recruitment of TRP channels in the trigeminal neuropathic pain.

Neuropeptides expression

After nerve injury, inflammatory process leads to the release of many pro-inflammatory mediators with consequent decrease in nociceptors activation threshold and increase in nervous fiber excitability. Together with the inflammatory process, neuropeptides and degenerative nervous fibers changes are also crucial peripheral mechanisms. Inflammatory mediators participate in peripheral sensitization promoting an excessive release of neurotransmitters. Here, although a tendency towards a decrease was seen in the ipsilateral

side compared to the contralateral side in the IoN-CCI rats, no significant differences in neuropeptides protein expression at the NTC level were seen, neither between groups nor between sides. It is possible that the immunohistochemical technique did not fully highlighted the changes occurred, thus additional experiments with a different technique will be achieve to provide clear results.

Similarly to the results obtained in this study, Lynds et al. (2017) reported no differences in neuropeptides (CGRP and SP) levels between ipsi- and contralateral TG 2 weeks after IoN transection injury. Conversely, Xu et al, (2008) reported a reduction of CGRP and SP protein levels in the ipsilateral caudal medulla 8 days after partial IoN ligation. These observations suggest that the neuropeptides release likely takes place at early time points after surgery (Costa et al., 2016), and that they are mostly involved at the peripheral terminals (Costa et al., 2016). However, the mRNA expression levels of genes coding for these neuropeptides are highly increased in the central areas containing the NTC, as well as the TG ipsilateral to the IoN ligation, thus suggesting a probable role for the neuropeptides even after 4 weeks from the injury. Interestingly, we found an increased expression of Calca (CGRP) mRNA in IoN ligated rats even at the contralateral TG. It has been shown that projections from the TG reach the medullary and cervical dorsal horns on both sides (Pfaller and Arvidsson, 1988; Jacquin et al., 1990), and that unilateral TG stimulation activates neurones in both ipsi- and contralateral NTC (Ingvardsen et al., 1997; Samsam et al., 2001).

One of the mechanisms that could contribute to neuropeptides expression is the Ca(2+)/calmodulin-dependent protein kinase (CaMK) - cAMP response element-binding protein (CREB) cascade, which is probably triggered following TRP channels activation (Nakanishi et al., 2010). Possibly, this effect is reverted by administration of the TRPA1 antagonist ADM_12, which markedly reduced mRNA expression levels of CGRP and SP. Similarly to the migraine model, the blockade of TRP channels, which co-localize with CGRP and SP in the trigeminal neurons (Story et al., 2003; Huang et al., 2012), can inhibit Calca and PPT-A mRNA expression thus reducing the neuropeptides release and the trigeminal sensitization process. The data support the pivotal involvement of CGRP and SP in the delivery and transmission of pain sensation to the central nervous system, and their role in trigeminal pain syndrome. In fact, an increased concentration of neuropeptides was

found in cerebrospinal fluid and venous blood of patients with trigeminal neuralgia compared to healthy controls (Qin et al., 2016; Strittmatter et al., 1997).

6. CONCLUSION

Migraine and trigeminal neuropathic pain share a lot of similarities; actually, some authors suggest migraine to behave like a neuropathic pain disorder (Chakravarty and Sen, 2010; Biondi, 2006). Both are characterized by sensitization and hyperexcitability of the trigeminal system, with consequent allodynic and hyperalgesic phenomena associated to dural neurogenic inflammation (Chakravarty and Sen, 2010; Filipović et al., 2014). These disorders share as well some therapeutic strategies, such as treatment with antiepileptic drugs (Sidhu e Sadhotra, 2016). Moreover, it has been shown in experimental studies that CGRP receptor antagonists (efficacious in migraine pain) reduced the mechanical allodynia induced by IoN ligation (Michot et al., 2015). Taken together these observations reveal common pathophysiological mechanisms that are still under intense investigation. Understanding the pathophysiology underlying migraine and trigeminal neuropathic pain, will shed light on the complex mechanisms that take part in the trigeminal pain.

Our data show that TRPA1 blockade by ADM_12 treatment exerted an anti-allodynic effect in the rat model of trigeminal neuropathic pain and an anti-hyperalgesic effect in the animal model of migraine, suggesting that it might be useful in the treatment of these trigeminal pain disorders. Moreover, our data suggest an important role also for TRPV1 channels, which could be differently involved depending on the type of pain. Further exploration on the mechanisms underlying the antinociceptive effects of TRPA1, and studies directed to better understand the TRP-related pathways as well as the relationship between TRPA1 and TRPV1 should improve our understanding of nociceptive processing in trigeminal pain.

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8. ABBREVIATIONS

5-HT: 5-hydroxytryptamine

AITC: allyl isothiocyanate

CGRP: calcitonin gene-related peptide

CNS: central nervous system

CSC: cervical spinal cord

DAB: 3′3′-diaminobenzidine tetrahydrochloride

DMSO: dimethylsulfoxide

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GBD: Global Burden of Disease

GPCRs: G protein–coupled receptors

IASP: International Association for the Study of Pain

ICHD: International Classification of Headache Disorders

IHC: immunohistochemistry

IL-6: interleukin-6

iNOS: inducible nitric oxide synthase

IoN-CCI: constriction injury of the infraorbital nerve

KPBS: potassium phosphate buffered saline

LC: locus coeruleus

MST: mechanical stimulation testing

NF-κB: nuclear factor-κB

NMDA: N-methyl-D-aspartate

NO: nitric oxide

NOS: nitric oxide synthase

NSAIDs: non-steroidal anti-inflammatory drugs

NTC: nucleus trigeminalis caudalis

NTG: nitroglycerin

OD: optical density

PAG: periaqueductal grey

PB: parabrachial area

PGE2: prostaglandin E2

PLC: phospholipase C

RT-PCR: real time polymerase chain reaction

S1: primary somatosensory cortex

SP: substance P

TG: trigeminal ganglia

TN: trigeminal nerve

TNF-α: tumor necrosis factor α

TRP: transient receptor potential channels

TRPA: TRP ankyrin

TRPC: TRP canonical

TRPM: TRP melastatin

TRPML: TRP mucolipin

TRPP: TRP polycystin

TRPV: TRP vanilloid

VPM: ventral posteromedial nucleus

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