CROSS-TALK BETWEEN SENSORY NEURONS AND NON-NEURONAL CELLS IN TRIGEMINAL GANGLIA OF A MIGRAINE MOUSE MODEL

Thesis submitted for the degree of
"Doctor Philosophiae"

S.I.S.S.A
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To Liliana and Renzo

To Andrea

Bonum certamen certavi,
cursum consummavi,
fidem servavi

Declaration

The work described in this thesis was carried out at the International School for Advanced Studies, (Trieste, Italy) between November 2007 and December 2011.
The data reported in the present thesis have been performed by the candidate in collaboration with the electrophysiologist students in our lab for patch clamp technique (Hullugundi S.K. and Nair A.). This data are going to be published in different articles, which are listed below, in preparation. In all cases the candidate contributed to data analysis, the interpretation and discussion of the results and to the writing of manuscripts.

Manuscripts in preparation:

Franceschini A., Vilotti S., Ferrari M.D., van den Maagdenberg A.M.J.M., Nistri A. and Fabbretti E.
"TNFα levels and macrophage activation reflect an inflammatory potential of trigeminal ganglia in transgenic mouse model of Familial Hemiplegic Migraine"

Franceschini A., Hullugundi S., van den Maagdenberg A.M.J.M., Fabbretti E. and Nistri A.
"Acute inflammatory stimulus by LPS differentially activates P2X3-sensitive sensory neurons and macrophages of cultured trigeminal ganglia from WT and R192Q KI mice."

Franceschini A., Hullugundi S., Abbate R., Nair A., van den Maagdenberg A.M.J.M., Nistri A. and Fabbretti E.
"Functional crosstalk between sensory neurons and macrophages in trigeminal ganglia in relation to migraine pain".

CONTENTS

List of Abbreviations

5-HT	serotonin;
α,β-meATP	α, β methylene - adenosine 5'-triphosphate;
ATP	adenosine-5'-triphosphate;
APCs	antigen presenting cells;
ASIC	acid-sensing ion channels;
BBB	blood-brain barrier;
BDNF	brain-derived neurotrophic factor;
BOLD fMRI	blood oxygenation level-dependent functional magnetic resonance imaging;
BSA	bovine serum albumin;
cAMP	cyclic adenosine monophosphate;
CALCRL	calcitonin receptor-like receptor;
CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit;
$Ca_V2.1$	voltage activated calcium channel 2.1;
CBF	cerebral blood flow;
CCL2	chemokine (C-C motif) ligand 2;
CCR2	chemokine (C-C motif) receptor 2;
CD	cluster of differentiation;
CGRP	calcitonin gene relates peptide;
CNS	central nervous system;
CSD	cortical spreading depression;
CX3CR1	CX3C chemokine receptor 1;
DAG	diaglycerol;
DAPI	4',6-diamidino-2-phenylindole;
DED	death effector domain;
DD	death domain;
DRG	dorsal root ganglia;
F4/80	mouse homologue of EGF-like module-containing mucin-like hormone receptor-like 1;
FADD	fas-associated death domain;
FBS	fetal bovine serum;
FHM-1	Familial Hemiplegic Migraine type 1;

FITC	fluorescein isothiocyanate;
GAPDH	glyceraldehyde 3-phosphate dehydrogenase;
GFAP	glial fibrillary acidic protein;
GS	glutamine synthetase;
Iba1	ionized calcium binding adaptor molecule 1;
IFN-γ	interferon gamma;
IFNGR	interferon gamma receptor;
IKK	I-kappaB kinases;
IL	interleukin;
IL1R	interleukin-1 receptor;
IL1RAcP	IL1R accessory protein;
IRAK	IL1 receptor-associated kinases;
i.p.	intraperitoneal injection;
i.v.	intravenous injection;
JNK	c-Jun N-terminal kinases;
MA	migraine with aura;
MHC	major histocompatibility complex;
MO	migraine without aura;
KI	knock-in;
LIF	leukemia inhibitory factor;
LPS	lipopolysaccharide;
M0	non-polarized macrophage;
M1	classically activated macrophage;
M2	alternatively activated macrophage;
MΦ	peritoneal macrophage;
MAP	mitogen activated protein;
MAP2	microtubule-associated protein 2;
MAPK	MAP kinase;
MCP-1	monocyte chemotactic protein 1;
MMP	matrix metallopeptidase;
mRNA	messenger ribonucleic acid;
NSAIDs	non-steroidal anti-inflammatory drugs;
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells;
NGF	nerve growth factor;
NIK	NF-κB-inducing kinase;

P*X*	post natal day *X*;
P2X	purinergic 2X receptor;
P2Y	purinergic 2Y receptor;
PAG	periaqueductal gray;
PAMPs	pathogen-associated molecular patterns;
PBMc	peripheral-blood mononuclear cells;
PBS	phosphate buffered saline;
pERK	phospho extracellular-signal-regulated kinases
PET	positron emission tomography;
PGE_2	prostaglandin E_2;
PI3K	phosphatidylinositol 3-kinases;
PKC	protein kinase C;
PNS	peripheral nervous system;
RAMP	receptor activity modifying protein
rCBF	regional cerebral blood flow;
RCP	receptor component protein;
RIP	receptor interacting protein;
ROI	region of interest;
RT-PCR	real time PCR
SEM	standard error of the mean;
SGC	satellite glial cells;
SP	substance P;
STAT	signal transducer and activators of transcription;
TACE	TNF-α converting enzyme;
TG	trigeminal ganglia;
TGF-β	transforming growth factor beta;
Tc	cytotoxic T cells;
Th	T-helper cells;
TLR	toll-like receptor;
TNG	trigeminal nucleus caudalis;
TNFα	tumor necrosis factor alpha;
TNFR	tumor necrosis factor receptor;
TOLLIP	tool-interacting protein
TRADD	TNFR associated domain;
TRAF	TNF receptor associated factors;

Tregs	regulatory T cells;
TrkA	tyrosine kinase receptor type A;
TRPV1	transient receptor potential vanilloid 1;
VNUT	Cl^--dependent vesicular nucleotide transporter;
WT	wild-type;
Zy-FITC	zymosan A FITC-conjugated.

Chapter 1: ABSTRACT

Latent changes in trigeminal ganglion structure and function may predispose to acute attacks of migraine pain. In this thesis, we investigated whether changes in trigeminal ganglia may contribute to produce an inflammatory background phenotype compatible with sensitization of sensory neurons.

To this aim, we used a knock-in (KI) mouse model for Familial Hemiplegic Migraine type 1 (FHM-1) expressing the R192Q mutation in the α1 subunit of $Ca_V2.1$ (P/Q-type) calcium channels, known to have enhanced released of migraine mediators like CGRP, and higher P2X3 receptor function. We found that R192Q KI gain-of-function stimulated the occurrence of a global inflammatory background in the trigeminal ganglia that appear to be readily activated by inflammatory stimuli. In selected experiments, we tested *in vivo* and *in vitro* how a well-known systemic inflammatory stimulus like LPS, might change inflammatory markers (specific immune antigens and cytokine expression) in trigeminal ganglia and functional responses of nociceptive neurons. In addition, trigeminal ganglia were co-cultured with peritoneal macrophages to obtain functional evidence for the crosstalk between neurons and non-neuronal cells of inflammatory lineage (macrophages).

We found that R192Q KI ganglia were characterised by a stronger presence of active macrophages and higher release of TNFα that might contribute to migraine pain. Our data support the hypothesis that soluble factors like peptides, ATP, cytokines and chemokines, released from sensitised neurons as well as from active macrophages, are essential elements in the cellular cross-talk necessary to modulate ganglia hypersensitivity and chronic pain progression.

Chapter 2: INTRODUCTION

2.1 Migraine

2.1.1 - Definition and prevalence

Migraine is disabling, costly, under-diagnosed, and undertreated disease with incompletely understood pathophysiology. Epidemiologic studies on migraine and headache in Europe revealed that this pathology constitutes a major public health problem (Stovner et al., 2006). The economic impact of migraine disorder, in terms of lost productivity and increased health care, continues to be of great concern (Berry, 2007).

There are different clinical diagnostic criteria to distinguish headache and migraine, although the definition of the term "migraine" derives from the word hemicrania and refers to the unilateral localization of pain, even if bilateral pain is often present (Moskowitz and Buzzi, 2010). Considering all headaches, the prevalence is 51% (men 41% and women 58%) (Stovner et al., 2006).

Migraine is characterized by severe, recurrent stereotype chronic headaches associated with neurological, gastointestinal and autonomic changes. Migraine prevalence is 12%, in particular 16% in women and 8% in men (Stovner et al., 2006).

Migraine has different occurrence depending by ages (Fig. 2.1): the highest prevalence is from the age group 25-50 years old (Stovner et al., 2006).

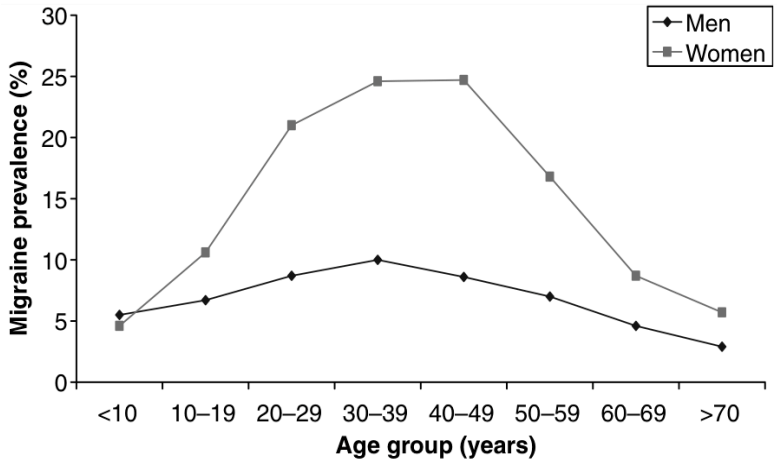


Fig. 2.1 Migraine prevalence related to age

(Stovner et al., 2006)

2.1.2 - Subtypes of migraine

Migraine is divided in two major subtypes: migraine without aura (MO) and migraine with aura (MA) with further subdivisions (Table 2.1).

Table 2.1 - Migraine classification according to the International Classification of Headache Disorders, 2nd edition (ICHDII) (Headache Classification Subcommittee of the International Headache Society, 2004)

1. Migraine
1.1 Migraine without aura
1.2 Migraine with aura
1.2.1 Typical aura with migraine headache
1.2.2 Typical aura with non-migraine headache
1.2.3 Typical aura without headache
1.2.4 Familial hemiplegic migraine
1.2.5 Sporadic hemiplegic migraine
1.2.6 Basilar-type migraine
1.3 Childhood periodic syndromes that are commonly a precursor of migraine
1.3.1 Cyclical vomiting
1.3.2 Abdominal migraine
1.3.3 Benign paroxysmal vertigo of childhood
1.4 Retinal migraine
1.5 Complications of migraine
1.5.1 Chronic migraine
1.5.2 Status migrainosus
1.5.3 Persistent aura without infarction
1.5.4 Migrainous infarction
1.5.5 Migraine-triggered seizures
1.6 Probable migraine
1.6.1 Probable migraine without aura
1.6.2 Probable migraine with aura
1.6.3 Probable chronic migraine

(Moskowitz and Buzzi, 2010)

The diagnostic criteria for migraine remained fairly stable from the first edition to the second edition of migraine classification, with the most prominent changes including the addition of criteria for the diagnosis of chronic migraine and the separate categorization of hemiplegic migraine (Moskowitz and Buzzi, 2010).

What a patient experiences before, during and after an attack cannot be defined exactly. During a migraine attacks it is possible to distinguish 4 different phases of migraine (Fig. 2.2).

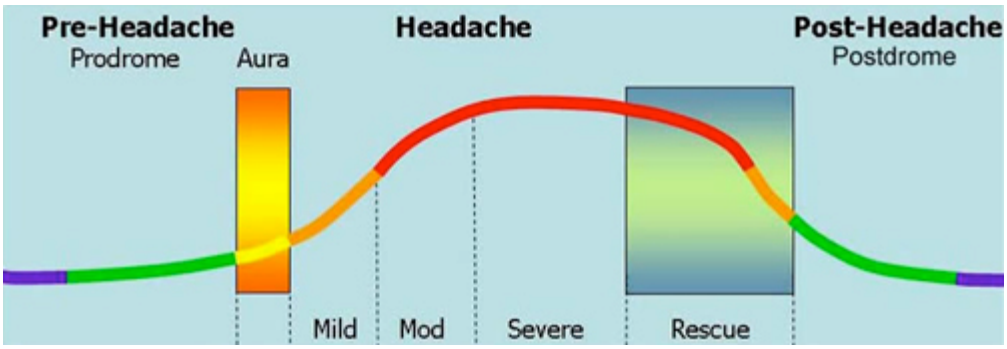


Fig. 2.2 - The different phases of migraine

(http://www.headachecare.com)

The first phase is the *Prodrome*, which occurs hours or days before the headache and consists of altered mood and other general symptoms.

The second possible phase is the *Aura*, which immediately precedes or accompanies the headache attack. Symptoms of migraine aura can be visual, sensory or motor in nature.

After that there is the *Pain phase*, also known as headache phase, typically characterized by unilateral, pulsating, moderate to severe headache lasting between 4 and 72 hours accompanied by other symptoms such as nausea photophobia and phonophobia.

The last phase is the *Postdrome*, were the patient might feel tired, have head tenderness, cognitive difficulties, mood changes and weakness.

Migraine without aura (MO) is quite common and affects 80% of migraineurs (Evans and Mathew, 2004) with the following characteristics:

- duration of 4 – 72 h;
- two of more of the following headache characteristics: unilateral (60%) or bilateral (40%) location, pulsating quality, moderate to severe intensity, aggravation by physical activity;
- one or more associated symptoms occurring during the attack: nausea/vomiting or both photophobia and phonophobia;
- attacks must not be attributable to another disorder.

The frequency of attacks is extremely variable. In 85% of cases there is throbbing pain, indeed if the patients are not under pharmacological treatment, 80% of them have moderate-severe intensity pain. The signs and symptoms of migraine vary among patients (Cutrer and Martin, 2010).

2.1.3 – Migraine with Aura

Migraine with aura (MA) affects the 20% of migraineurs. It is characterized by an additional typical phase of headache attack: the aura. "Aura" is defined by International Headache Society (IHS) as a "recurrent disorder manifesting in attacks of reversible focal neurological symptoms that usually develop gradually over 5–20 minutes and last for less than 60 minutes". Aura phase generally precedes or accompanies the appearance of migraine headache, and it is predominantly visual (Cutrer and Martin, 2010). To fulfill the IHS diagnostic criteria for migraine with aura, an individual must have had at least two attacks involving an aura consisting of at least one of the following: a) fully reversible visual symptoms, including positive features (flickering lights, spots, or lines) and/or negative features (loss of vision); b) fully reversible sensory symptoms, including positive features (pins and needles) and/or negative features (numbness); c) fully reversible dysphasic speech disturbance. Furthermore, visual aura is the most common aura symptom, occurring in 99% of patients, followed by sensory, language, and motor auras. Visual aura occurs without any other aura symptoms in 64% of cases, whereas other aura types usually occurr in association with another aura type (Cutrer and Martin, 2010).

The phenomenon of aura is related to spreading depression, namely a wave of complete neuronal and glial depolarization found in migraine patients (Ayata et al., 2010, Pietrobon, 2010a). Today, aura is defined as an alteration in cortical function that usually begins in the occipital lobe and spreads forward. The mechanism by which aura transduces the headache of migraine remains to be definitively determined. Although not all studies agree, the majority suggests that hyper excitability and cortical depression could be a model for aura (Cutrer and Martin, 2010; Pietrobon, 2010a).

Interestingly, in the later migraine classification, motor symptoms are included as an aura subtype and considered as diagnostic of hemiplegic migraine. However, the typical aura symptoms (visual, sensory, and language) very frequently occur in hemiplegic migraine (Cutrer and Martin, 2010; Pietrobon, 2010a).

2.1.4 – The pathophysiology of migraine

The mechanisms of migraine remain incompletely understood. However, different studies and new technologies have allowed formulation of current concepts that may explain parts of the migraine syndrome. The various theories generally agree about the involvement of trigemino vascular system.

2.1.4.1 – The trigeminal vascular system

The trigeminal system has been postulated to play a significant role in the pathophysiology of migraine. In fact, the most outstanding feature that distinguishes migraineurs from normal subjects

is the susceptibility to recurrent activation of the trigeminovascular and upper cervical system (Moskowitz and Buzzi, 2010).

The trigeminal pathway involves trigeminal ganglia (TG), trigeminal nucleus caudalis (TNC) in the brainstem, some thalamic nuclei and the somato sensory cortex. The trigeminal vascular system consists of the neurons innervating the cerebral vessels whose cell bodies are located in the trigeminal ganglion. The ganglion contains bipolar cells: the peripheral fibres make synaptic connection with vessels, and other cranial structures, particularly the pain-producing large cranial vessels and dura mater; the centrally projecting fibres make synapses with neurons in the caudal brain stem or high cervical cord. Tracing studies have identified the trigeminal nerve as the major afferent pathway for pain from the vessels and dura mater (Thalakoti et al., 2007).

These fibres are predominantly found in the first (ophthalmic) division of the trigeminal nerve and have widely ramifying axons that may innervate several vessels ipsilaterally. The trigeminal innervation is predominantly to the forebrain, but it extends posteriorly to the rostral basilar artery, whereas the more caudal vessels are innervated by the C2 and C3 dorsal roots, which also synapse with the central trigeminal neurons (May and Goadsby, 1999).

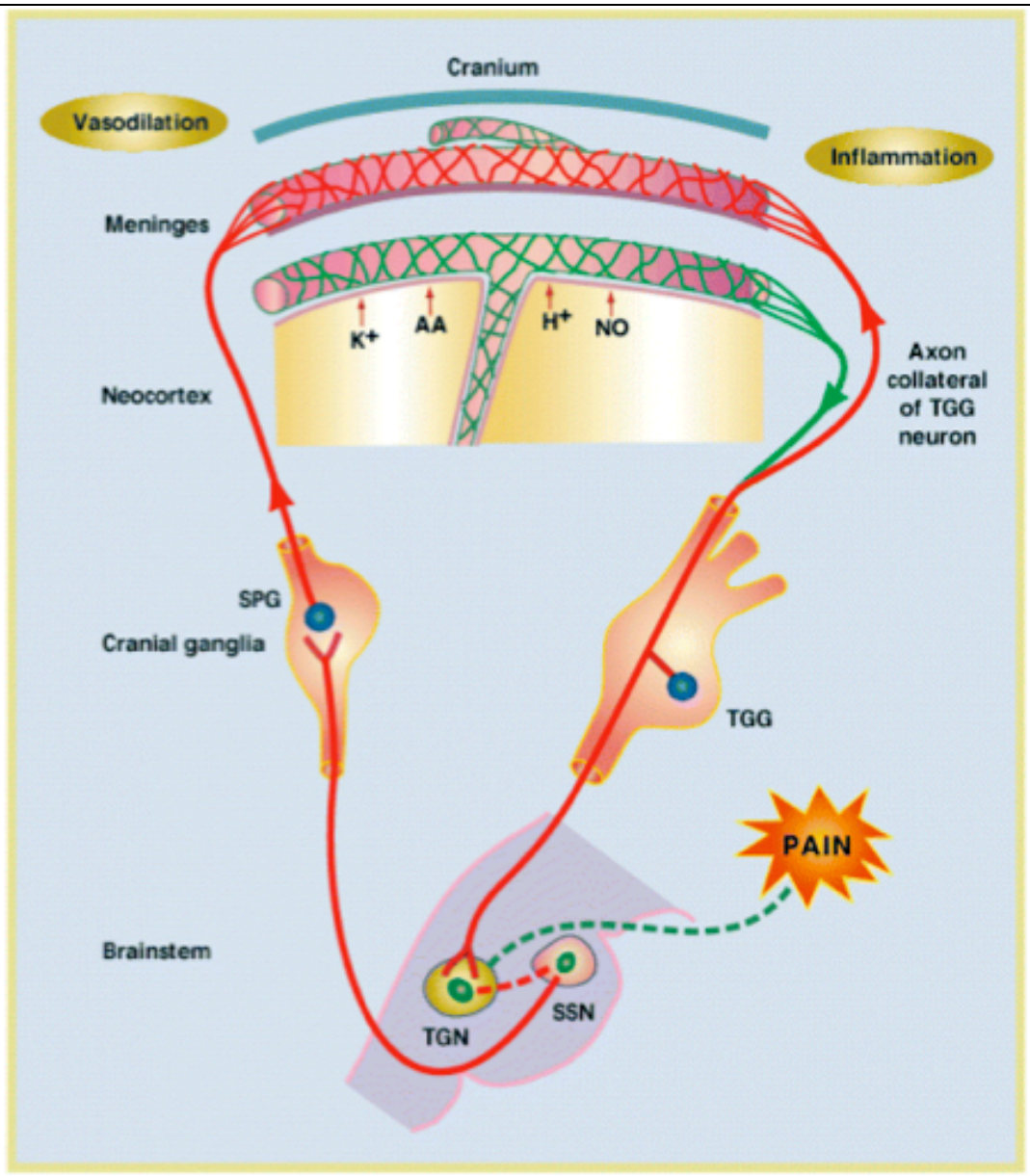


Fig. 2.3 - Relationship between the trigeminal nerve, meninges, brainstem, and parasympathetic efferents.

(Moskowitz, 2007)

Although the brain tissue is insensitive to pain, the trigeminal nerve innervates blood vessels in the meninges, circle of Willis, and extracranial arteries, and can carry pain impulses from these structures.

2.1.4.2 – The vascular theory

The vascular theory was proposed in the '40-50s principally by Wolff in order to explain migraine pathophysiology: he believed that ischemia induced by intracranial vasoconstriction is responsible for the aura of migraine, the subsequent vasodilation and activation of perivascular nociceptive nerves resulted in headache. The idea of Wolff was originated by the isolation and clinical introduction of ergotamine (vasoconstrictor) as anti-migraine drug at the beginning of 20th century

(Tfelt-Hansen and Koehler, 2011).

This theory was based on the following observations: extracranial vessels become distended and pulsatile during a migraine attack, stimulation of intracranial vessels in an awake person induces headache and vasodilators provoke an attack, whereas vasoconstrictors have anti-migraine effects. However, this theory has few weak points: the efficacy of some drugs used to treat migraines that have no effect on blood vessels, and recent studies found that intracranial blood flow patterns were inconsistent with the vascular theory (Tfelt-Hansen and Koehler, 2011).

This theory was recently reconsidered and revised, even if it is not more considered by many headache specialists. The possibility can not be exclude that extracranial vasodilatation is a source of pain in migraine, albeit accompanied by lowered pain threshold and concurrent local sterile inflammation (Shevel, 2011).

2.1.4.3 – The neurologic theory

In the 80's, the vascular theory led researchers to study regional cerebral blood flow (rCBF): during the aura phase all patients developed rCBF reduction (oligemia). Oligemia gradually spread anteriorly in the course of 15 to 45 minutes. These results were interpreted by Lassen indicating "that the vasospastic model of the migraine attack is too simplistic. Alteration in neuronal function, in the blood–brain barrier (BBB), or in some other brain process, is more likely to be the primary event of the attack" (Tfelt-Hansen and Koehler, 2011). Thus, it was considered the Cortical Spreading Depression (CSD) as a primary neuronal process underlying the spreading oligemia.

2.1.4.3.1 – Cortical Spreading Depression

Cortical Spreading Depression (CSD), discovered by Leão in 1944, is a self-propagating depolarisation of neurons and glia associated with depressed neuronal electrical activity and transient loss of membrane ionic gradients, and massive surges of extracellular potassium (K^+), intracellular calcium (Ca^{2+}) as well as neurotransmitters. Leão first observed that CSD leads to transient dilatation of pial arteries (Dalkara et al., 2006).

The depolarization wave is followed by repolarization, which once initiated by a variety of chemical, electrical, and mechanical stimuli progresses outward across the surface of the cerebral cortex at a speed of approximately 2-5 mm/min (Leão, 1944; Lauritzen, 1987; Busija et al., 2008). CSD has been demonstrated in almost all the grey matter regions of the central nervous system (CNS) (Dalkara et al., 2006), and is associated with major changes in extracellular levels of ions (K^+ concentration), neurotransmitters (glutamate) and cerebral blood flow (CBF) (Busija et al., 2008). In 2001, Hadjikhani using imaging studies (blood oxygenation level-dependent functional magnetic resonance imaging - BOLD fMRI) demonstrated that an electrophysiological event such

as CSD generated the aura in the visual cortex (Tfelt-Hansen and Koehler, 2011). In 1996 a study by Shimazawa and Hara in rats demonstrated that CSD caused a long-lasting blood flow enhancement selectively within the middle meningeal artery (Tfelt-Hansen and Koehler, 2011). In addition, CSD provoked plasma protein leakage within the dura mater. The results provided a neural mechanism, dependent on trigeminal and parasympatic activation, by which extracerebral cephalic blood flow couples to CSD and it was suggested that a similar mechanism in man explains the headache in migraine with aura. Auras with motor or other sensory symptoms probably also result from CSD-like events within primary motor or sensory cortices (Pietrobon and Striessng, 2003). Indeed, CSD influences the expression of many genes associated with inflammation even in healthy brain (Thompson and Hakim, 2005).

2.1.4.3.2 – Brainstem generator

Some researcher postulated that migraine originates in brainstem. The hypothesis of the presence of a "brainstem generator" emphasizes the key role for descending modulation, and a change in modulation from rostral structures, activating the brainstem to initiate of a migraine attack. PET studies demonstrated strong brainstem activation in association with an acute, spontaneous attack in patients with migraine without aura (Pietrobon and Striessnig, 2003). The authors stated, "it is tempting to consider the observed activation in the brainstem as the visualization of the postulated migraine centre in humans" (Tfelt-Hansen and Koehler, 2011). This view proposes that an episodic dysfunction in brainstem nuclei involved in the central control of nociception is the primary cause of migraine headache. In support of the theory, perturbation of PAG produces migraine-like headaches in non-migraineurs, and blood flow increases in several areas of the dorsal rostral brainstem during migraine attacks. However, the theory doesn't explain how the trigeminovascular afferents become activated as a consequence of brainstem dysfunction (Pietrobon and Striessnig, 2003). Moreover, not in all patients it was observed an activation of brainstem nuclei involved in the central control of nociception.

2.1.4.4 – The neurogenic inflammation theory

Moskowitz postulated the neurogenic inflammation theory in 1984. He proposed that the propagation of the migraine pain results from the action of inflammatory neuropeptides (like calcitonin gene related peptide – CGRP, and substance P - SP), released by sensory fibers activated by different triggers (CSD, brainstem dysfunction, altered cortical excitability) that may active on specific centers in the brain (Moskowitz, 1984; Moskowitz, 1993; Tfelt-Hansen and Koehler, 2011). In particular, neural activation releases vasoactive neurotransmitters from their afferent processes, which in turn provokes inflammatory changes in peripheral target tissues (in this instance, cerebral

blood vessels). In addition, the release of substance P from sensory fibers is important in mediating changes in vessel permeability, explaining the unilateral distribution of migraine pain and directing the pain to dilating blood vessels (Tfelt-Hansen and Koehler, 2011). Moskowitz suggested that there is a "sterile inflammation" of cranial blood vessels during migraine attacks: "the relationship of trigeminovascular fibers to the pathogenesis of vascular head pain sheds light on possible mechanisms of migraine and other central nervous system conditions associated with headache and inflammation" (Moskowitz, 1984; Tfelt-Hansen and Koehler, 2011).

This theory can integrate different theories and previous findings in patients: the decrease in cerebral blood flow is then thought to trigger cortical spreading depression, which in turn stimulates trigeminal nerve fibers, which then release neuropeptides such as substance P, neurokinin A, and CGRP. These neuropeptides then promote vasodilatation and a sterile inflammatory response around nearby meningeal blood vessels, eliciting neurogenic perivascular inflammation, vasodilatation, and headache pain (Pulley et al., 2005). In addition, these neuropeptides may sensitize nerve endings, which may result in prolongation of the headache.

2.1.4.5 – Sensitization

Clinical studies underline the development of peripheral and central sensitization during migraine. If activation of the trigeminal system is transient and relatively brief, other mechanisms probably intervene to sustain the headache. Two processes may be important: (1) peripheral sensitization of the primary afferent neuron; and (2) central sensitization of higher-order neurons within the spinal cord and brain (Moskowitz and Buzzi, 2010).

Among the common symptoms of peripheral sensitization during migraine one is the throbbing feeling of the headache and its aggravation during routine physical activities that increase intracranial pressure. Such intracranial hypersensitivity involves the sensitization of nociceptors that innervate the meninges (Strassman et al., 1996). Peripheral sensitization is characterized by increased excitability of primary afferents in response to external mechanical or thermal stimuli at the original site of inflammation. A large number of mediators produced at the site of tissue injury and inflammation can promote the excitation and sensitization of nociceptors (Burstein and Jakubowski, 2010). Mediators such as bradykinin, histamine, serotonin (5-HT) and prostaglandin E_2 (PGE_2) can promote both excitation and mechanical sensitization at meningeal level; other mediators such as cytokines, interleukins 1, 6, and 8 (IL1, IL6, IL8) and tumor necrosis factor-alpha (TNFα) can promote peripheral sensitization (Sachs et al., 2002; Burstein and Jakubowski, 2010). Additional inflammatory mediators are protons, proteases, and nitric oxide (Strassman et al., 1996).

A classical symptom of central sensitization during migraine is the phenomenon of allodynia (a painful response to a non-painful stimulus), when patients become irritated by mundane mechanical

and thermal stimulation of the scalp and facial skin. Such allodynia involves the sensitization of nociceptive trigeminovascular neurons of the medullary dorsal horn that receive converging sensory inputs from the dura and the skin (Burstein et al., 1998). Central sensitization is triggered by sensory inputs arriving from sensitized nociceptors that supply the affected site. Sensitized dorsal horn nociceptors become responsive to innocuous (previously sub-threshold) sensory signals that arrive from areas outside the affected site. Burstein posited that central sensitization could also be produced in trigeminovascular neurons because topical applications of inflammatory agents on the exposed rat dura activate and sensitize the trigeminovascular pathway for many hours (Burstein and Jakubowski, 2010). Accordingly, innocuous skin stimuli evoke dramatic activity in central trigeminovascular neurons during migraine, when they are sensitized, but produce little or no response in the absence of migraine, when they are not sensitized (Burstein and Jakubowski, 2010).

Despite the growing body of evidence regarding migraine pathophysiology, an attempt to identify a single unifying theory to explain susceptibility continues to be unsuccessful, partially because there is a wide genetic heterogeneity combined with wide variations in migraine phenotype (Cutrer, 2006).

However, the "final common pathway" involves the activation of the trigeminocervical pain system, which may arise from more than one initiating process (Fig. 2. 4; Charles and Brennan, 2010; Moskowitz and Buzzi, 2010).

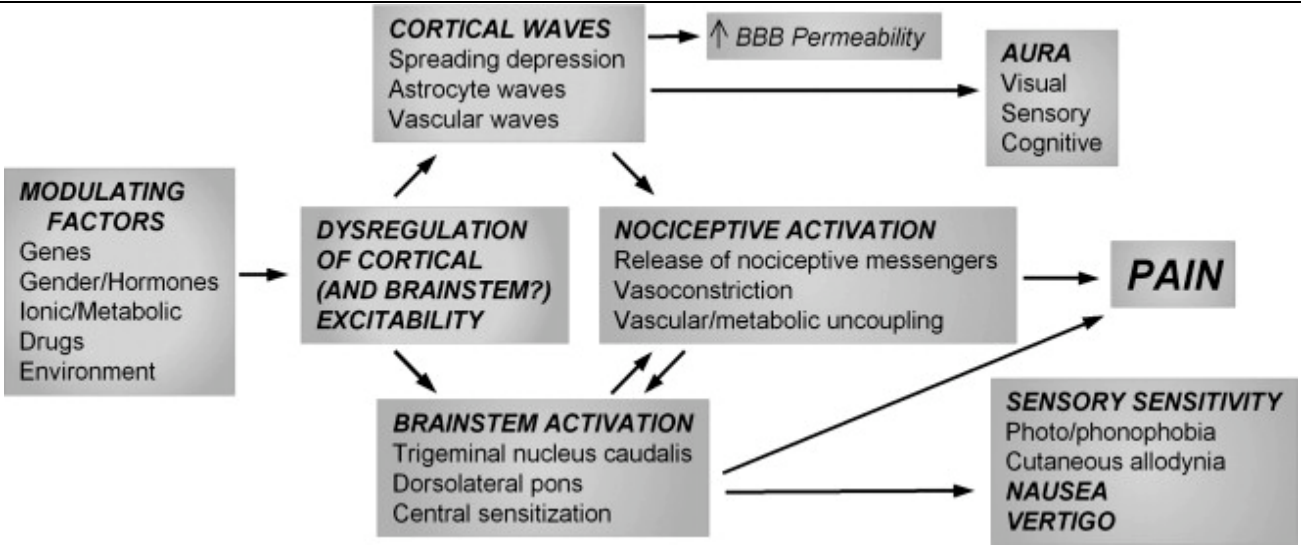


Fig. 2.4 - Schematic of hypothesized sequence of events in migraine

(Chares and Brennan, 2010)

2.1.5 – Migraine mediators

Migraine mediators are a heterogeneous group of substances released from the trigeminal neurovascular system during neurogenic inflammation (Giniatullin et al., 2008; Burstein and Jakubowski, 2010). As described above, a large number of chemical mediators (SP, CGRP, serotonin, bradykinin, histamine) have been shown to produce trigeminal sensitization (Burstein and Jakubowski, 2010). These extracellular mediators are the same main players during inflammation processes (Fischer et al., 2010; Burstein and Jakubowski, 2010).

2.1.5.1 – Bradykinin

Bradykinin is among the most potent sensitizing agents and is responsible of acute sensitisation and activation (Fischer et al., 2010). Bradykinin decreases the threshold of thermally-activated ion channels such as TRPV1 (Transient Receptor Potential Vanilloid 1); it also sensitizes nociceptors by modulating other ion channels, for example by reducing the activity of potassium channels, which makes cells more excitable by decreasing the resting potential and increasing their resistence. It is an important inflammatory mediator, which causes the release of transmitters from neurons, including CGRP and SP, but it also promotes the release of further inflammatory mediators from immune cells, including NGF, interleukins, TNFα and prostaglandins (Fischer et al., 2010).

2.1.5.2 – Serotonin

Serotonin (5-HT) is involved in trigeminal pain. Sensitization by 5-HT has been demonstrated for sodium channels; indirectly, serotonin sensitizes by activating monocytes and by interleukin 6 release from endothelial cells (Fischer et al., 2010). Sumatriptan and other triptans, selective agonist of serotonin $5\text{-HT}_{1B/D}$ receptors specifically designed for the acute treatment of migraine headache, act thought different pathways: causing vasoconstriction in meningeal and cerebral blood vessels with subsequent inhibition of pro-inflammatory neuropeptide release; causing central effects involving decreased pain transmission (Bolay and Durham, 2010). This leads to a decrease in the release of several peptides, including CGRP and substance P (Zhang Z et al., 2007).

2.1.5.3 - Protons

Also protons are known to be implicated in migraine. Commonly they act as sub-threshold sensitizing agents, acting cooperatively with bradykinin and inflammatory mediators (Fischer et al., 2010).

2.1.5.4 – Calcitonin gene-related peptide

The neuropeptide CGRP is implicated in the pathophysiology of migraine. High concentrations of CGRP (but not of other neuropeptides) have been found in plasma, jugular venous blood, and saliva patients during the headache phase of migraine (Sarchielli et al., 2004; Raddant and Russo, 2011).

In humans, CGRP exists in two forms, α-CGRP and β-CGRP, which differ by three amino acids, yet they exhibit similar biological functions (Bolay and Durham, 2010). α-CGRP is preferentially expressed by sensory neurons and this isoform is predominant in trigeminal ganglia (Boley and Durham, 2010). It is the most potent vasodilatatory peptide in the cerebral circulation and at peripheral nerve endings (Bolay and Durham, 2010; Raddant and Russo, 2011). In addition to vasodilation, peripheral release of CGRP also triggers mast cell degranulation, which contributes to neurogenic inflammation together with substance P.

CGRP mediates its effects though a heteromeric receptor composed of a G protein-coupled receptor called calcitonin receptor-like receptor (CALCRL) and a receptor activity-modifying protein (RAMP). A cytoplasmic protein, receptor component protein (RCP), enhances receptor coupling to the G-protein signaling machinery (Raddant and Russo, 2011). CGRP receptors are found throughout the body; relevant to migraine, receptors are located on cerebral vascular smooth muscle, on dural mast cells and on a subset of trigeminal ganglion neurons and satellite glia of the trigeminal ganglion (Raddant and Russo, 2011).

Even though the activation of CGRP G-protein-coupled receptors is usually followed by cyclic adenosine monophosphate (cAMP)-dependent intracellular Ca^{2+} rise, this phenomenon is rarely observed on trigeminal neurons (Giniatullin et al., 2008).

2.1.5.5 – Substace P

Substance P (SP) induced vasodilatation is nitric oxide release dependent. Substance P is involved in nociception, transmitting information about tissue damage from peripheral receptors to the central nervous system to be converted to the sensation of pain. The effects produced by substance P released from peripheral endings of capsaicin-sensitive primary sensory neurons are particularly prominent on the vasculature where they cause vasodilatation of arterioles, plasma protein extravasation in post-capillary venules and leukocyte adhesion to endothelial cells of venules. Peptide-containing primary sensory neurons are characterized by their unique sensitivity to capsaicin; subsets of primary sensory neurons are stimulated selectively by capsaicin that causes the release of sensory neuropeptides, thus, promoting neurogenic inflammation (Harrison and Geppetti, 2001).

2.1.5.6 – Neurotrophins

Neurotrophins, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), activate the tyrosine kinase-couples receptors of the Trk family. Short-term effects of neurotrophins are mediated by phosphorylation and trafficking of ion channels to the membrane, and long-term changes mainly caused by changes in gene expression (Fischer et al., 2011).

NGF promotes hyperalgesia mediated via TrkA receptors activating the PI3K-Src signalling pathway. Application of NGF sensitizes spike firing and TRPV1 receptor activity of dorsal root ganglion (DRG) neurons and facilitates release of algogenic substances like CGRP from TG neurons (Simonetti et al., 2006). In particular, on trigeminal ganglion neurons, NGF produces rapid upregulation of P2X3 receptor function through a molecular pathway essentially different from CGRP (Giniatullin et al., 2008). NGF is also an important inflammatory mediator; it is retrogradely transported to the cell nucleus, where it induces a long-term up-regulation of gene expression, including TRPV1, acid-sensing ion channels (ASIC) and sodium channels, neuropeptides such as substance P and calcitonin gene-related peptide (CGRP), and bradykinin receptors (Fischer et al., 2011).

BDNF, brain-derived neurotrophic factor, is a secreted protein, found in a range of tissue and cell types, not just in the brain. It is a mediator of plasticity at central synapses and modulator of nociceptive signalling (Ba□kowiec-Iskra et al., 2011). BDNF is a mediator of plasticity at first-order synapses in trigeminal nociceptive pathways, with implications for pathophysiology of migraine and other primary headaches (Balkowiec-Iskra et al., 2011). Tarsa et al. (2010) showed that inflammation within peripheral endings of trigeminal nociceptors dramatically up-regulates BDNF in trigeminal ganglion (TG) neurons *in vivo*, even if the cellular mechanisms remain unknown. It was demonstrated that TNFα stimulates de novo synthesis of BDNF in TG neurons, acting at the transcriptional level in a promoter-specific manner (Balkowiec-Iskra et al., 2011).
BDNF is also an intermediate of the CGRP-induced events and is, per se, an adequate stimulus for P2X3 gene transcription in TG neurons as much as CGRP itself. Neutralization of endogenous BDNF cause a strong reduction in the ability of CGRP to generate larger electrophysiological responses mediated by P2X3 receptors (Giniatullin et al., 2008; Simonetti et al., 2008).

2.2 Neuroinflammation

2.2.1 – Inflammation in neurodegenerative diseases

Recently, many chronic neurodegenerative diseases (Multiple Sclerosis, Parkinson's disease, Alzheimer's disease and Amyotrophic Lateral Sclerosis) have been associated to inflammatory responses. Not surprisingly, therefore, the notion of neuroinflammation has become a dominant theme in contemporary neuroscience, and it has been used as a "catch-all" term to describe the role of inflammatory processes (O'Callaghan et al., 2008). In all these diseases, neuroinflammatory processes are the cause or consequence of neurologic diseases.

Neuroinflammation is not easy to define. Traditionally, neuroinflammation was considered to be a process accompanied by a huge invasion of the CNS by blood leukocytes, involving the synthesis/release of proinflammatory mediators, such as cytokines and chemokines (Infante-Duarte et al, 2008). In canonical inflammatory response (outside CNS), neuroinflammation involves monocytes, neutrophils and macrophages. Recently, also other cell types, like microglia and astrocytes, are considered part of immune cells, because they are source and target of pro-inflammatory mediators in response to damaged neuronal targets (O'Callaghan et al., 2008).

The role of neuroinflammation is controversial: it could either be beneficial or detrimental to the injured nervous system (Donnelley and Popovich, 2007; Pineau and Lacroix, 2009). In fact, acute inflammation that occurs immediately after insult to the CNS activates different cell types and mediators in order to limit the proliferation of invading pathogens and promotes the regeneration of the tissue. Viceversa, in chronic inflammation states, the sustained activation of immune cells is highly detrimental and causes cell damage (Bernardino and Malva, 2007).

Even if it is well demonstrated that inflammation occurs in CNS, we can't forget that the inflammatory process in CNS is different in comparison to other tissues. So the old concept of "immune privilege" within the CNS has been substantially revised in the last decades, but it does not decay: CNS is "immune-privileged" organ due to its isolation from the peripheral immune system by the blood-brain barrier (BBB). Insult to the brain induces expression and release of small BBB permeable cytokines and chemokines that can activate resident immune cells and attract peripheral inflammatory immune cells to the site of injury (Williams et al., 2007). Even if the immune cells recruitment is a rapid mechanism in other tissues, in CNS is modest and delayed (Bernardino and Malva, 2007).

2.2.2 – Innate and adaptive immunity response

Immediately after an insult, the innate immunity response is activated. Phagocytic cells, like macrophages and microglia are involved. These cells immediately produce and release the

principally pro-inflammatory cytokines, in order to recruit other immune cells. Pathogens are characterized by conserved elements, called pathogen-associated molecular patterns (PAMPs). These elements are rapidly recognized by macrophage and microglia thanks to Toll-like receptors (TLRs). For example, lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is one of the most characterized PAMP, causing a robust innate immunity response (Bernardino and Malva, 2007).

If the inflammation is transient, the innate response is enough to contrast a simple infection. If the innate immune response failed to eliminate the source of inflammation, following TLRs/PAMPs interaction, adaptive immunity response is activated. In particular, immune cells and neurons begin to produce inflammatory substances attracting T and B lymphocyte to the site of inflammation. In that case, antigen presenting cells (APCs), a highly specified group of cells, process and expose antigens with major histocompatibility complex (MHC) molecules on their surface. Thus, T lymphocytes can recognize it and became activated and also involving B lymphocyte into adaptive immunity response. APCs in neuroinflammatory process include microglia, macrophages and dendritic cells (Bernardino and Malva, 2007).

2.2.3 – Immune- like cells in central and peripheral nervous system

Different cell types are involved in innate and adaptive immune responses. In this section, the most important cells involved in the neuroinflammatory process are described.

2.2.3.1 – Mast cells

Mast cells are generally considered to be critical effector cells in allergic disorders; however, they are also important initiators and effectors of innate immunity, and are resident in many tissues, including nerves (Austin and Moalem-Taylor, 2010).

It has been demonstrated that mast cells degranulate at the site of a nerve lesion or, in case of migraine, at the dura mater level, stimulated by metalloproteinase, releasing mediators such as histamine, serotonin, proteases, prostaglandins and cytokines (Austin and Moalem-Taylor, 2010; Guillot et al., 2011). Mast cell releasing mediators can elicit hyperalgesia by direct activation of nociceptors (histamine, bradykinin, TNFα), they can also have chemoattractant properties (histamine, TNFα and transforming growth factor beta - TGF-β)(Austin and Moalem-Taylor, 2010; Guillot et al., 2011).

2.2.3.2– Neutrophils

Neutrophils granulocytes are the most abundant type of white blood cells in mammals and form an essential part of the innate immune system. During the beginning (acute) phase of inflammation,

neutrophils are one of the first-responders of inflammatory cells to migrate towards the site of inflammation. They play a very early role because infiltration is short-lived and confined, peaking at 24 h (Austin and Moalem-Taylor, 2010; Guillot et al., 2011). Neutrophil recruitment is mediated by the release of chemoattractants, such as nerve growth factor beta (NGF-β), monocyte chemoattractant protein-1 (MCP-1). Neutrophils contribute to the inflammatory response by the release of superoxide and other reactive oxygen species (causing neurotoxic effect on neurons) and different cytokines and chemokines (facilitating macrophage activation and recruitment)(Austin and Moalem-Taylor, 2010).

2.2.3.3 – T lymphocytes

T lymphocytes have an important role in cell-mediated immunity in neuropathic pain. They can be sub-divided in T helper (Th) cells, cytotoxic T (Tc) cells and regulatory T cells (Tregs), with several subpopulations (Austin and Moalem-Taylor, 2010). In particular, Th1 cells promote the development of pain hypersensitivity by releasing pro-inflammatory cytokines (IL2 and interferon gamma - INFγ, TNFα), whereas Th2 cells produce anti-inflammatory cytokines (IL4, 5, 10 and 13) (Austin and Moalem-Taylor, 2010; Guillot et al., 2011).

Recently, a new class of T cells, Th17, have been implicated in pain hypersensibility. This subclass of T cells produce IL17, a potent pro-inflammatory cytokines, that contributes to the regulation of immune cell infiltration and glial cell activation after peripheral nerve injury and the ensuing neuropathic pain (Kim and Moalem-Taylor, 2010).

2.2.3.4 – Glial cells

Glial cells are non-neuronal cells that maintain homeostasis, form myelin, and provide support and protection for neurons in CNS and peripheral nervous system (PNS). In CNS glial cells include astrocytes, oligodendrocytes and microglia; in PNS they are Schwann and satellite cells. Glial cells are crucial for onset and progression of neuroinflammatory process and neuropathic pain.

2.2.3.4.1 – Schwann cells

Schwann cells are responsible for providing trophic support, maintaining the local environment, as well as providing myelin sheath to large myelinated axons and enveloping small bundles of unmyelinated C-fibre axons. In the early phases of the inflammatory process, Schwann cells undergo a phenotypic switch (Austin and Moalem-Taylor, 2010). They proliferate and release mediators that contribute to regeneration (nerve grow factor beta – NGF-β, glial cell line-derived neurotrophic factor and BDNF) or macrophage recruitment (matrix metallopeptidase 9 - MMP-9 in response to TNFα) (Chattopadhyay et al., 2007). Later, Schwann cells begin to secrete other

mediators including cytokines (TNFα, IL1β, IL6), chemokines (LIF, MCP-1) and ATP (Adenosine-5'-triphosphate), sensitising and activating the axon nociceptors (Austin and Moalem-Taylor, 2010). Schwann cells are compared with the CNS oligodendrocytes in terms of function, but are very different in terms of their morphology and myelination properties (Ohara et al., 2009).

2.2.3.4.2 - Satellite glial cells

Satellite glial cells (SGCs) are glial cells that line the exterior surface of neurons in the peripheral nervous system (PNS); in particular, in sensory ganglia, they wrap completely around the sensory neurons and are connected to each other by gap junctions (Hanani, 2005).

The SGC is often thought of as the PNS equivalent of the CNS astrocyte, even if the morphology of the two types of cells is different: astrocytes are commonly multipolar with very attenuated cytoplasmic extensions, indeed, they have different marker expression. Common features consist on functions such as insulation and neurotransmitter recycling (Ohara et al., 2009).

In models of neuropathy and peripheral inflammation, damaged neurons release ATP that activate SGCs. Activated SGCs start to proliferate, increase expression of GFAP, release of pro-inflammatory cytokines, such as IL1β, and alter the normal potassium and glutamate buffering (Hanani, 2005; Austin and Moalem-Taylor, 2010). In their activated state, they can enhance neuronal firing through both direct (IL1β) and indirect (potassium and glutamate) mechanisms (Hanani, 2005; Takeda et al., 2008).

2.2.3.4.3 - Astrocytes

Astrocytes, also known collectively as astroglia, are characteristic star-shaped glial cells in the brain and spinal cord and represent the largest cell population in the CNS. Their function is believed to be nutritional and structural; indeed, during the early stages of injury they have protective role and are responsible for regulating extracellular ions, protons and neurotransmitter concentrations in their microenvironment. One of the most important role is in the removing of excess of glutamate from the synapses.

Peripheral nerve injury leads to central activation of astrocytes. Astrocyte activation is morphologically characterised by hypertrophy, a modest increase of the number of astrocytes, increased production of intermediate filaments, glial fibrillary acidic protein (GFAP), and functionally by increased production of a variety of pro-inflammatory substances in the spinal cord and the brain. This phenomenon is linked to the maintenance of pain sensitivity, because astrocyte release nitric oxide, ATP and cytokines (Austin and Moalem-Taylor, 2010).

Astrocytic Ca^{2+} waves may be an alternative mechanism to explain the spreading symptoms of migraine aura, albeit with a number of caveats: the propagation rate is slower and it typically decays

over a distance of a few hundred microns (CSD and aura propagate over many millimetres). Indeed this interpretation doesn't explain the hemodynamic changes recorded during spreading depression and migraine aura as summarized above (Ayata, 2010).

2.2.4 – Microglia and Macrophages

A separate section is reserved to microglia and macrophages, to describe in details their phenotype and their role in neuroinflammation and in neuropathic pain.

Macrophages are distributed throughout the body and can be broadly grouped into tissue and circulating macrophages. They monitor the tissue environment for pathogens, maintain tissue homeostasis, phagocyte dead and dying cells, and respond rapidly to perturbations in the local environment (Thacker et al., 2007; David and Kroner, 2011; Guillot et al., 2011). Macrophages have remarkable plasticity that allows them to efficiently respond to environmental signals and change their phenotype (Mosser and Edwards, 2008). As previously reported, they are main player in the innate immune response.

A common progenitor, the peripheral-blood mononuclear cell (PBMc), gives rise to the different macrophages. PBMcs, in fact, migrate into tissue under steady state (giving rise to resident macrophages) or in response to inflammation (recruited macrophages) (Fig. 2.5). Also in the blood, monocytes are not a homogeneous population of cells: there is some evidence that supports the fact that tissue macrophages are originated by a specific monocyte population (Mosser and Edwards, 2008).

Resident macrophages have different characteristics to tissue environment, including secreted substances and surface proteins. Tissue-resident macrophages, in normal conditions, cease to proliferate, but they have active messenger RNA (mRNA) and protein synthesis. They can die in situ or be induced to migrate to draining lymph nodes, where they are filtered from the afferent lymph (Gordon, 2003).

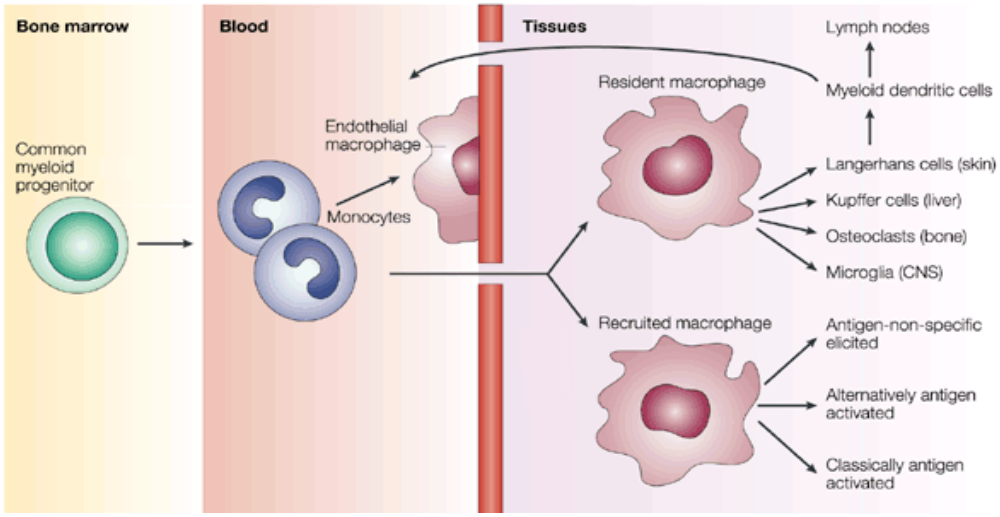


Fig. 2.5 Differentiation, distribution and activation of macrophage in vivo

(Adapted from Gordon, 2003)

In the case of inflammation and/or neurodegeneration, macrophage counts increase at the specific site involved (Hanish, 2002; Scholz and Wolf, 2007; David and Kroner, 2011). They are recruited in response to peripheral nerve injury, such as inflammation of and/or loss of axons, myelin, or both. Their main function of recruited macrophages is to phagocyte foreign material, microbes, and other leukocytes as well as removing injured and dying tissue debris or dying remnants of injured Schwann cells and axotomised axons. Indeed, macrophages are also implicated in pain-related behaviour (Thacker et al., 2007; Austin and Moalem-Taylor, 2010; David and Kroner, 2011; Guillot et al., 2011).

It was observed an early and robust accumulation of macrophages not only in the injured brain or spinal cord but also in the PNS (Leskovar et al., 2000). In the CNS, after insults, different types of macrophages are found in different locations and they differ in their morphology and some of their functions (Fig. 2.6; David and Kroner, 2011). All of them are derived from microglia and haematogenous macrophages and cannot be distinguished by their morphology or antigenic markers (David and Kroner, 2011).

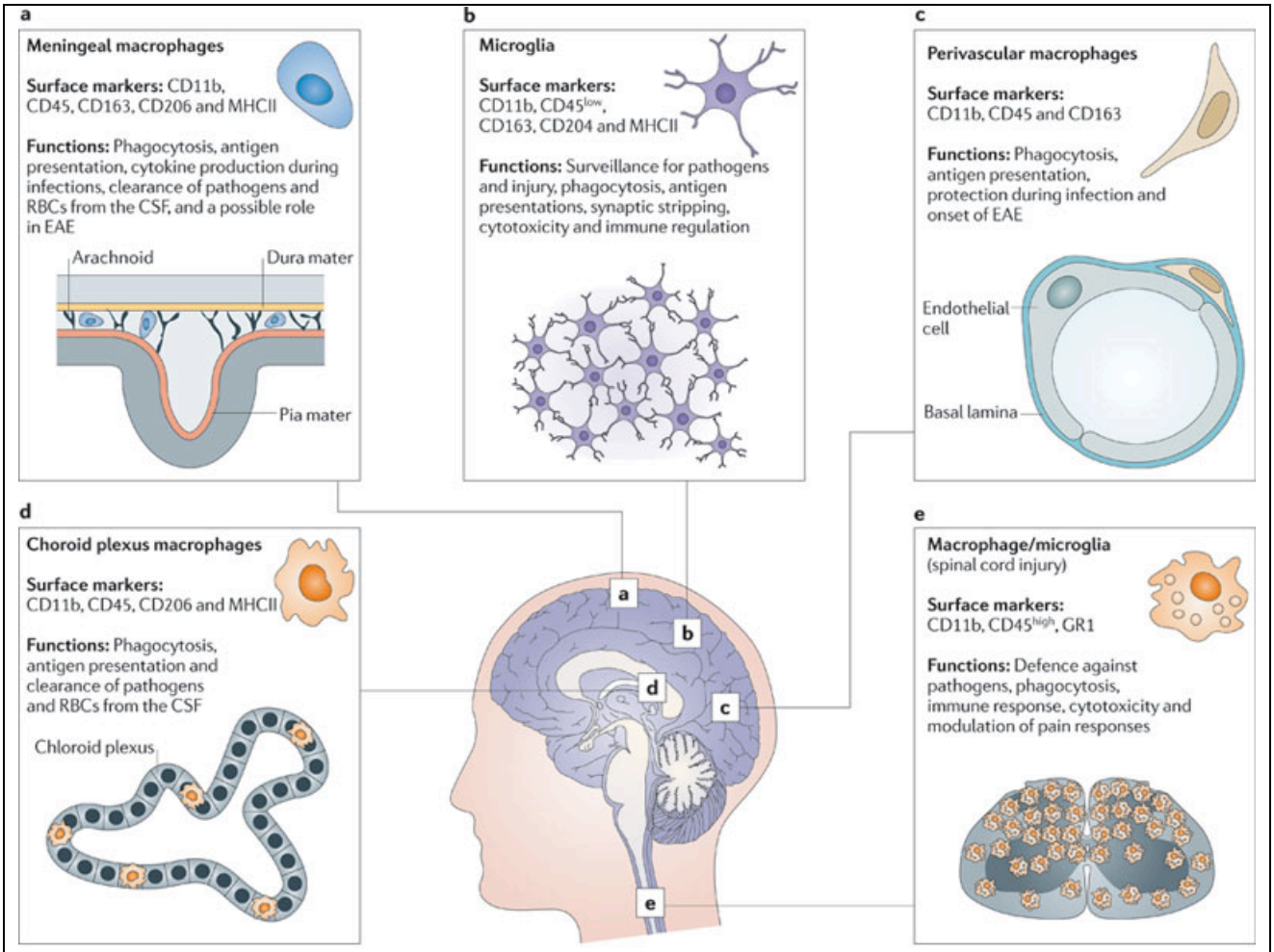


Fig. 2.6 - Macrophages and microglia in the CNS

A, C, D Macrophages and *B* microglia in different regions of the uninjured CNS show differences in morphology and surface markers. *E* In the injured CNS (the spinal cord is shown) macrophages and microglia have a different morphology from those seen in the uninjured CNS, and as they cannot be distinguished from each other, they are referred to as macrophages/microglia.

(David and Kroner, 2011)

2.2.4.1 - Microglia

Microglia cells are a particular population of normally quiescent resident macrophages, which perform immune surveillance in the CNS. In the healthy mature CNS (including the brain, spinal cord, as well as the eye and optic nerve) they have a ramified morphology, and a small soma with fine cellular processes (Kettenmann et al. 2011). This state of microglia is normally called "resting" microglia, but recently it has been demonstrated that, in this state, microglia have many tasks. They carry out homeostatic surveillance and are sensors of pathological changes, that's why "resting" microglia is called also "surveying" microglia. Thus, the "resting" state may actually reflect a defined mode of an active cell (Hanish and Kettenmann, 2007)

Microglia cells can recognize a wide range of mediators (Table 2.2), and respond promptly to signals independently if they are coming from damaged cells, or immune signals (like cytokines and T cells), as well as from pathogens (Aloisi, 2001; Austin and and Moalem-Taylor, 2010; Hanish and Kettenmann, 2007)

Table 2.2 Examples of signals and modulators of microglial activation

Class of compound	Examples
Surface structures and DNA/RNA of viral, bacterial or fungal origin	Agonists of members of the pattern recognition receptor families, notably TLR1/2, TLR3, TLR4,TLR6/2 and TLR9, such as bacterial LPS or cell wall proteoglycans and lipoteichoic acid (LTA), gp41, gp120 (the TLR4-agonistic LPS serving as a common model agent)
Abnormal endogenous proteins	β-amyloid (aggregates), Aβ25–35, Aβ40, Aβ42, prion protein (PrP)
Complement	Complement factors C1q, C5a
Antibodies	Immunoglobulin of various classes and isotypes (IgA, IgG, IgM), presented in immune complexes
Cytokines	Colony stimulating factors (M-CSF, GM-CSF), IL1, IL2, IL4, IL6, IL10, IL12, IL15, IL18, IFN-γ, TGF-β, TNFα
Chemokines	Ligands for chemokine receptors: CCR3, CCR5, CXCR2, CXCR, CXCR4, CX3CR1, IL-8R
Neurotrophic factors	Brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), NT-4
Plasma components	Albumin, fibronectin, fibrinogen, thrombin
Other proteins and peptides	Apolipoprotein E (ApoE), heat shock proteins hsp60 and hsp70, CD40L, melanocyte-stimulating hormone (MSH), endothelin, S100 proteins, vasoactive intestinal peptide (VIP)
Neurotransmission-related compounds	ATP (and related purines), -adrenergic agonists, glutamate, kainate, NMDA
Ions	K+, Mn2+
Other compounds	Cannabinoids, ceramide, gangliosides, lysophosphatidic acid (LPA), melatonin, opioids (endomorphines), platelet-activating factor (PAF), prostaglandin E2 (PGE2), steroid hormones, vitamin D3

(Adapted from Hanish and Kettenmann, 2007)

Microglia cells respond to these mediators by undergoing rapid and profound changes in cell shape, gene expression and functional behavior, which summarily is defined as “microglial activation” (Kettenmann et al., 2011). Microglia reverts to an amoeboid appearance (Fig. 2.7), increased size and motility to a site of infection following chemotactic gradients (Austin and and Moalem-Taylor, 2010; Kettenmann et al., 2011). The activation state is characterized by expression of several makers as well as activation of the p38-mitogen activated protein (MAP) kinase pathway (Austin and and Moalem-Taylor, 2010).

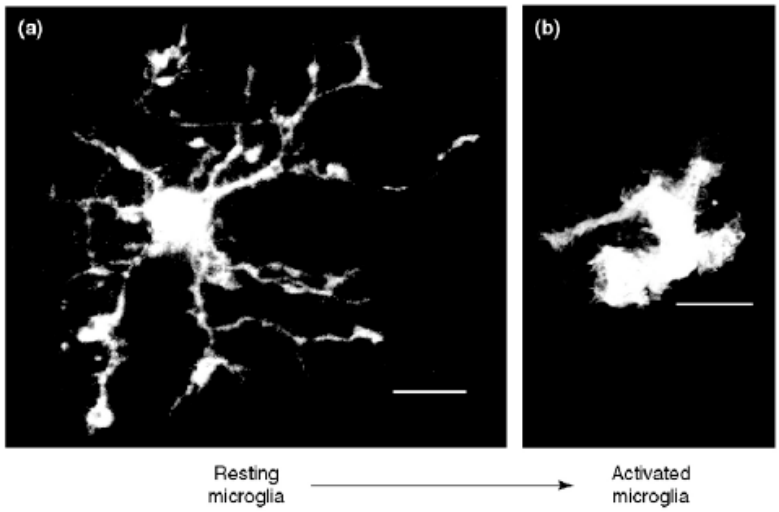


Fig. 2.7 - Microglial activation: changes in morphology

(Adapted from Kettenmann, 2011)

Local densities of microglia can also increase by proliferation or recruitment of macrophages and differentiated into microglia to organize the protection and restoration of tissue homeostasis (Austin and Moalem-Taylor, 2010; Kettenmann et al., 2011).

However, microglia activation is no more considered an all-or-none event or monophasic process, as the responses to pathologic events and the mediators released by microglia are context dependent and adapt to the microenvironment changes, introducing the concept of "provincially adapted" microglia (Hanish and Kettenmann, 2007; Austin and and Moalem-Taylor, 2010; Kettenmann et al., 2011). In activated state, microglia release of multiple factors and compounds with proinflammatory and immunoregulatory effects are additional elements to the activation process, like phagocytosis (Kettenmann et al., 2011).

2.2.4.2 – Macrophages in the PNS

The macrophage population in the peripheral nerve consists of resident cells and hematogenously derived macrophages, only seen after tissue damage. In contrast to other tissue systems, resident macrophages do not require the activation of precursor cells, and respond extremely rapidly to nerve damage (Donnelly and Popovich, 2007; Thacker et al., 2007).

Few days after trauma or infection in the PNS, circulating macrophages that flood across the leaky blood–nerve barrier reach the site thanks to three main signaling pathways: interaction of the chemokine fractalkine with the CX3CR1 receptor, interaction of MCP-1 with the CCR2 receptor, and activation of the TLRs (Donnelly and Popovich, 2007; Thacker et al., 2007; Guillot et al., 2011). Some pro-inflammatory cytokine such as TNFα released from immune cells induces metalloproteinase such as MMP-9 that, in turn, promotes migration of macrophages to the injured

site via breakdown of the blood-brain barrier (Shubayev et al., 2006; Ren and Dubner, 2011).
Recruited macrophages quickly outnumber the resident cells and are thought to be vital for degeneration and subsequent regeneration of the peripheral nerve, with a specific and well-modulated mechanism, involving several pro-inflammatory mediators and other cell types (Donnelly and Popovich, 2007; Thacker et al., 2007).
As described for microglia cells, macrophage activation can be observed as a change in their morphology (Glenn et al., 1993; Lynch, 2009; Perego et al., 2011).
Macrophage function and substances released by macrophages are specific for different type of nerve damage or neuropathology. Different studies suggest that macrophages may contribute to the generation of neuropathic pain through several mechanisms, including the release of pro-nociceptive mediators (Guillot et al., 2011). A reduction in neuropathic pain behaviours has been correlated to an attenuation of macrophage recruitment into the damaged nerve (Thacker et al., 2007).
The unique position of macrophages between the inflammatory response and the adaptive immune system makes them an obvious target but also the main players of neuroimmune cross-talk that lead to neuropathic pain.

2.2.4.3 – Microglial and Macrophage effects: detrimental or beneficial?

Activated microglia/macrophages can have detrimental or beneficial effects at the site of inflammation or injury (Benardino and Malva, 2007; Donnelly and Popovich, 2008; David and Kroner, 2011; Guillot et al., 2011).

2.2.4.3.1 - Detrimental effects

Activated microglia/macrophages produce various pro-inflammatory cytokines, proteases and other factors. These released substances are the first to respond to injury or inflammation, within minutes. If the pro-inflammatory response is sustained or excessive, these cells may cause detrimental consequences and can be associated with the onset and/or exacerbation of neuronal death.
Over-expression of pro-inflammatory mediators leads to spontaneous demyelination or cause cytotoxic effect directly on neurons. Indeed, different studies on spinal cord injury and stroke animal models have proved that pro-inflammatory cytokines such as IL1β and TNFα produced by microglia/macrophages may contribute to neurondegeneration. Cytokines or mediators that are released after pro-inflammatory stimulation have been implicated in the secondary phase that occurs after the first insult. Administration of anti-inflammatory drugs can contribute to functional recovery and confer neuroprotection (Donnelly and Popovich, 2008; David and Kroner, 2011).
Neutrophils, microglia and macrophages produce superoxide anion and nitric oxide which combine

to form the highly reactive and toxic compound peroxynitrite. Production of free radicals induce apoptosis in neurons and glia via the irreversible oxidation of proteins, lipids and nucleic acids (Donnelly and Popovich, 2008; David and Kroner, 2011). These substances may disrupt glutamate metabolism by astrocytes, causing glutamate levels to increase to damage neurons (Donnelly and Popovich, 2008).

2.2.4.3.2 - Beneficial effects

Microglia/macrophages are intrinsically neuroprotective: they regularly survey and provide trophic support to neurons and glia; they phagocyte and eliminate invading pathogens, cell debris and several neurotoxins; they produce neuroprotective cytokines and growth factors (TGF-β, NGF, BDNF) that have beneficial effects on neurons and limits oligodendrocyte toxicity (Donnelly and Popovich, 2008).

Pro-inflammatory cytokines, like TNFα, have also beneficial effects; in fact, TNFα signalling through tumour necrosis factor receptor superfamily member 1B (TNFR2) is required for proper remyelination (David and Kroner, 2011). Macrophages also release anti-inflammatory cytokines (IL10 and IL1 receptor antagonist) (Bernardino and Malva, 2007).

2.2.4.4 – Macrophage polarization

Macrophages are classified in two main groups: M1 reserved for classically activated macrophages and M2 identified as alternatively activated macrophages. The M2 has rapidly expanded to include essentially all other types of macrophage. In addiction, recently a new sub-type of resting, non-polarized macrophage called M0 was identified (Mosser and Edwards, 2008; David and Kroner, 2011).

Th1 cells activate classically activated macrophages (M1) through Toll-like receptors (TLRs) and interferon gamma. M1 macrophages produce pro-inflammatory cytokines (IL12, IL1β and TNFα), and cytotoxic mediators (reactive oxygen and nitrogen species), as well as they increase their phagocytic and antigen-presenting capacity. Macrophages activated in this way are effective in killing intracellular pathogens macrophage (Mosser and Edwards, 2008; David and Kroner, 2011).

Th2 cells activate M2 macrophage through interleukin 4 (IL4) and 13 (IL13). M2 macrophages express high levels of anti-inflammatory cytokines, such as IL10 and TGF-β, show defective nuclear factor-κB (NF-κB) activation, upregulate arginase 1 and down-regulate expression of pro-inflammatory cytokines. M2 are active principally against extracellular pathogens and parasitic infections (Mosser and Edwards, 2008; David and Kroner, 2011).

The two sub-groups of activated macrophages are time and tissue dependent. For example, after an insult or injury, at early stages due to the release of pro-inflammatory cytokines that remain up

regulated in the first few days, M1 are the most macrophage class involved. These macrophages seem responsible for detrimental effects both *in vitro* and *in vivo* (David and Kroner, 2011).

The M2 sub-groups are activated later and are often described as anti-inflammatory cells. Microglia in CNS and macrophage recruit from the periphery belongs to M2 phenotype. Even if M2 have beneficial effects when they reach the inflamed sites, their prolonged presence may also have detrimental effects (David and Kroner, 2011).

M2 macrophages are not a homogeneous population. Three different subtypes are indentified (M2a, M2b and M2c) based on particular polarizing signals and different functional properties. M2a and M2c macrophages have anti-inflammatory and reparative properties. M2b macrophages produce high amounts of the anti-inflammatory cytokine IL10 and pro-inflammatory cytokine TNFα, IL1β IL6 and low levels of IL12. For that, M2b are considered to modulate different aspects of the inflammatory response (Mosser and Edwards, 2008; David and Kroner, 2011).

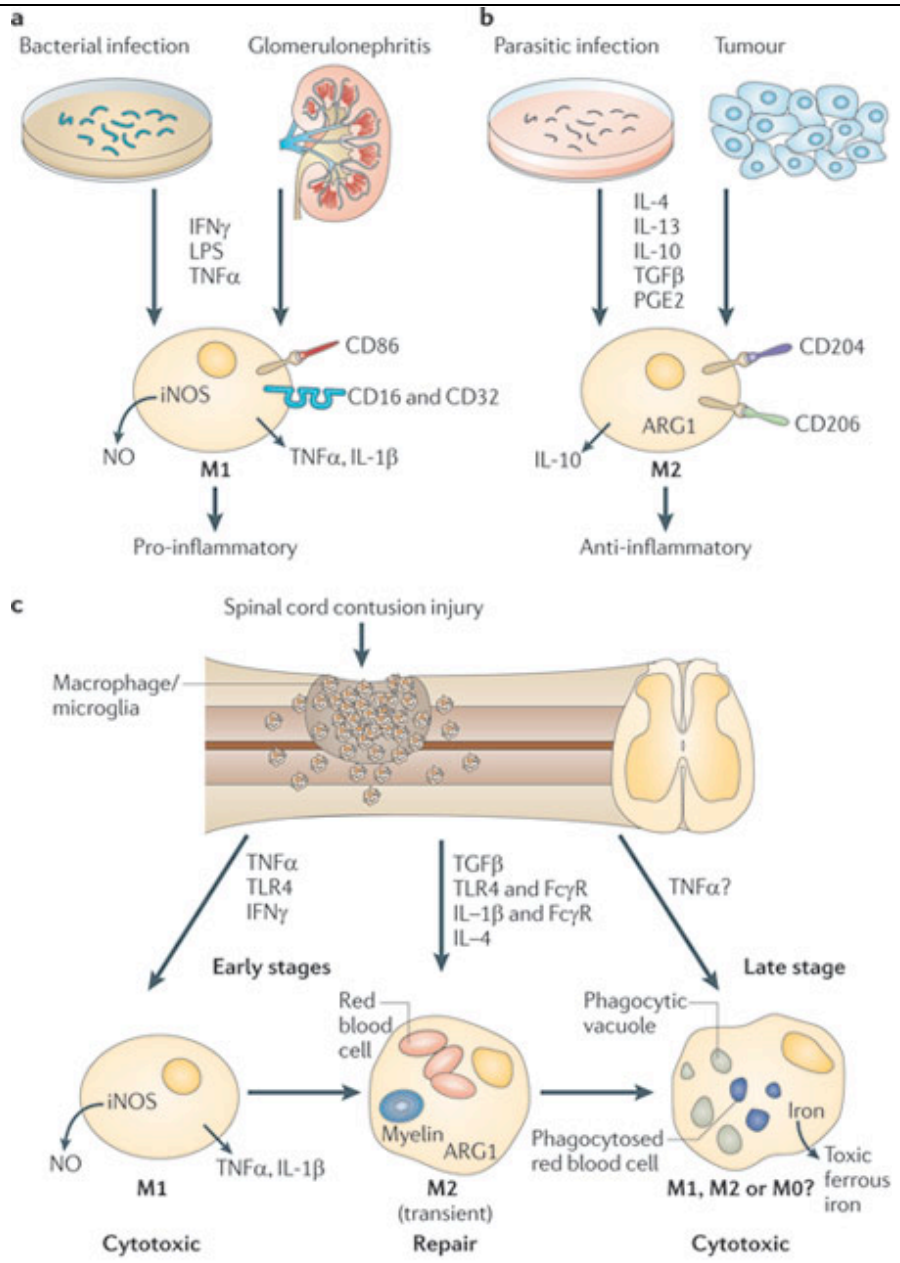


Fig. 2.8 - Macrophage polarization

a, Some of the cell surface markers and pro-inflammatory cytokine and cytotoxic molecules that are expressed by M1 cells. *b,* Some of the characteristic cell surface markers and anti-inflammatory and protective molecules that are expressed by M2 cells. *c*, Macrophage polarization in the injured spinal cord. In the early time points after spinal cord contusion injury, macrophages acquire a predominately M1 polarized state. A small number of macrophages, probably M1 macrophages that have phagocytosed cellular material, transiently acquire M2 polarization. At later stages after spinal cord injury, a larger proportion of the cells are M1 and a smaller number are M2 cells. It is possible that some of these cells acquire a resting M0 state as expression of pro-inflammatory cytokine and the inducible isoform of nitric oxide synthase (iNOS) is not maintained. However, macrophages at this later stage may still be cytotoxic as there is evidence that they release toxic iron acquired from haemoglobin in the phagocytosed red blood cells. ARG1, arginase 1; Fc□R, Fc receptor for immunoglobulin G; IFNγR, interferon-γ receptor; LPS, lipopolysaccharide; IL1β, interleukin 1β; TGFβ, transforming growth factor β1; TLR4, Toll-like receptor 4; TNFα, tumour necrosis factor.

(David and Kroner, 2011)

2.2.4.5 – Macrophage/microglia markers

There are different and numerous markers that help to define the different subgroups of microglia/macrophages (Fig. 2.6). Unfortunately, as previously reported, it is not possible to distinguish microglia and macrophages, and some markers recognize different subtypes of immune cells. Here there are the main macrophage/microglia markers used in literature.

Iba1 (ionized calcium binding adapter molecule 1) is specifically expressed in macrophages/microglia and is up-regulated during the activation of these cells (Imai et al., 1996; Ito et al., 1998). The anti-Iba1 polyclonal antibody commercially avaible recognizes a synthetic peptide corresponding to the Iba1 carboxy-terminal sequence that is completely conserved among human, rat, and mouse Iba1 proteins (Ito et al., 1998). Iba1 expression levels increase in macrophage/microglia cells when there are activated, due to the fact that this protein is involved in phagocytosis and in reorganization of actin filaments (Sasaki et al., 2011).

Leukocyte CD11/CD18 adhesion molecules are a family of cell-surface glycoproteins, consisting of 3 heterodimers sharing a common *beta* subunit (CD18) with a distinct *alpha* subunit (CD11a, CD11b, CD11c), called integrins (Mazzone and Ricevuti, 1995). CD11b mRNA or protein expressions can be up-regulated by different factors, like chemoattractants and cytokines, causing a migration or activation of immune cells in inflammation (Mazzone and Ricevuti, 1995; Simon et al., 2000; Scholz et al, 2008).

ED1 (CD68) is a 110 kDa transmembrane glycoprotein of unknown function highly expressed by human monocytes and tissue macrophages (Holness and Simmons, 1993). It is a marker of phagocytic activity (Holness and Simmons, 1993; Scholz et al., 2008)
Scholz et al. (2008) showed that, in the dorsal horn, ED1 positive cells occur with the same distribution as microglia, but recognized a sub-population characterized by an amoeboid shape lacking the fine ramification of resting microglia or the thick cell processes of activated microglia. So ED1 seems to identify microglial cells that have transformed into phagocytes, infiltrating perivascular cells, or circulating macrophages invading the spinal cord across the blood-brain barrier (Scholz et al., 2008).

F4/80, also known as EMR1, is a 160 kDa extensively glycosylated protein of the EGF-TM7 family of adhesion molecules. It is primarily expressed by many resident tissue macrophages, including microglia in the brain, but several macrophage populations lack this marker, e.g. marginal zone

metallophils and osteoclasts, or express it only weakly, including myeloid dendritic cells (Gordon and Mantovani, 2011). The significance of F4/80 expression heterogeneity by resident macrophages remains unclear even if some studies found it is tightly regulated by the state of activation of these cells: in bacillus Calmette-Guérin–infected animals it was demonstrated that F4/80 is expressed at lower levels in activated macrophages than in unstimulated resting macrophages (Lin et al., 2005). These studies point to a function for F4/80 in selected tissue macrophage populations.

2.2.5 – Inflammatory mediators

After trauma or neuroinflammatory process, in CNS as well as PNS, inflammatory mediators (like cytokines, chemokines and other soluble medators) play a key role in development, maintenance and termination of the inflammatory process. They are also crucial for the cross-talk between neurons and non-neuronal cells. A part from inflammatory process, they are involved into development, tissue repair, and hemopoiesis (Haddad, 2002).

2.2.5.1 – Cytokines

Cytokines are small, non-structural proteins constitutively expressed on the cell surface in precursor form, which can be cleaved to allow rapid release, whereupon they can diffuse over a relatively short distance to act on another cell for auto and paracrine signalling (Haddad, 2002; Hanish, 2002; Austin and Moalem-Taylor, 2010). Cytokines interact with specific receptors located on the cell membrane or with soluble receptors. Cytokines expression is normally transient and trascriptionally/translationally regulated, but there are some cytokines that are constitutively expressed (Bernardino and Malva, 2007).

Interleukins (IL), the initial name, derive from leukocytes: they are identified as molecules produced by or acting on leukocytes. Nowadays, different types of cells (immune cells and also neurons) are found as source or target of cytokines (Bernardino and Malva, 2007; Austin and Moalem-Taylor, 2010).

There are two main subgroups in which cytokines are divided: pro- and anti-inflammatory cytokines. Membership of these categories depends on the final balance of their effects on the immune system (Bernardino and Malva, 2007). Obviously depending on concentration, type of cell and site of release, cytokines can have beneficial and detrimental effects. Moreover, cytokines exhibit pleiotropy and redundancy: different cytokines can have the same role and act on the same cell population, but, at the same time, an individual cytokine can have multiple functions on different cells (Bernardino and Malva, 2007; Austin and Moalem-Taylor, 2010).

Cytokines have different and important roles: initiate, propagate and regulate an inflammatory reaction, controlling immune cells and neuronal activity and plasticity. At the beginning of the

inflammatory process, pro-inflammatory cytokines are essential, stimulating immune cells itself, moreover causing release of other cytokines (pro- but also anti-inflammatory) and chemokines. A simplified description of the general phenomenon is the following. TNFα and IL1β are the first cytokines released at the site of inflammation. They activate different pathways, which culminate with the increased expression of target genes involved principally in immune and inflammatory response (Bernardino and Malva, 2007). The mechanism of maintenance of pain is not investigated in details. Recent studies support the theory that also in chronic pain a pivotal role is done by pro-inflammatory mediators, such as IL6. Differently from the early phase of inflammation, in chronic pain phase sensory neurons themselves may up-regulate both cytokines/chemokines and their receptors (Miller et al., 2009).

In order to modulate or conclude the inflammatory response, anti-inflammatory cytokines are involved. They regulate pain mediators and down-regulate pro-inflammatory cytokines determinating M2 polarization of macrophages, and the release of other mediators, which control the proceeding or the ending of inflammation. (Uçeyler et al., 2009; Austin and Moalem-Taylor, 2010; Ren and Dubner, 2010; David and Kroner, 2011; Guillot et al., 2011).

Cytokines regulate the bidirectional crosstalk between neuronal and non-neuronal cells. At the same time, cytokines activity are modulated by neurotransmitters, neuropeptides, grow factors and hormones (Hanish, 2002; Bernardino and Malva, 2007).

Here I will report the most important cytokines involved in neuroinflammatory process, in particular for migraine pathophysiology.

2.2.5.1.1 - Tumor necrosis factor

Tumor necrosis factor (TNF, cachexin or cachectin formerly known as tumor necrosis factor-alpha or TNFα) is known as a major pro-inflammatory cytokine. It is a pleiotropic cytokine with the ability to induce a cascade of additional cytokine production. It is expressed as a 26 kDa transmembrane precursor from which a soluble 17 kDa polypeptide is released after proteolytic cleavage, mainly by the metalloprotease TNFα converting enzyme (TACE) (Bernardino and Malva, 2007; Austin and Molaem-Taylor, 2010). TNFα is released by macrophages, microglia and glial cells, but there is evidence that also neurons (in particular DRG ones) produce TNFα (Schafers et al., 2003; Li et al., 2004; Thaker et al., 2007).

TNFα seems to have an important role in the devolpment of allodynia/hyperalgesia in several neurophatic pain models, and it is considered as “initiator” of neutopathic pain (Thacker et al., 2007). In fact, neutralising antibodies or receptor antagonists can reverse pain hypersensitivity in models of peripheral nerve injury (Austin and Molaem-Taylor, 2010).

In the case of damage to a peripheral nerve, a first wave of TNFα with mRNA peak occurs in the

first hour immediately after inflammation released by resident active macrophages, Schwann cells and mast cells, as well as damage neurons. Subsequently, recruited macrophages and neutrophils release additional TNFα, resulting into a second peak 3-5 days later (Austin and Molaem-Taylor, 2010). TNFα is crucial also in migraine, because TNFα levels increase after migraine pain onset and decrease progressive over time after the onset of attack (Robbins and Maides, 2011).

TNFα exerts its actions via two distinct receptors: the constitutively expressed TNFR1 and the inducibly expressed TNFR2 (Thacker et al., 2007). TNFR1 is expressed in most tissues, and can be fully activated by both the membrane-bound and soluble trimeric forms of TNFα, whereas TNFR2 is found only in cells of the immune system, and responds to the membrane-bound form of the TNFα homotrimer.

The main difference between the two receptors is the presence in the TNFR1 of intracellular regions so-called death domains (DD) linked to proteins primarily involved in cell death signalling. TNFR1 recruits a cytoplasmic protein called TNFR associated domain (TRADD) that interacts with DD regions. TRADD-DD complex can recruit fas-associated death domain (FADD) and Receptor interacting protein (RIP).

FADD contains the death effector domain (DED) motif that activates caspases inducing cell death. TRADD/FADD complex recruits the cysteine protease caspase-8. A high concentration of caspase-8 induces its autoproteolytic activation and subsequent cleaving of effector caspases, leading to cell apoptosis. (Fig. 2.9 A).

TNFR1/TRADD associates to RIP when the complex is translocated to cholesterol and sphingolipid enrich membrane microdomain (known as lipid rafts). RIP is a potent inducer of apoptosis, it enhances gene expression through NF-κB pathways, and in part through JNK pathways.

Indeed, the TNFR1/TRADD complex can interact directly with TRAF2 (TNF receptor associated factor 2 proteins) that as RIP is involved in apoptosis and gene expression activating JNK pathways, and secondarily NF-κB pathways. These pathways, in particular NF-κB, are responsible for the expression of inflammatory genes and several cytokines and chemokines.

NF-κB is a heterodimeric transcription factor that translocates to the nucleus and mediates the transcription of a vast array of proteins involved in cell survival and proliferation, inflammatory response, and anti-apoptotic factors.

Another mediated pathways of TNFR1 involves a distinct domain of the receptor complex in order to activate different molecules (diaglycerol –DAG, protein kinase C – PKC, etc) causing activation of the NF-κB cascade (Fig. 2.9 C).

The overall function of TNFR2 is largely unknown. Its pathway depends on TRAF-family member activation and the subsequent activation of signalling pathways such as NF-κB, JNK, pERK and p38 pathways. These pathways increase cytokines expressions but also the expression, of regulatory

proteins with anti-apoptotic activity (Bernardino and Malva, 2007; Thacker et a., 2007; Leung and Cahill, 2010).

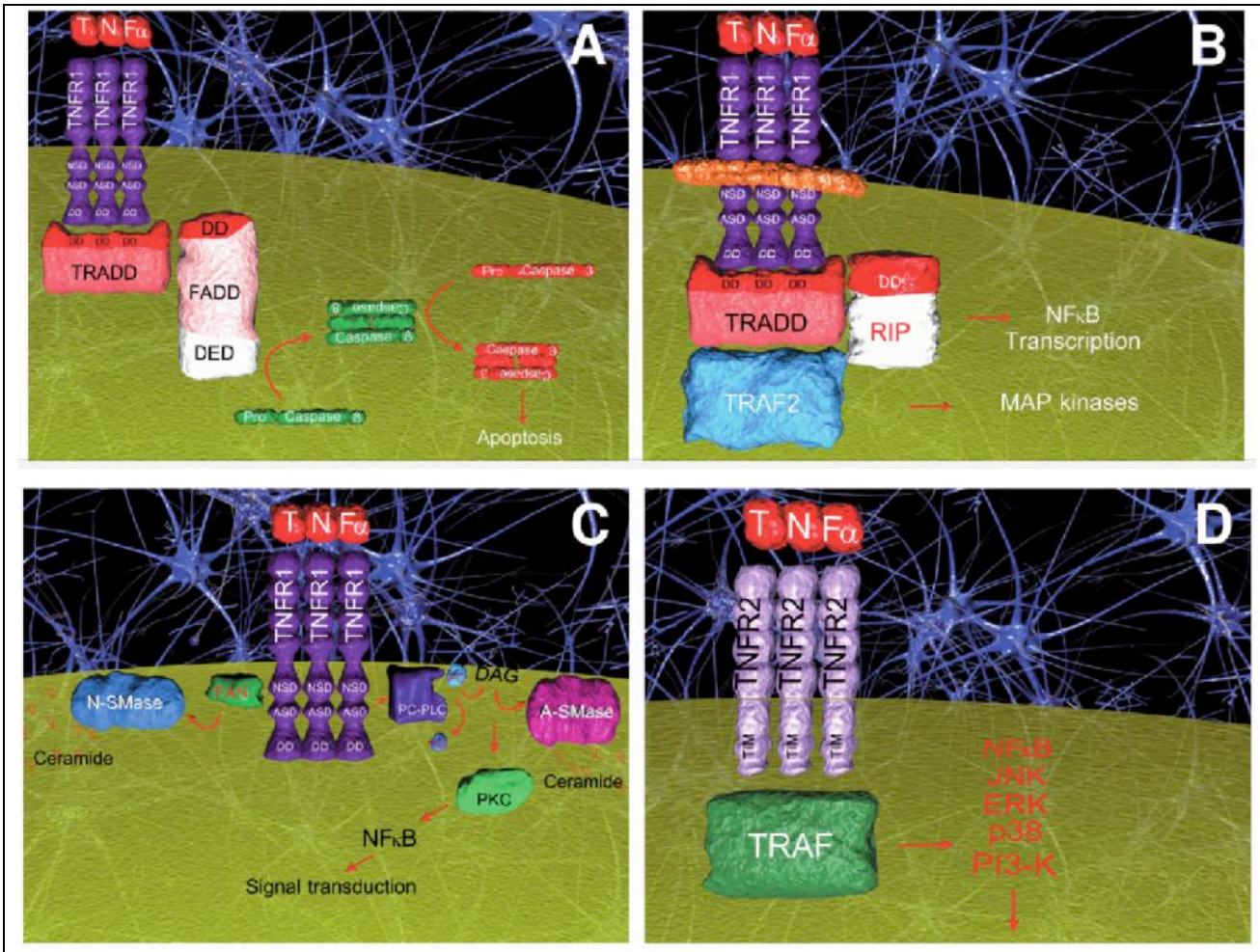


Fig. 2.9 - The major signal transducing pathways activated by TNFR1 and TNFR2

A, *B*, *C*, TNFR1 triggers signalling pathways via three different functional domains. *D*, TNFR2 signalling pathways.

(Bernardino and Malva, 2007)

In the naïve system, stimulation of TNFR1, but not TNFR2, induces pain-associated behaviour in vivo; after nerve injury, TNFR1 and TNFR2 activation leads to pain hypersensitivity and ectopic firing (Uçeyler et al., 2009; Austin and Molaem-Taylor, 2010). Different studies confirmed the theory that in the intact nervous system TNFR1 is capable of mediating the excitation of sensory neurons and induction of pain hypersensitivity while, following nerve injury, there is induction of TNFR2, which along with continuing presence of TNFR1, contributes to ongoing excitation of sensory neurons associated with neuropathy (Austin and Molaem-Taylor, 2010).

In the PNS, in particular in DRGs, topical application of TNFα to rat nerve root increases phosphorylation of extracellular signal-related kinase (pERK) with an onset time of several hours, while acute application of TNFα to cultured DRG neurons induces phosphorylation of c-Jun

terminal kinase (JNK) and proteinkinase p38 (p38), but not ERK (Uçeyler et al., 2009). Thus, the same cytokines can activate different pathways depending on the microenviroment of the tissue/culture examined.

2.2.5.1.2 – Interleukin 1 beta

Interleukin 1 is a crucial cytokine with immunostimulatory/pro-inflammatory signal, mediator of innate defense and immune responses, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The main sources of IL1 are immune cells including macrophages, monocytes, and glial cells, as well as neurons. IL1 family consist in IL1α, IL1β and IL1-receptor antagonist (IL1ra) with 2 specific receptors (IL1RI and II) with different binding affinities (Hanish, 2002; Thacker et al., 2007; Austin and Molaem-Taylor, 2010).

In pro-inflammatory pathways and neuropathic pain, IL1β is released in early phases by immune and neuronal cells. The inactive form (31 kDa) is cleaved by IL1β converting enzyme (ICE or caspase-1) into 17 kDa mature protein. Normally it binds IL1RI that mediates the biological effects, vice versa IL1RII is not capable to transduce the IL1β signal, but it acts as decoy receptor (Hanish, 2002; Thacker et al., 2007; Austin and Molaem-Taylor, 2010). Fig. 2.10 shows a schematic representation of the downstream signalling of IL1RI. Briefly, IL1RI forms a heterodimer with IL-1 receptor accessory protein (IL-1RAcP), leads to the activation of the transcription factor NF-κB through different signaling mechanisms, two IL1 receptor-associated kinases: IRAK-1 and IRAK-2. Also TRAF6 is implicated: it interacts with IL-1RAcP with subsequent NF-κB activation through NF-κB-inducing kinase (NIK) to two I-kappaB kinases (IKK-1 and -2) (Bernardino and Malva, 2007).

Other mitogen activated protein kinases, including JNKs and p38 MAPK through various MAP2Ks are participating in IL1 responses by activating transcription (such as MCP-1 and IL6) through the AP-1 transcription factor. In the cell, IL-1R binds to toll-interacting protein (TOLLIP), which results in the inhibition of IRAK1 and by promoting efficient degradation of IL-1R) (Bernardino and Malva, 2007).

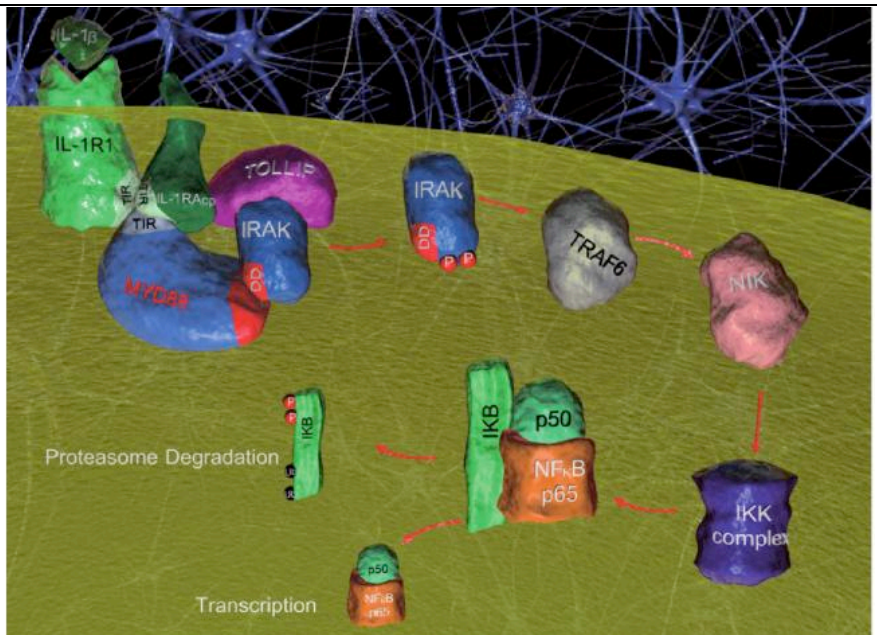


Fig. 2.10 - Schematic representation of IL1β pathways

(Bernardino and Malva, 2007)

IL1β has been identified as one of many algogenic agents that may play a role in neuropathic pain. In the periphery, IL1β itself evokes in prolonged hyperalgesia and allodynia after peripheral nerve injury (Thacker et al., 2007; Austin and Molaem-Taylor, 2010). IL1β exposure has been shown to enhance voltage-dependent sodium currents in trigeminal ganglion neurons, which was dependent on IL1R, and PK-C signalling (Liu et al., 2006). In the spinal cord, IL1β application enhances synaptic transmission and neuronal activity (Thacker et al., 2007; Kawasaki et al., 2008; Uçeyler et al., 2009; Austin and Molaem-Taylor, 2010). Considering migraine pathophysiology, IL1β facilitates CGRP release from peptidergic neurons (Uçeyler et al., 2009).

2.2.5.1.3 – Interleukin 10

IL10 is a powerful anti-inflammatory cytokine produced primarily by activated T, B cells, macrophages and mast cells. This cytokine has pleiotropic effects in immunoregulation and inflammation. It down-regulates the expression of Th1 cytokines, MHC class II antigens, and stimulatory molecules on macrophages. It blocks NF-κB activity, and is involved in the regulation of the JAK-STAT signaling pathway (Uçeyler et al., 2009; Austin and Moalem-Taylor, 2010). The mechanism of action of IL10 is predominantly through suppression of pro-inflammatory cytokines, and the resulting decrease in recruitment and activation of further immune cells at the injury site and in peripheral nerve injury (Austin and Moalem-Taylor, 2010). Different studies support the anti-inflammatory effects of IL10, because increasing expression of IL10, by gene therapy or pharmacologically, have substantial inhibitory effects on acute pain and established painful

neuropathy in animal models.

2.2.5.1.4 – Interleukin 6

IL6 is a predominantly pro-inflammatory cytokine controlling the inflammation in the acute phase, released by many cell types, including mast cells, macrophages, lymphocytes, neurons and glial cells (Hanisch, 2002; Austin and Molaem-Taylor, 2010). Targets are T, B cells, plasma, bone marrow, and liver cells, microglia, Schwann cells, macrophages as well as neurons that show IL6 receptor (IL6R).

Like IL1β and TNFα, IL6 is considered a pro-inflammatory cytokine; but, unlike other, it helps to initiate and regulate acute-phase responses, a complex of adjustments in metabolic and executive organ functions (liver, immune cells) and circulating serum components that assist in host defence (Hanisch, 2002).

Different studies regarding the role of IL6 in the PNS showed that it is predominantly pro-inflammatory in neuropathic pain, it can modulate acute nociception and increase the CGRP release. However, IL6 can have also opposing inflammatory responses in different conditions determined by the simultaneous presence of other factors (cytokines) (Hanisch, 2002; Austin and Molaem-Taylor, 2010). In fact, at the site of injury it may be anti-nociceptive, as well as promoting peripheral axonal regeneration. In studies on peripheral nerve injury, IL6 and IL6R normally activate Janus kinases (JAK) and signal transducers and activators of transcription (STAT) transcription factor pathways (Austin and Molaem-Taylor, 2010).

2.2.5.1.5 – Interferon gamma

IFNγ, a dimerised soluble cytokine, is originally called macrophage-activating factor, a term now used to describe a larger family of proteins to which IFNγ belongs. IFNγ interacts with two different receptors (Interferon gamma receptor 1 and 2 – IFNGR1/2) activating the JAK-STAT pathway.

IFNγ is a potent pro-inflammatory cytokine implicated in the pathogenesis of neuropathic pain. Different immune cells produce IFNγ, but it can modulate both neurons and immune cells. Regarding immune cells, it has a pivotal role in the regulation of immune activity, in particular in the decision of whether Th1 or Th2 immune responses are favored (Hanisch, 2002; Austin and Molaem-Taylor, 2010). It promotes up-regulation of several proteins: notably iNOS, the purinergic receptor 4 (P2X4), and chemokine receptor CCR2, all of which contribute to the development of neuropathic pain states (Hanisch, 2002; Austin and Molaem-Taylor, 2010).

2.2.5.1.6 – Other important cytokines implicated in migraine

Other important cytokines mainly implicated in neuroinflammatory processes and even in migraine

are IL2, IL4, IL5 and IL12p70.

IL2 is produced mainly by T-cells and drives T- and B-cell proliferation and differentiation. In combination with LPS or IFN-γ, IL2 can enhance microglial NO production (Martelletti, 1991; Hanisch, 2002; Perini et al., 2005).

IL4 is an anti-inflammatory cytokine, released by activated T cells, mast cells and granulocytes. There are several studies suggesting analgesic IL4 actions: it is anti-nociceptive in models of acute and chronic pain, it attenuates mechanical allodynia and thermal hyperalgesia. IL4 anti-nociceptive effects are largely due to the suppression of pro-inflammatory cytokines (in specific IL1β and TNFα) and macrophage/microglia activation; additionally, promoting the differentiation of the anti-inflammatory Th2 cell population (Uçeyler et al., 2009; Austin and Moalem-Taylor, 2010).

IL5 is produced by T helper-2 cells and mast cells; it stimulates B cell growth and eosinophil activation (Haddad, 2002). Interleukin 5 has long been associated with the cause of several allergic diseases including allergic rhinitis and asthma, but recently studies on migraneurs reveal that also this cytokines is involved in migraine (Munno et al., 1998; Munno et al., 2001).

IL12p70 is a heterodimeric cytokine composed of two covalently linked chains, p40 and p35. It is produced by macrophages, dendritic cells and B cells in response to antigenic stimulation. It acts on NK cells and T cells, regulating growth and development of these cells. It is the major inducer of IFN-γ production, and at the same time IFN-γ itself is capable of substantially increasing the IL12 production via transcriptional regulation (Haddad, 2002; Müller-Berghaus et al., 2004).

2.2.5.2 – Chemokines

Chemokines (CHEMOtactic cytoKINES) are small, secreted cytokines that are important because of their chemoattractant effects that control the migration of different classes of immune cells in association with the development of inflammation. These cytokines has been shown to contribute directly also to nociception, in the induction and maintenance of chronic pain (Bernardino and Malva, 2007; White et al., 2007; Austin and Moalem-Taylor, 2010).

Chemokines are classified according to the number and the relative position of the first N-terminal cysteine residues in their primary structure; so four different subgroups are indentified. Alfa (CXC) are chemoactractant for lymphocytes, monocytes and neutrophils; beta (CC) for monocytes, macrophages, lymphocytes, bosophils, eosinophils, dendritic and natural killer; gamma (XC) and delta (CXC3) for mononuclear inflammatory cells. Chemokine receptors are G-coupled proteins (Bernardino and Malva, 2007).

Chemokines and their receptors can be divided in two main categories: (1) homeostatic chemokines, secreted constitutively involved in basal leukocyte trafficking, developmental organization of the brain, regulation of neurotransmitter release, modulation of ion channel activity

and cell death or survival; (2) inflammatory/secreted, up-regulated or produced by cells during infection, injury or inflammation. The main aim of the latter chemokines is to attract leukocytes, microglia and astrocytes to an injured or infected site, to regulate and influence acute and chronic pain. Chemokines are important molecules involved in the cross-talk between neurons and non-neuronal cells (Bernardino and Malva, 2007; White et al., 2007; Austin and Moalem-Taylor, 2010).
In particular, MCP-1, also known as Chemokine (C-C motif) ligand 2 (CCL2), and its receptor, CCR2, have been implicated in macrophage recruitment to sites of tissue injury, infection, and inflammation. CCR2 exhibit an exceptionally prolonged up-regulation in the injury-associated DRG and the trigeminal ganglion following peripheral nerve injury, supporting the possibility that this type of signaling could contribute to the chronic nature of neuropathic pain (White et al., 2007; Austin and Moalem-Taylor, 2010). At the injury site and in the DRGs, MCP-1 was released by damaged neurons. The activation of CCR2 sensitizes nociceptors via transactivation of TRPV1. The up-regulation in DRG neurons is TNFα dependent. Thus, TNFα may be the upstream regulator of chemokine signaling in these cells (White et al., 2007; Austin and Moalem-Taylor, 2010).

2.2.5.3 – Metalloproteinases

Metalloproteinases (MMPs) are produced by different cells including neurons, glia, leukocytes and macrophages. MMPs have many roles in CNS ranging from remodelling tissues during development to a critical role in multiple phases of neuroinflammation. One of these proteolityic enzymes, MMP-9, has been shown to be up-regulated in plasma of migraineurs (Leira et al., 2007). In particular, MMP-9 induces BBB disruption, is implicated in changes in vascular permeability and macrophage recruitment. Recently, MMP-9 gene deletion or pharmacologic inhibition has been shown to reduce injury-induced macrophage recruitment and protects nerves from axonal degeneration (Chattopadhyay et al., 2007). Indeed, MMP-9 and TNFα seem have a synergic effect for macrophage recruitment in PNS (Shubayev et al., 2006). In cortical spreading depression, neuronal and glial depolarization initiates a cascade that disrupts the BBB via an MMP-9-dependent mechanism (Leira et al., 2006).

2.3 Cross-talk between neurons and immune cells: implication for migraine pathophysiology

There is emerging evidence from animal models that sustained inflammatory responses involving microglia, macrophages and astrocytes contribute to neurological disease progression (Glass et al., 2010). Studies of spinal cord or nerve injury have elucidated the time profile of neuroinflammation in acute and chronic pain as a consequence of the bidirectional cross-talk between immune cells and neurons (Fig. 2.11; Allan and Rothwell, 2001; Watkins and Maier, 2002; Donnelly and Popovich, 2007; Thacker et al., 2007; Miller et al., 2009; Takeda et al., 2008; Austin and Moalem-Taylor, 2010; Ren and Dubner, 2010; Guillot et al., 2011).

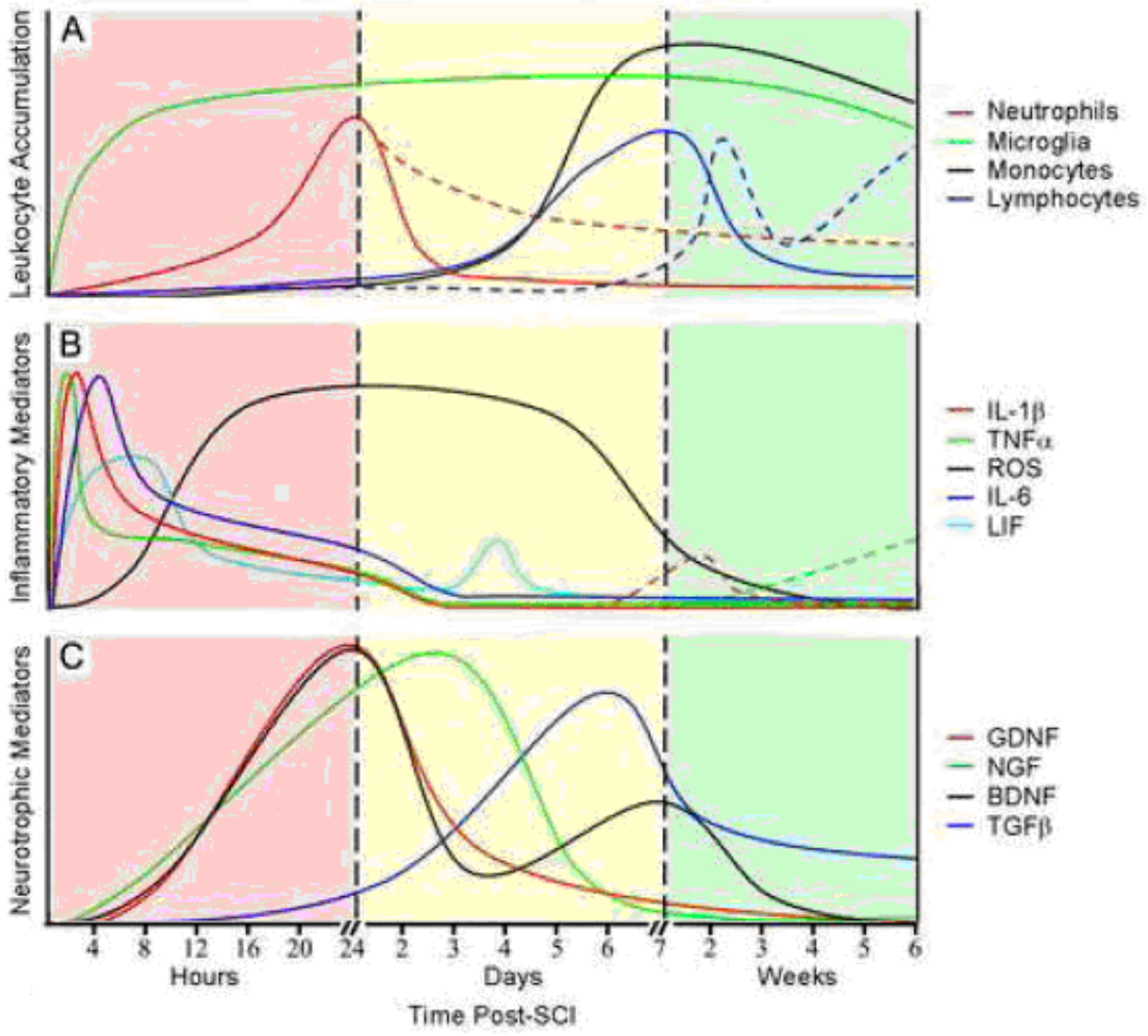


Fig. 2.11 - Temporal correlation between inflammatory cascades, secondary neurodegenerative events and functional recovery in SCI rodents

A, Activation of resident microglia and intraspinal accumulation of circulating leukocytes. Dashed lines departing from

solid curves depict data from SCI mice whereas continuing solid curves indicate data from SCI rats. Solid curves before these break points are from both species. *B*, Expression of proinflammatory cytokines and reactive oxygen species (ROS). *C*, Expression of neurotrophic cytokines. Values on the vertical axis represent relative changes and are not to scale.

(Adapted from Donnelly and Popovich, 2007)

As described before, there is a theory that involves neuroinflammation in migraine pathophysiology (Moskovitz and Buzzi, 2010).

Following the Moskowitz's theory, a sterile inflammation is caused by neuropeptides (substance P and CGRP) at meningial levels, evoking mast cell degranulation and the release of pro-inflammatory mediators, causing plasma protein extravasation and vasodilatation (Williamson and Hargreaves, 2001; Ren and Dubner, 2010; Robbins and Maides et al, 2011). These substances causing activation of resident macrophages that respond in 1-2 hours releasing pro-inflammatory cytokines, beginning with TNFα, followed by the stimulation of IL1β, and subsequent release of IL6 (Allan and Rothwell, 2001; McMahon et al., 2005; Austin and Moalem-Taylor, 2010; David and Kroner, 2011; Guillot et al., 2011).

At the same time also neurons became active through ATP and purinoreceptors releasing other immune and migraine mediators (Suadicani et al., 2010; Kristiansen and Edvinsson, 2010; Ren and Dubner, 2010; Fumagalli et al, 2011). The neuronal responses to immune mediators was evaluated with application of different immune mediator to sensory neurons: LPS evokes a concentration-dependent increase in intracellular calcium accumulation (Ca^{2+}_i) and inward currents, sensitizing TRPV1 responses via TLR4 (Acosta and Davies, 2008; Chung et al., 2011; Diogenes et al., 2011). The same pathway seems activated on sensory neurons by TNFα: acute application of TNFα increases nociception-related ion currents in DRG neurons via an up-regulation of TRPV1 receptor (Li et al., 2004; Schaible et al., 2010). Indeed, TNFα increases BDNF mRNA and protein expression via p38 pathways in trigeminal ganglia neurons (Balkowiec-Iskra et al., 2011).

These studies prove that immune cell mediators can modulate trigeminal ganglion neurons with implication for pathophysiology of migraine and for trigeminal neurophatic and neuroinflammatory pain (Sommer and Kress, 2004; Takeda et al., 2007; Schaible et al., 2010; Balkowiec-Iskra et al., 2011).

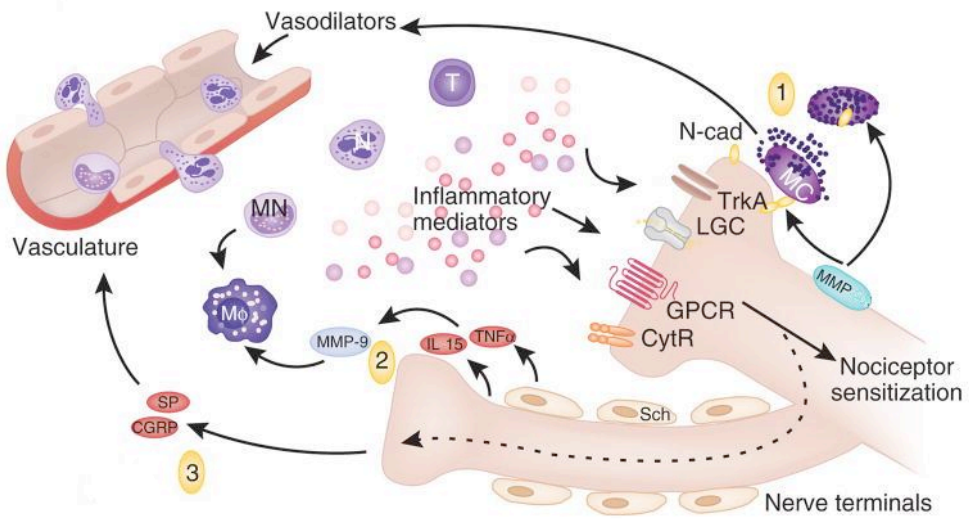


Fig. 2.12 - Immune activation and nociceptor sensitization after injury.

Migraine caused mast cells (MC) degranulation and vasodilators released, promoting adhesion and transmigration of immune cells including T cells (T), neutrophils (N) and monocytes (MN), and recruitment of macrophages (MΦ). These cells, once activated, release a battery of inflammatory mediators that act on receptors expressed on adjacent nociceptor nerve terminals, leading to peripheral nociceptor sensitization. Targets include cytokine receptors (CytR), G protein–coupled receptors (GPCR), ligand-gated channels (LGC) and tyrosine kinase receptor type 1 (TrkA). Three examples of interactions between immune cells and nerve terminals are depicted. (1) Mast cell degranulation requires direct contact between mast cells and nerve terminals, mediated by N-cadherin (N-cad). The metalloproteinase MMP prevents mast cell degranulation by digesting N-cad. (2) Release of TNFα and IL15 by peripheral nerves and Schwann cells activates MMP-9 and facilitates recruitment of macrophages. (3) Nociceptive nerve terminals can secrete substance P (SP) and CGRP through activation of neighboring nerve terminal branches. Substance P and CGRP promote vasodilation and extravasation of immune cells.

(modified from Ren and Dubner, 2010)

2.4 Purinergic signalling in neuroinflammation and in migraine pain

2.4.1 – ATP and the purinergic theory of neuroinflammation

As mentioned above, one pathway of immune cell-to-neuron communication involves different purinergic receptors. The importance of ATP and purinergic receptor was understood in the 90's when different studies showed the involvement of them in pain and in inflammation.

Although extracellular actions of ATP have been known since the late 1920s, its involvement in immunomodulation has been considered only during the last 10 years (Di Virgilio and Lemaire, 2006). Indeed, ATP is also associated to pain (Inoue, 2006).

ATP was identified as a co-transmitter in sympathetic and parasympathetic nerves and it acts as either sole transmitter or a co-transmitter in most nerves in both the peripheral nervous system and central nervous. ATP is present in synaptic and/or secretory vesicles and can be co-stored and co-released with other neurotransmitters (e.g. γ-aminobutyric acid - GABA, noradrenaline or glutamate; Abbracchio et al., 2009). Intracellular concentriation of ATP is in the millimolar range, and due to its negative charges cannot passively diffuse across the plasma membrane. As the extracellular ATP concentration is normally in nanomolar range, even a small ATP release can cause high signal-to-noise ratio (Di Virgilio and Lemaire, 2006).

ATP and other nucleotides are conserved in secretory and synaptic vesicles. Uptake of ATP is mediated by a Cl^--dependent vesicular nucleotide transporter (VNUT), which belongs to the SLC17 anion transporter family, which includes also vesicular glutamate transporters (Abbracchio et al., 2009). There is evidence for exocytotic neuronal vesicular release of ATP and also vesicular release of ATP from astrocytes. ATP can be released through other mechanisms such as ATP-binding cassette transporters, connexin or pannexin hemichannels, plasmalemmal voltage-dependent anion channels, in addition to P2X7 receptors (Fig. 2.13; Abbracchio et al., 2009; Burnstock et al., 2011).

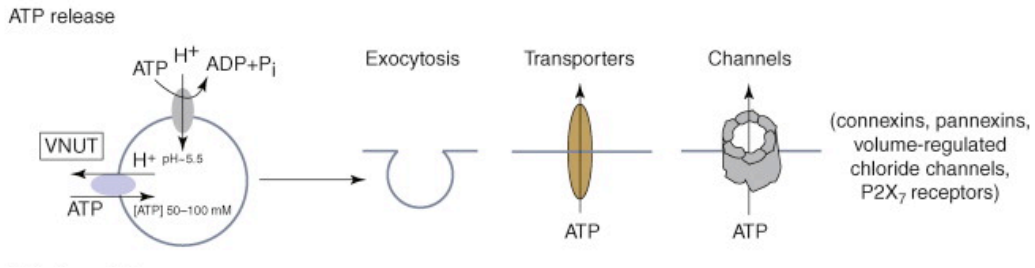


Fig. 2.13 – ATP release

(Adapted from Abbracchio et al., 2009)

ATP acts through P2 purinergic receptors that are classified in two main sub-groups: P2X, ionotropic, and P2Y, metabotropic, receptors on the basis of mechanism of action, pharmacology and molecular cloning (Fig. 2.13; Abbracchio et al., 2009).

The term purinergic receptor was originally introduced to illustrate specific classes of membrane receptors that mediate relaxation of gut smooth muscle as a response to the release of ATP (P2 receptors) or adenosine (P1 receptors). Various purinergic receptor subtypes have been shown to be widely distributed throughout the nervous system and the PNS in neurons and immune cells (Abbracchio et al., 2009). P2X receptors (P2X1–P2X7) are coupled to nonselective cation channels, allowing the influx of Na^+ and Ca^{2+}, whereas P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14) are G-protein coupled, and their activation leads to inositol lipid hydrolysis, intracellular Ca^{2+} mobilization, or modulation of adenylate cyclase activation (Inoue, 2006).

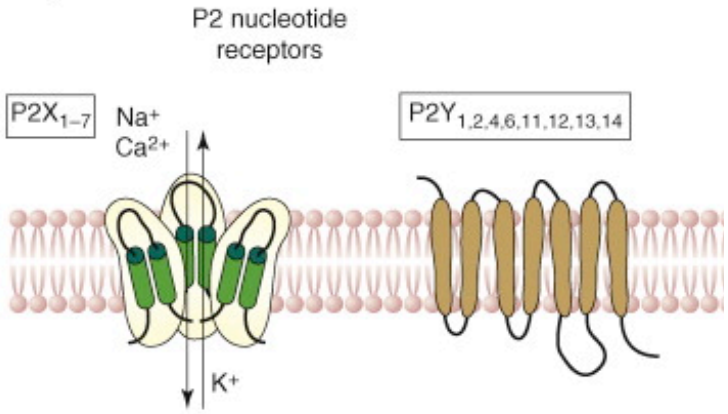


Fig. 2.13 – Purinergic receptos P2X and P2Y

(Adapted from Abbracchio et al., 2009)

The concept of purinergic neurotransmission was introduced in the 70's by Burstock (Burnstock, 1976; Abbracchio et al., 2009). Purinergic receptors are a family of characterized plasma membrane molecules involved in several cellular functions such as vascular reactivity, apoptosis and cytokine secretion. In neuropathic pain or neuroinflammatory diseases, ATP is a crucial cell-to-cell mediator. Although it was thought that the main source of extracellular ATP was from damaged or dying cells, it is now well estabilished that ATP is released in physiologic conditions by healthy cells via several mechanisms, acting as a fast excitatory neurotransmitter or playing long-term (trophic) roles in cell proliferation, growth and development (Inoue, 2006; Abbracchio et al., 2009).

Apart from the demonstration that P2 receptors are expressed in all the cell types involved in inflammatory reactions, different studies performed on spinal cord injury and inflammation are at the basis of the "purinergic theory of inflammation" (Marchand et al., 2005; Fumagalli et al., 2011;

Ren and Dubner, 2011). P2X receptor antagonists have been demonstrated to reduce nociceptive sensitivity in a wide variety of animal models, including tail-flick, chemically induced persistent and inflammatory pain, and neuropathic pain (Inoue, 2006; Donnelly-Roberts et al., 2008).

ATP can act either directly on neurons (e.g., P2X3, P2X2/3, and P2Y receptors) or indirectly through neural-glial cell interactions (P2X4 and P2X7 receptors) (Donnelly-Roberts et al., 2008). Suadicani et al. (2010) demonstrated bidirectional calcium signaling between neurons and satellite glial cells in sensory ganglia cultures, via activation of purinergic P2 receptors, and, to some extent, gap junctions. Furthermore, they propose that not only sensory neurons, but also SGCs release ATP. This form of intercellular calcium signaling likely plays key roles in the modulation of neuronal activity within sensory ganglia in normal and pathological states (Suadicani et al., 2010)

ATP strongly activates microglia/macrophages P2 receptors stimulating the release of IL6, TNFα, and IL1β and affecting their responses (proliferation, process motility, migration, phagocytosis) (Fumagalli et al., 2011).

2.4.2. – Purinergic receptor in microglia/macrophages

Different purinergic receptors are found on microglia/macrophages, among them P2X4, P2X7, P2Y2 and P2Y6, and there are also differently expressed in the different phase of activation of immune cells (Inoue, 2006; Abbracchio et al., 2009).

2.4.2.1 – P2X4

P2X4 receptors are widely expressed in a variety of cell types, including both neurons and microglia in the central nervous system. Homomeric P2X4 subunits constitute slow desensitizing calcium-permeable cationic channels that can be activated by ATP but are less sensitive to α,β - meATP and BzATP. P2X4 receptors play a role in the development of neuropathic and inflammatory pain. After spinal nerve injury, P2X4 receptor protein expression increases in spinal microglia, but not in neurons or astrocytes, but it remaines unchanged in DRG neurons (Donnelly-Roberts et al., 2008).

The up-regulation of P2X4 receptor expression in microglia is therefore a key process in neuropathic pain, but its mechanism is not clearly understood. P2X4 mRNA levels can be raised by activating toll-like receptors (TLRs) and an extracellular matrix protein, fibronectin. The last one seems very important for the enhancement of nerve injury-induced P2X4 up-regulation and allodynia (Tsuda et al., 2010). P2X4 mRNA and protein up-regulation is regulated by different pathways: the phosphatidylinositol 3-kinase (PI3K)-Akt and mitogen-activated protein kinase kinase (MAPK kinase) extracellular signal-regulated kinase (ERK) signaling cascades (Tsuda et al., 2010).

2.4.2.2 – P2X7

Among P2X receptors, one of the most studied candidates for inflammatory modulation is the P2X7 one. Homomeric P2X7 receptors are activated by high concentrations of ATP (>100 μM): prolonged agonist activation leads to the formation of large cytolytic pores in the cell membrane allowing entry of non-selective large hydrophilic cations (North, 2002; Burnstock, 2007; Donnelly-Roberts et al., 2008; Fumagalli et al., 2011). P2X7 receptors are expressed by cells of hematopoietic lineage, including mast cells, lymphocytes, erythrocytes, and highly expressed by peripheral macrophages, while in the CNS, P2X7 receptors are localized mainly to microglia as well as astrocytes and Schwann cells (North, 2002; Burnstock, 2007; Donnelly-Roberts et al., 2008).

P2X7 receptor activation supports inflammatory and neuropathic pain. The first evidence indicating the involvement of P2X7 receptors in pain was provided by a study using P2X7-deficient mice. These mice exhibited reduced thermal and mechanical hypersensitivities after partial sciatic nerve ligation (Tsuda et al., 2010). P2X7 receptor-activation has a role in cytokines release: P2X7 activates of caspase-1 and the rapid maturation and release of the proinflammatory cytokine, IL1β (Donnelly-Roberts et al., 2008; Fumagalli et al., 2011).

Studies with P2X7 receptor-selective ligands provide direct evidence that acute in vivo block of P2X7 receptors significantly reduces nociception in animal models of persistent neuropathic and inflammatory pain. ATP induces the release of IL1β from microglia in a manner that requires purinoreceptor 7 (P2X7) and a priming of TLRs by LPS, suggesting that this occurs only upon inflammation (Donnelly-Roberts et al., 2008; Ren and Dubner, 2010; Tsuda et al., 2010; Fumagalli et al., 2011). IL1β released after stimulation by sensory ganglion satellite glial cells can modify neuronal excitability (Takeda et al., 2007) or activity, modulating neuronal mediator release (Capuano et al., 2009).

Interestingly, recent evidence has indicated a structural and functional interaction between P2X7 and P2X4 receptors, in particular a functional heteromeric combination of P2X4 and P2X7 (P2X4/7) receptors has been described in mouse macrophages (Donnelly-Roberts et al., 2008; Tsuda et al., 2010). The receptor P2X7 also modulates the P2X3 receptor: satellite cell P2X7 tonically inhibits P2X3 receptor expression in DRG neurons (Ren and Dubner, 2010).

2.4.3. – P2X3

Peripheral neurons (both sensory and autonomic) predominantly express P2X3 and P2X2/3 receptors, which are implicated in pain and temperature perception (Abbracchio et al., 2009). P2X3 is selectively expressed by a large population of DRG and TG sensory neurons (Simonetti et al.,

2006).

While the P2X3 receptor is thought to be strongly implicated in acute, chronic or inflammatory pain, contribution of P2X2/3 seems to be limited to chronic pain. Up to now it is unclear what signals are responsible for heteromeric assembling in vivo, even if heteromeric channel formation could modify pain transmission. Direct peripheral and spinal application of the P2X3/P2X2/3 antagonist, A-317491, attenuates hyperalgesic responses in complete Freund's adjuvant-inflamed animals and reduces formalin-induced nocifensive behaviors (Donnelly-Roberts et al., 2008).

An interesting electrophysiological property of homomeric P2X3 receptor is fast and long-lasting receptor inactivation in the continuous agonist presence (desensitization). In fact, repetitive activation P2X3 receptors by ATP cause a marked desensitization of ATP-induced responses. Conversely, decreasing desensitization of P2X3 receptors or accelerating recovery from it may represent an efficient mechanism for sensitization of nociceptive neurons to ATP in chronic pain (Jarvis, 2003; Giniatullin et al., 2008).

P2X3 receptors are suggested to contribute to migraine pain because some migraine mediators decrease receptor desensitization and, in particular, accelerate their recovery, thus ensuring more efficient transmission of nociceptive signals. Furthermore, enhanced responses of trigeminal neurons mediated by P2X3 receptors in the presence of migraine mediators suggests that the phenotype of such cells has become a more effective transducer of ATP-mediated signalling (Giniatullin et al., 2008).

2.5 Genetics in migraine

Both familial clustering and twin studies suggest that significant genetic mechanisms underlie migraine. Based on different genetic approach studies, it is hypothesized that rare monogenic subtypes and common multifactorial types of migraine share common genes and related biochemical pathways for the trigger threshold and initiation mechanisms of attacks. Several studies have indicated various susceptibility genes for migraine with and migraine without aura associated with variants in oestrogen, progesterone, and insulin receptor genes, as well as promoter variants cytokines (Wessman et al., 2007). In fact, Yilmaz et al. (2010) have shown possible contribution of TNFα and IL1β gene polymorphisms to migraine headache generation.

The rare mendelian form of migraine, familial hemiplegic migraine (FHM), is the most successful model for the identification of migraine-associated cellular mechanisms (Pietrobon, 2007; Wessman et al., 2007; Pietrobon, 2010a; van den Maagdenberg et al., 2010).

2.5.1 – Familial Hemiplegic Migraine

FHM is a rare, severe, monogenic subtype of migraine with aura, characterized by transient weakness (hemiparesis) (Headache Classification Subcommittee of the International Headache Society, 2004). Apart from the hemiparesis, the headache and aura features of the FHM attack are identical to common migraine.

Thus, from a clinical point of view, FHM seems a representative of the migraine spectrum, and a valid model to study migraine (van den Maagdenberg et al., 2010).

Three genes (two ion-channel genes and one encoding an ATP exchanger) have been found to underlie FHM. These mutations probably contribute to hyperexcitability of neurons by either an increased release or an inefficient clearing of synaptic glutamate (Fig. 2.14) (Pietrobon, 2007; Wessman et al., 2007; Pietrobon, 2010a; van den Maagdenberg et al., 2010).

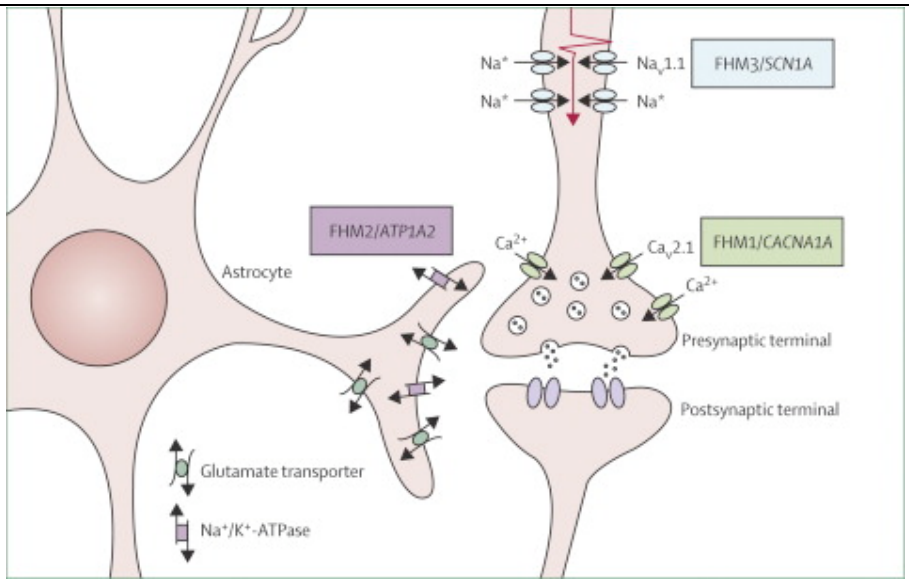


Fig. 2.14 - Functional roles of the proteins coded by known FHM

(Wessman et al., 2007)

2.5.1.1 – FHM-1

In 1996 Ophoff and co-workers were able to show that FHM, linked to chromosome 19p13 is caused by missense mutations in the CACNA1A gene, encoding the pore-forming α1 subunit of a P/Q type voltage-gated neuronal Ca^{2+} channel, $Ca_V2.1$ (Wessman et al., 2007; van den Maagdenberg et al., 2010). The main function of neuronal $Ca_V2.1$ calcium channels is to modulate release of neurotransmitters, at peripheral neuromuscular junctions as well as central synapses, mainly within the cerebellum, brainstem, and cerebral cortex (van den Maagdenberg et al., 2010). $Ca_V2.1$ channels are expressed in all brain structures that have been implicated in the pathogenesis of migraine, including the cerebral cortex, the trigeminal ganglia, and brainstem nuclei involved in the central control of nociception (Pietrobon and Striessnig, 2003; Pitetrobon, 2010a; Nair et al., 2010).

CACNA1A causes about 50% of all cases of FHM, but different *CACNA1A* mutations have been associated with a wide range of clinical phenotypes including pure forms of FHM or combinations with various degrees of cerebellar ataxia or fatal coma (Wessman et al., 2007; Pietrobon, 2010a; Van Den Maagdenberg et al., 2010). $Ca_V2.1$ is connected to inflammatory and neuropathic pain, because a 50% loss of $Ca_V2.1$ channels leads to reduced inflammatory responses (Pietrobon, 2010a).

Numerous different missense mutations associated with FHM1 have been described (Fig. 2.15). All produce substitutions of conserved amino acids in important functional regions, including the pore lining and the voltage sensors (Pietrobon, 2010a)

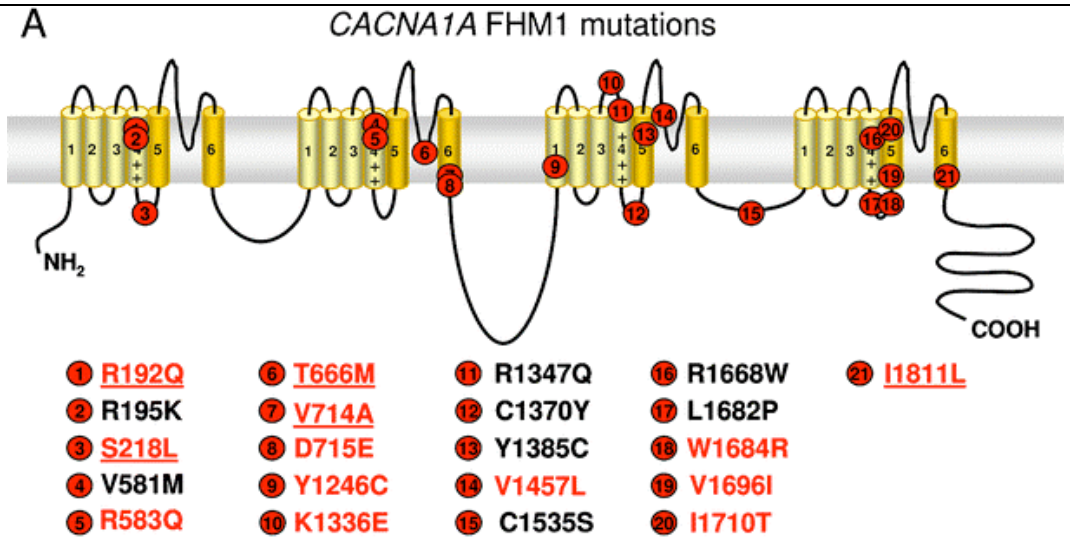


Fig. 2.15 - Stuctures of P/Q type voltage gated calcium channel. Mutations responsible of FHM-1

(Pietrobon, 2010b)

CACNA1A mutations affect the biophysical properties of individual channels; channel gating, and density of functional channels on the cell surface. The channel dysfunction allows it to open in response to smaller depolarisations than does the wild-type channel (Wessman et al., 2007; Pietrobon, 2010b). The enhanced channel activity at negative potentials leads to an increased local Ca^{2+} influx and gain of function at the single-channel level, which could contribute to the neuronal hyperexcitability. Moreover, mutant channels stay open for a larger fraction of time, thus allowing more Ca^{2+} influx than WT channels (Wessman et al., 2007; Pietrobon, 2010b). The shift to lower voltages of $Ca_V2.1$ channel activation, was measured in cerebellar and cortical neurons of knockin mice carrying two different FHM-1 mutations: R192Q and S218L (van den Maagdenberg et al., 2004; Pietrobon, 2010b). While the R192Q mutation produces a pure FHM phenotype characterized by typical attacks, the S218L mutation produces a severe clinical phenotype, in which typical attacks of FHM triggered by minor head trauma are frequently followed by deep coma and other common symptoms are ataxia and cerebral and/or cerebellar atrophy. The homozygous R192Q knockin mice appear healthy; the homozygous S218L mice are prone to sudden death for seemingly unknown reasons (Pietrobon, 2010b).

Cacna1a knock-in mice homozygous for the human FHM-1 R192Q mutation have enhanced neurotransmission at the neuromuscular junction and, most interestingly, increased susceptibility to cortical spreading depression (van den Maagdenberg et al., 2004; Wessman et al., 2007).

2.5.2 – *Cacna1a* R192Q KI mice

Natural mutants and conventional knockout (KO) mice of $Ca_V2.1$-α_1 exist with phenotypes ranging from severe ataxia, dystonia, and premature death (leaner, $Ca_V2.1$ KO) to ataxia or epilepsy (tottering, rolling Nagoya, and rocker). The role of this channel is also supported by the fact that the $Ca_V2.1$ KO mice die at an early age, because *Cacna1a* is broadly expressed in CNS (Todorov et al., 2006).

Loss of function of $Ca_V2.1$ channel affects mainly cerebellar function, whereas gain of function leads to cortical dysfunction. These mutations were studied in heterologus systems, but this can give artifacts due to overexpression. To better understand the functional properties of the mutated channel, van den Maagdenberg et al. (2004) developed a transgenic mouse strain carrying the human FHM-1 mutation R192Q to provide a well defined genetic mouse model of migraine. Functional analysis revealed a pure gain-of-function effect on Ca^{2+} channel current, characterized by increased neurotransmission at the neuromuscolar junction, and increased susceptibility to cortical spreading depression, with a reduced threshold and increased velocity. In fact, electrophysiological recordings from cortical slices show a gain of function phenotype: as a consequence of this mutation the channel opens more readily at more negative voltage than wild type channels leading to increased Ca^{2+} influx (van den Maagdenberg et al., 2004).

These results from R192Q KI mice may explain the underlying mechanism for the increased susceptibility to migraine aura and can be useful in vivo models to study migraine pathophysiological processes.

Chapter 3: AIMS OF THE STUDY

Even if the neuroinflammatory theory proposed by Moskowitz has been confirmed by different studies, it is important to understand if neuroinflammation, there is in migraine animal models. Moskowitz and collaborators have proposed that during the migraine attack occurs a condition of sterile inflammation, a process of acute inflammation with recruitment of inflammatory cells and release of local pain mediators in the meninges. Previous studies have suggested that meningeal tissue of migraine animal models and migraneurs show histological changes typical of neuroinflammation, while it is unclear whether trigeminal ganglia are also affected by such neuroinflammatory process. This seems to be a likely possibility because antigen-presenting immune cells have been characterized in human trigeminal ganglia and certain chronic pain models are associated with neuroinflammatory changes in dorsal root ganglia. Hence, it is not known if, at the level of trigeminal ganglia, a FHM-1 mouse model with a R192Q missense mutation in the $\alpha 1$ subunit of voltage-gated $Ca_V2.1$ calcium channels might show changes consistent with this hypothesis.

In this contest my project was focused on understanding whether, during the latent period of migraine attack, there is a neuroinflammatory constitutive pathology that may predispose to the migraine attack by facilitating the activation of nociceptive sensory neurons.

First, immune-derived non-neuronal cells in TG ganglia from WT and *Cacna1A* KI mice were characterized. In particular, macrophages in WT and R192Q KI trigeminal ganglion were studied by evaluating their expression markers and their state of activation. Inflammatory mediators, such as cytokines and chemokines, were investigated in basal condition and after inflammatory stimuli.

Subsequently, the crosstalk between sensory neurons and macrophages in trigeminal ganglion cultures from WT and R192Q KI mice was studied. The effects of non-neuronal cells on TG ganglion neurons were evaluated through changes in P2X3 function after inflammatory stimuli or macrophage addition to trigeminal ganglion cultures. Viceversa, the effects of neurons on non-neuronal cells was investigated by estimating the state of activation of macrophages.

Chapter 4: MATERIALS AND METHODS

4.1 - Animal procedures

$Ca_V2.1$ R192Q knock-in (R192Q KI) and wild-type (WT) female littermates (P10, P30 and P90) were used. Animals were maintained in accordance with the guidelines of the Italian Animal Welfare Act and their use was approved by the Local Authority Veterinary Service. Genotyping was performed by PCR as previously reported (van den Maagdenberg et al., 2004).

Trigeminal ganglion cultures were obtained from animals at the age of P12-14 as described before (Simonetti et al., 2006) and employed after 1 or 2 days from plating. Briefly, primary cultures of TG ganglion sensory ganglia were obtained from WT or R192Q KI mice (P12–14). Animals were anesthetized by diethyl ether and decapitated (in accordance with the Italian Animal Welfare Act and approved by the Local Authority Veterinary Service). TG were rapidly excised and enzymatically dissociated in F12 medium (Invitrogen Corp, S.Giuliano Milanese, Italy) containing 0.25 mg/ml trypsin, 1 mg/ml collagenase and 0.2 mg/ml DNAse (all from Sigma, Milan, Italy) at 37°C. Cells were plated on poly-L-lysine-coated petri dishes in F12 medium with 10% fetal calf serum. For patch clamp experiments, cells were diluted twice. For ELISA experiments, each Petri dish contains ganglia from 2 mice. For 2 days in culture experiments, after 24 h from plating, half of the medium was changed.

4.2 - Protocol for macrophage-trigeminal ganglion co-cultures

To explore the potential interaction between macrophages and trigeminal ganglion cells, we tested the protocol to supplement standard trigeminal ganglion cultures with the addition of host macrophages. To this end, mouse host macrophages (MΦ) were extracted from the peritoneal cavity of WT mice 72 h after a single intraperitoneal injection (i.p.) of Brewer thioglycollate medium (0.4 g/kg, 3 % wt/vol) (Ray and Dittel, 2010). Primary macrophages were plated in DMEM/10% FBS medium and kept for 14 days in culture (with change of medium every 48 h). Thereafter, macrophages were collected, counted in a Burker chamber, and transferred, for co-cultures experiments, to Petri dishes containing trigeminal ganglia (from WT or R192Q KI mice) dissociated on the same day. Experiments were performed after 2 days of co-culturing. Preliminary experiments were run to estimate the number of host macrophages to be added for co-culturing. Thus, we used patch-clamp currents generated by P2X3 receptor activation as a reliable index of nociceptive sensory neuron activity (see below; Simonetti et al., 2006) to study whether such functional responses were influenced by the number of host macrophages (120,000 or 300.000 or 1 million). Under these conditions, while control WT currents had an average amplitude of -290 ± 30 pA (*n* =

15), co-culturing with 120,000 host macrophages raised the average amplitude of P2X3 currents to -500 ± 60 pA (n = 24; p = 0.05). Larger macrophage numbers (300,000 or 1 million) led to current amplitudes of -660 ± 90 (n = 6) or -540 ± 70 pA (n = 7) that were not significantly different from the value observed with 120,000 macrophages. The latter value was then employed for subsequent experiments. We next investigated how addition of 120,000 host macrophages changed the global number of such cells in co-culture: thus, in WT culture, endogenous macrophages per ROI were 10 ± 1, while in KI culture the corresponding value was 18 ± 1 (n = 3, $p < 0.05$). With 120,000 macrophages, the number of Iba1 cells per ROI was 23 ± 2 in WT co-cultures and 38 ± 4 in KI co-culture (n = 3, WT+MΦ vs KI+MΦ, $p < 0.05$ co-cultures vs controls, $p < 0.01$).

4.3 - Immunohistochemistry

4.3.1 - Immunohistochemistry of trigeminal ganglia tissue

WT or R192Q KI mice were deeply anesthetized with i.p. urethane (0.3 ml of 1 gr/ml; Sigma).

For total macrophage and DAPI cell counting, intact trigeminal ganglia from WT or R192Q KI female mice of different ages (P10, P30, P90) were fixed in 4% paraformaldehyde in phosphate saline buffer (PBS) at 4°C for at least 24h and cryopreserved in 30% sucrose (in PBS) at 4°C for up to 48 h. Serial longitudinal sections (1 over 3) of 14 micron-thick trigeminal ganglia were collected on SuperFrost glass specimens (Microm, Thermo Fisher Scientific, Waltham, MA, USA),

For neuronal areas (V1, V2, V3; Thalakoti et al., 2007) cell counting, P30 female mice were perfused transcardially with PBS followed by 4% paraformaldehyde. Trigeminal ganglia were removed, postfixed for 1 h at room temperature and cryoprotected overnight in 30% sucrose at 4°C. Each immunohystochemistry experiment was performed on an average of 7 cryostat-cut serial longitudinal slices (14 µm-thick) sampled every ~70 µm, and thus covering the entire ganglion.

Samples were incubated in a blocking solution containing 5% bovine serum albumin (BSA), 1% fetal bovine serum (FBS) and 0.5% Triton X-100 in PBS for 1 h at room temperature (RT) and immunostained with primary antibodies (over night at 4°C) in blocking solution and secondary antibodies (2 h at RT, Table X.1) in PBS solution. After that, nuclei were counterstained with DAPI (1:1.000 in PBS; Sigma). Immunohistochemistry data were obtained by at least 3 independent experiments, where WT and R192Q KI trigeminal ganglia were processed in parallel.

4.3.2 - Immunohistochemistry of trigeminal ganglia cultures

Immunocytochemistry of trigeminal ganglia in culture from WT or KI mice was performed as already described (Simonetti et al., 2006). Briefly, TG culture cells were fixed in 4% paraformaldehyde for 20 min at RT. Sample were incubated in a blocking solution containing 5% BSA, 1% FBS and 0.1% Triton X-100 in PBS for 1 h at room temperature and immunostained with

primary antibodies (2 h at RT, Table 4.1) in blocking solution and secondary antibodies (1 h at RT) in PBS solution. After that, nuclei were counterstained with DAPI (1:1.000 in PBS; Sigma). Immunohistochemistry data were obtained from at least 3 independent experiments, in which WT or R192Q KI samples were processed in parallel.

Table 4.1 – Primary and Secondary antibodies used

Antibodies	Diluition used	Species	Source
anti-Iba1	1:300	rabbit	Wako, Richmond, VA, USA
anti-β-Tubulin III	1:1000	mouse	Sigma, Milan, Italy
anti-CD11b	1:50	rat	eBioscience, S.Diego, CA, USA
anti-CD31	1:100	rat	BD Pharmingen, Milan, Italy
anti-ED-1	1:50	mouse	AbD Serotec, Oxford, UK
anti-biotin-F4/80	1:50	rat	eBioscience, S.Diego, CA, USA
anti-glutamine synthetase	1:150	mouse	Millipore, Milan, Italy
anti-MAP2	1:300	mouse	Alomone, Jerusalem, Israel
anti-P2X3	1:300	guinea pig	Neuromics, Edina, MN, USA
anti-P2X3	1:300	rabbit	Alomone, Jerusalem, Israel
anti-P2X4	1:300	rabbit	Sigma, Milan, Italy
anti-P2X7	1:300	rabbit	Alomone, Jerusalem, Israel
anti-pro-TNFα	1:500	rabbit	AbCam, Cambridge, UK
anti-TNFα antibody	1:100	rat	eBioscience, S.Diego, CA, USA

Secondary antibodies used were: goat-anti-mouse / rabbit / rat / guinea pig AlexaFluor488- or 594-conjugated antibodies (1:500; Invitrogen) or donkey-anti-mouse / rabbit AlexaFluor488- or 594-conjugated antibodies (1:500; Invitrogen) and streptavidin-AlexaFluor 647 antibodies (1:100, Invitrogen).

4.4 - Image acquisition and definition of Region of Interest (ROI) for each experiments

We used different strategies of image acquisition depending on the purpose of the experiments.

For *in vivo* experiments, to evaluate the total number of Iba1 and DAPI cells, we acquired images from entire ganglia with Zeiss Axioskop fluorescence microscope (Zurich, Switzerland) and the region of interest (ROI) considered was 640 x 480 μm.

To evaluate different compartimentalization of macrophages in trigeminal ganglia or for reconstruction of longitudinal section of a trigeminal ganglion, we acquired Z-stack images with Leica confocal microscope (LEICA TCS SP2, Wetzlar, Germany). ROIs used to evaluate number of macrophage in fiber and neuronal areas were 300 x 300 μm.

To evaluate co-expression of different macrophage markers, we acquired Z-stack images (with Leica confocal microscope) of 3 different ROIs (370 x 370 μm) in each V areas.
Macrophage volumetric analysis was performed on 3D reconstruction (Z-stack; 0.5 μm steps) of high magnification confocal images (Leica).
For *in vitro* experiments, we acquired images of 320 x 240 □m with a Zeiss Axioskop fluorescence microscope.

4.5 - Cell counting and volume quantification

Different software was used for cell counting: DAPI counting *in vivo* was evaluated with Volocity 5.5 software (Perkin Elmer, Waltham, MA, USA), and *in vitro* with Image J and ITCN plugin (National Institutes of Health, U.S.A.); Iba1 cell counting in whole ganglia tissue was evaluated with CellCounter software (Glance Vision Technologies s.r.l., Trieste, Italy). For *in vitro* experiments and co-expression data, we used MetaMorph software (Molecular Devices, Downingtown, PA, USA).
Macrophage volume quantifications were obtained with ImageJ Voxel counter plugin (voxel, in μm^3).

4.6 - Drug treatment *in vivo* and *in vitro*

To evoke acute inflammation *in vivo*, WT or R192Q KI mice (P30) were injected i.p. with a single dose of saline (sham) or LPS (5 or 10 mg/kg, from E. coli 0111:B4; Sigma, Milan, Italy) 0.5, 1 or 5 h prior to sacrificing the animals. Ganglion tissue samples were collected and processed in parallel for WT and R192Q KI mice.
In trigeminal ganglia cultures, 0.5 μg/ml LPS was added to medium and experiments were carried out after 1, 5 and 24 h. CGRP (1μM) treatment was performed on trigeminal ganglion cultures (1 day *in vitro*) for 2 h. Parallel controls were treated with the same volume of saline in parallel.

4.7 - RNA isolation, reverse transcription and quantitative Real Time PCR

Total mRNA was extracted from primary cultures or intact ganglia as described before (Simonetti et al., 2006). For PCR experiments, total RNA was extracted from TG ganglia or from culture using Trizol reagent (Invitrogen). RNA quality was evaluated with formaldehyde gel and quantified with Nanophotometer (IMPLEN GmbH, Munich, Germany).
After Deoxyribonuclease I treatment (Invitrogen), cDNA synthesis and amplification were obtained using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacture's instructions.
Real time reactions were performed in duplicate in an iCycler IQ Real Time PCR System (Bio-Rad,

Hercules, CA, USA) using IQ SyBr Green Supermix Reactions (Bio-Rad) and primers listed in Table 4.2. All primer sequences were designed using Beacon designer (PREMIER Biosoft, CA, USA). The relative mRNA expression in the different samples was obtained after normalization based on glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-Tubulin III (β-Tub III) mRNA levels. All assays were validated for linearity of amplification efficiency and quantitative standard curves were obtained using serial dilutions of cDNA. To ensure absence of amplification artifacts, end point PCR products were initially assessed on ethidium bromide-stained agarose gels (2% in 0.5 X Tris-Borate-EDTA buffer) that gave a single band of the expected size for each assay (Table 4.2). Negative controls containing no template cDNA were run in each condition and gave no results. Calculations for relative mRNA transcript levels were performed using the comparative Ct method (ΔΔCt) between cycle thresholds of different reactions (Yuan et al., 2006). All steps were performed following the MIQE guidelines (Bustin et al., 2009).

Table 4.2 – Real time PCR primers

name	Primers	Locus	Length (bp)	Tm
GAPDH	Fw: 5'- AGAAGGTGGTGAAGCAGGCATC - 3' Rw: 5'- CGAAGGTGGAAGAGTGGGAGTTG - 3'	NM_008084	111	60°C
β Tub III	Fw: 5'- CGCCTTTGGACACCTATTC - 3' Rw: 5'- TACTCCTCACGCACCTTG- 3'	NM_023279	240	58°C
IL1β	Fw: 5'- TCTATACCTGTCCTGTGTAATGAAAG - 3' Rw: 5'- GGCTTGTGCTCTGCTTGTGAG - 3'	NM_008361	195	55.5°C
IL6	Fw: 5'- GAGCCCACCAAGAACGATAGTC - 3' Rw: 5'- CCAGCATCAGTCCCAAGAAGG - 3'	NM_031168	96	60°C
IL10	Fw: 5'- GGACTTTAAGGGTTACTTGGG - 3' Rw: 5'- AGAAATCGATGACAGCGCCT -3'	NT_078297	174	60°C
TNFα	Fw: 5'- GTGGAACTGGCAGAAGAG - 3' Rw: 5'- CCATAGAACTGATGAGAGG -3'	NM_013693	196	55.5°C
Iba1	Fw: 5'- GTCCTTGAAGCGAATGCTGG - 3' Rw: 5'- CATTCTCAAGATGGCAGATC -3'	NT_039662	185	60°C
CD11b	Fw: 5'- CCTTGTTCTCTTTGATGCAG - 3' Rw: 5'- GTGATGACAACTAGGATCTT -3'	NT_039433	217	60°C
P2X3	Fw: 5'- AGCGGTACTTCTCCTCATTCTC - 3' Rw: 5'- CAGGGCACTTCTGTCTTTGTC - 3'	NM_145526	94	58°C
P2X4	Fw: 5'- CATCCGCAGCCGTAAAGTG - 3' Rw: 5'- AGAGTCCGTTTCCTGGTAGC - 3'	NM_011026	112	55.5°C
P2X7	Fw: 5'- CCTCAGTGTTCCATCTTCC - 3' Rw: 5'- ATCCGTGTTCTTGTCATCC - 3'	NM_001038845	183	55.5°C
MCP-1	Fw: 5'- TTTTGTCACCAAGCTCAAGAGAG - 3' Rw: 5'- TCACTGTCACACTGGTCACTCC - 3'	NM_011333.3	248	60°C
MMP-9	Fw: 5'- CATAGAGGAAGCCCATTAC - 3' Rw: 5'- CCAACTTATCCAGACTCC - 3'	NM_013599.2	181	55.5°C

4.8 - Protein analysis

Western blotting was performed as described earlier (Nair et al., 2010). Briefly, TG ganglion or culture proteins were extracted in ice-cold lysis buffer containing TNE buffer (10 mM Tris-HCl at pH 7.5, 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 2% n-octyl β-D-glucopyranoside and 1% NP40) plus protease inhibitors (Sigma) and separated on 8-10% polyacrylamide gel. The following polyclonal antibodies were used: anti-P2X3 (1:300; Alomone), anti-P2X4 (1:300; Alomone), anti-P2X7 (1:300; Sigma); anti-β-tubulin III (1:2.000; Sigma); anti-pro-TNFα (1:200; Abcam), or anti-actin (1:3.000; Sigma).

To ensure correct equal loading reflecting the neuronal cell content in different lysates, protein extracts were quantified with bicinchonic acid (Sigma) and calibrated for the neuronal specific □-tubulinIII. The amount of loaded proteins was in the 20–50 μg/ml range. Western blot signals were detected with the enhanced chemiluminescence light system (GE Healthcare, Milano, Italy). Grey values were quantified with Scion Image software (Scion, Frederick, Maryland, USA) or with Uvi band software (Uvitec, Cambridge, UK). Total protein content of ganglia cultures was measured with the BCA kit (Bicinchoninic Acid Kit for Protein Determination, Sigma).

4.9 - ELISA analysis

Cytokines were quantified in lysates of trigeminal ganglia with SearchLight Mouse Cytokine Array I (Aushon Biosystem, Billerica, MA, USA) and the Mouse TNFα ELISA assay or Mouse IL1β ELISA assay (Thermo Scientific, Rockford, IL, USA). Tissue lysates and normalization over wet weight of the tissue were performed in accordance with the manufacturer's instructions. Each sample was run in duplicate.

Cytokine released by cultures were detected with Mouse TNFα ELISA assay or Mouse IL1β ELISA assay (Thermo Scientific). Medium was collected in accordance with the manufacturer's instructions. Each sample was run in duplicate.

Total protein content from cell culture or from trigeminal tissues was evaluated with BCA kit showing no difference between samples.

4.10 - Evans Blue experiments

Vascular permeability was visualized by Evans Blue extravasation. 0.5% Evans Blue (Sigma) dye was intravenous injected (i.v.) and after 30 min animals were sacrificed. Trigeminal ganglia were incubated 24 h at 60°C in formamide (2%, Sigma). Absorbances (610nm) were normalized over wet weight (Hakim et al., 2009). For an autofluorescence control, control animals were injected with saline.

4.11 - Patch-clamp recording

Functional responses of P2X3 receptors in trigeminal neurons from WT, R192Q KI or macrophages/trigeminal co-cultures were studied with patch clamp recording using the specific agonist α,β-methyleneadenosine 5'-triphosphate (α,β-meATP, Sigma), applied with a fast superfusion system (Rapid Solution Changer RSC-200; BioLogic Science Instruments, Claix, France). The time for solution exchange was about 30 ms (Sokolova et al., 2001). We measured current peak amplitudes, current rise-time (expressed as τ_{on}), onset of desensitization estimated by calculating the first-time constant of current decay, τ_{fast}, in accordance with our previous reports,

and recovery from desensitization with paired-pulse experiments in which α,β -meATP applications were spaced at 30 s interval and recovery was expressed as % of the first response in each pair (Simonetti et al., 2006; Nair et al., 2010).

4.12 - Phagocytosis assay

Macrophage phagocytosis tests were performed by incubating cultures with FITC-conjugated Zymosan A (Zy-FITC; 1 mg/ml, Sigma) for 10 min at 37 °C (Harrigan et al., 2008), fixed in 4 % paraformaldehyde and processed for immunofluorescence. Active macrophages were considered when taking up ≥ 1 granule of Zy-FITC. After counting the number of granules/active macrophage, the phagocytosis index was calculated as the percentage of Zy-FITC-positive macrophages multiplied by the number of Zy-FITC granules per single cell (Färber et al., 2009). The percentage of active macrophages was not significantly different between WT (49 ± 6 %) and KI (57 ± 6 %; $n = 5$; $p < 0.05$) cultures. Phagocytosis analysis was performed with Metamorph software.

4.13 - Statistical analysis

Data were collected from at least three independent experiments, and are expressed as mean ± standard error of the mean (SEM), where *n* indicates the number of independent experiments or the number of investigated cells, as indicated in the respective figure legend. Statistical analysis was performed using the Student's *t*-test or the Mann-Whitney rank sum test, depending on whether the data were normally distributed, or not. Multiple comparisons were analyzed with the One-way ANOVA and Tukey post-test. Sigma Stat and Sigma Plot (Systat Software Inc., San Jose, CA, USA) were used for statistical analysis. A p value of ≤ 0.05 was accepted as indicative of a statistically significant difference. Please note * $p \leq 0.05$; ** $p < 0.01$; *** $p<0.001$.

Chapter 5: RESULTS

5.1 Characterization of macrophages in WT and R192Q KI trigeminal ganglion tissue

5.1.1 - Iba1 cells in trigeminal ganglia of WT and R192Q KI mice

Previous studies identified functional and molecular properties of the different neuron sub-populations (Simonetti et al., 2006; Nair et al., 2010) and the role of satellite glial cells (Ceruti et al., 2011) in WT and R192Q KI trigeminal ganglia. We initially focus our attention on a particular population of non-neuronal cells that should have a crucial role in inflammation, namely, macrophages.

We identified macrophages using the Iba1 antibody, which is known to recognize macrophage/microglia cells (Imai et al., 1996; Ito et al., 1998), and we confirmed that, in trigeminal ganglion tissue, Iba1 is a specific marker of macrophages. In fact, we showed that Iba1 cells were distinct from satellite glial cells, as demonstrated by the lack of co-localization of Iba1 signal with glutamine synthetase signal (GS; Fig. 5.1), a known satellite cell marker (Hanani, 2010).

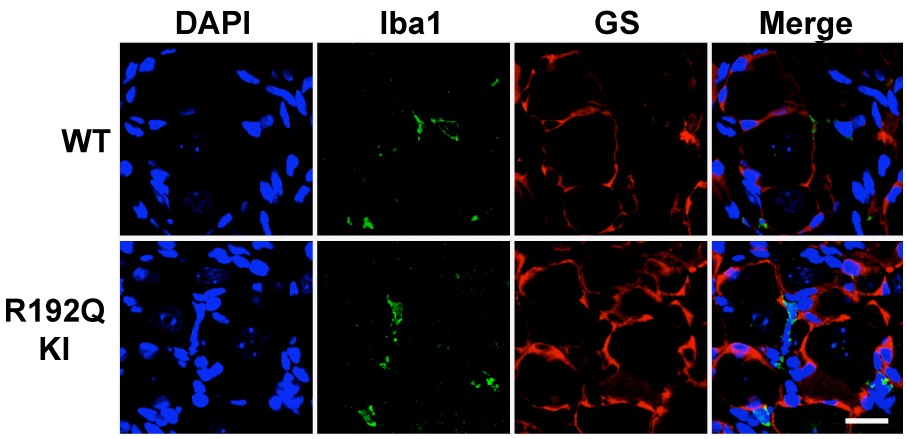


Fig. 5.1 - Iba1 is a macrophage marker

Panels show confocal images of WT (top row) and R192Q KI (bottom row) trigeminal ganglion sections immunostained with anti-Iba1 (green) and anti-glutamine synthetase antibodies (GS, red). Nuclei were labeled with DAPI (blue). Merged images are shown on the right panels. Note the differential morphology of Iba1-positive macrophages vs GS-positive satellite cells typically surrounding neurons. Scale bar: 15 μm.

Figure 5.2 A shows (P30) trigeminal ganglion (from WT or R192Q KI) reconstructions immunostained with antibodies recognizing the macrophage Iba1 protein (red), the microtubule-associated protein 2 (MAP2, blue), and the P2X3 receptor (green; expressed by a sub-population of nociceptive ganglion neurons) (Simonetti et al., 2006). A large number of macrophages was detected in trigeminal ganglia, in particular in R192Q KI TG. To quantify the differences between WT and R192Q KI trigeminal ganglia, we counted the number of Iba1 immunopositive cells in Regions of Interest (ROI). ROIs comprised the entire ganglion tissue, and for each experiment, we acquired images from different longitudinal sections of the ganglia to evaluate macrophage cells in the total ganglion. Fig. 5.2 B showed representative images of WT and R192Q KI trigeminal ganglia immunostained with Iba1 antibody at different ages (P10, P30 and P90). There were a small but significantly higher number of Iba1 immunoreactive cells throughout the tissue in R192Q KI trigeminal ganglia at different ages (Fig. 5.2 B, C). Such an increased of number of Iba1 cells was accompanied by a global increase in the number of cells counterstained with DAPI in young animals only (P10 and P30, Fig. 5.2 D).

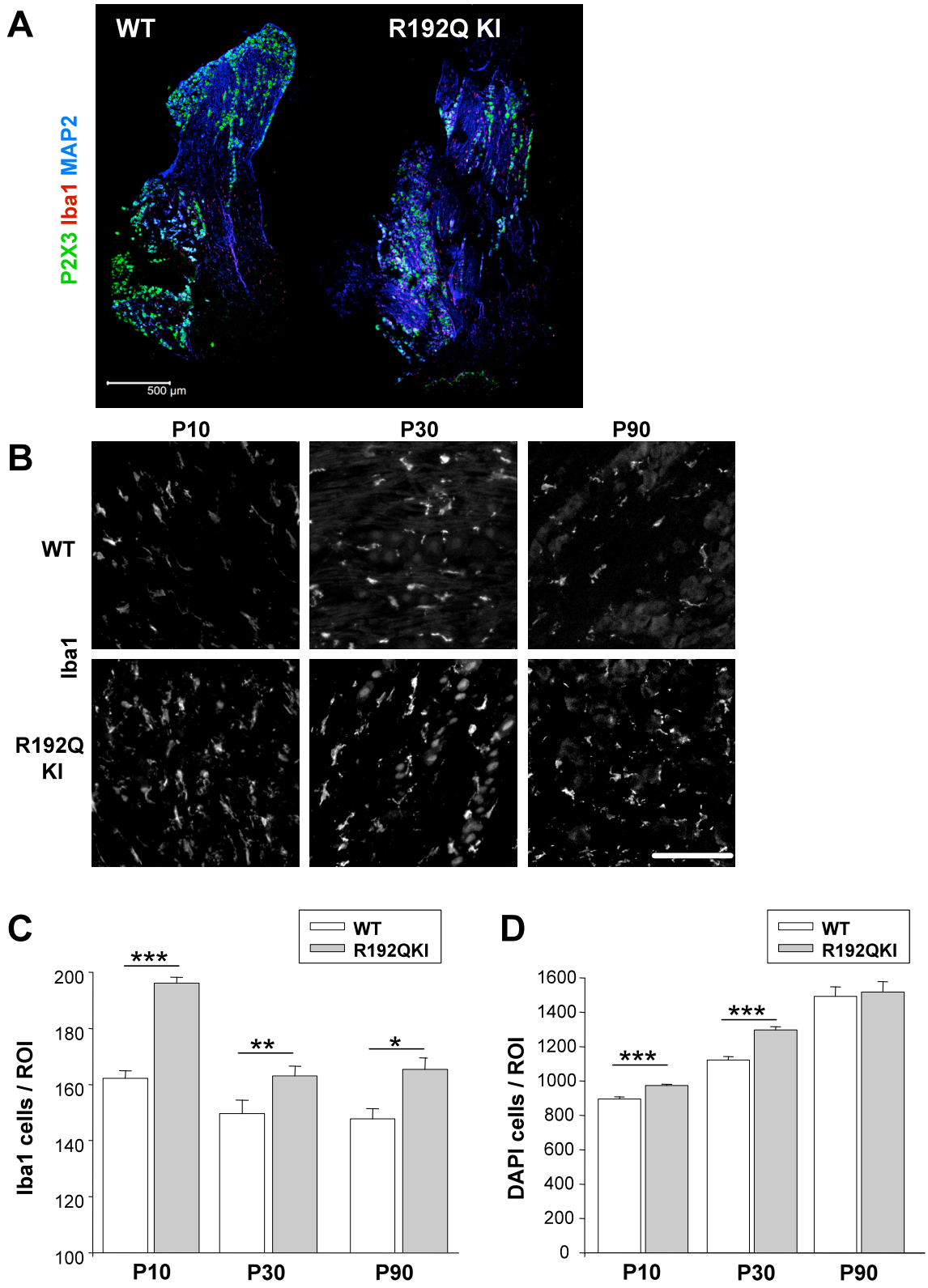


Figure 5.2 – Iba1 macrophages in WT or KI trigeminal ganglia

A, Reconstruction of a entire longitudinal sections of mouse trigeminal ganglia (from multiple confocal microscopy images taken in a single plane) from WT and R192Q KI immunostained with antibodies against Iba1 protein (red), MAP2 (blue), and P2X3 receptors (green). Scale bar: 500 µm. *B*, Example of miscropy images from WT and R192Q KI trigeminal ganglia at different ages (P10, P30, P90) immunostained with anti-Iba1. Data are from ROIs of 640 x 480 µm comprising the whole ganglion sections. Scale bar: 100 µm. $n = 4$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. *C*, Histograms quantify the number of Iba1-immunoreactive cells per ROI in WT and KI ganglia at different postnatal ages

(P10, P30 and P90). $n = 4$; *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$. *D*, Histograms quantify the number of cells (DAPI) per ROI in WT and KI ganglia at different postnatal ages (P10, P30 and P90). Data are from ROIs of 640 x 480 μm comprising the whole ganglion section. $n = 4$; *** $p < 0.001$.

Further experiments were done only with P30 female mice. Adult mice are possibly good models for studying the migraine pathology because, in patients, migraine typically occurs in the adult (Stovner et al., 2006). Moreover, at P10, mice do not have the complete expression and function of $Ca_V2.1$, which is fully manifested at P30 (Todorov et al., 2006).

We choose to perform our experiments on female mice for various reasons. First, migraine is a female-predominant disorder (Fig. 2.1, Stovner et al., 2007) and data obtained using male animals (in order to reduce variability resulting from fluctuations in female sex hormones) cannot be generalized to females (Bolay et al., 2011). Moreover, the ovarian steroids have been demonstrated to have direct effect on trigeminal ganglia causing different gene expression correlated to migraine pathophysiology, such as CGRP (Bolay et al., 2011). Differential sex studies have already performed on R192Q KI *Cacna1a* mice model of migraine (Eikermann-Haerter et al., 2009).

5.1.2 - Compartmentalization of Iba1 in Trigeminal Ganglia

Fig. 5.3 A compares confocal microscopy images of mouse trigeminal ganglion sections blue-labeled with DAPI (from WT or R192Q KI), and immunostained with the macrophage marker Iba1 and counterstained with the P2X3 antibody. A detailed investigation of these images demonstrated that, unlike in WT tissue, Iba1 positive cells were not equally distributed in the entire tissue, as macrophages were preferentially localized close to neurons rather than fibres in R192Q KI ganglia (Fig. 5.3 A, B; note that the ROI size for these calculations was half of the one shown in Fig. 5. 2). An accurate investigation of the distribution of Iba1 positive cell in the three branches of the ganglion (V1, V2 and V3, Fig. 5.3 C; Thalakoti et al. 2007), revealed that R192Q KI mice showed a higher number of Iba1 positive cells in neuronal-enriched regions (Fig. 5.3 D), with a significant increase of Iba1 cell density in R192Q KI with respect WT ones (23% increase in V2 and V3 areas, and up to 27% in the V1 region).

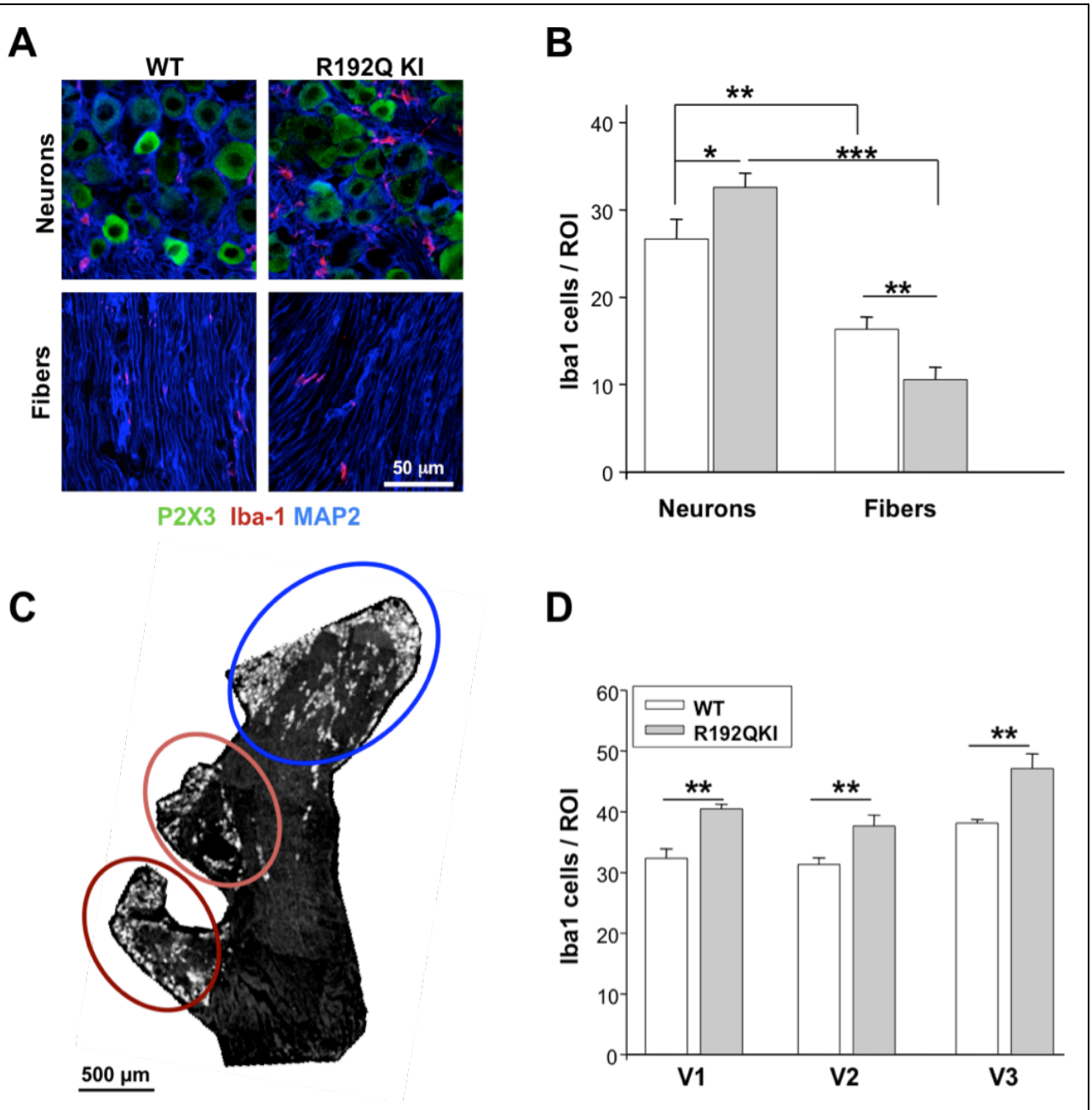


Figure 5.3 – Macrophage localization in WT or KI trigeminal ganglia

A, Representative confocal microscopy images of trigeminal ganglion sections from neuron (top row) and fiber (bottom row) areas in WT and R192Q KI trigeminal ganglia immunostained for the macrophage-specific marker Iba1 (red) and P2X3 (green) and MAP2 (blue). Scale bar: 50 µm. *B*, Iba1 positive cells were counted in neuronal or fiber rich ROIs of 300 x 300 µm; $n = 3$ mice; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. *C,* Reconstruction of an entire longitudinal section of mouse trigeminal ganglion immunostained for P2X3. Note discrete distribution of neuronal somata in three major subdivisions (i.e. V1, V2 and V3 indicated by ellipsoids) of the trigeminal ganglion. Scale bar: 500 µm. *D,* Histograms represent Iba1-positive cells in V1, V2 and V3 regions (in each one, the ROI was 370 x 370 µm) of WT and R192Q KI trigeminal ganglia. Data were collected in parallel from 3 WT and 3 KI mice; ** $p < 0.01$.

5.1.3 - Morphology of Iba1 cells

Because morphological changes of microglia/macrophages are associated with their activated state (Glenn et al. 1993; Inoue, 2006; Lynch, 2009; David and Kroner, 2011), we investigated morphology of Iba1 immunoreactive cells in the ganglia from WT and R192Q KI mice. We observed that R192Q KI ganglion macrophages displayed increased cell volume compared with WT ones and acquired an amoeboid morphology with limited number of processes (Fig. 5.4 A). 3D reconstruction of confocal images representative of Iba1-positive cells from WT and R192Q KI trigeminal ganglion was used to quantify the macrophage volume. Results are shown in Fig. 5.4 A, B.

On average, volumetric quantification of such cells confirmed a larger volume of Iba1 positive cells in R192Q KI ganglia with respect to WT (Fig. 5.4 B). In details, we analyzed the volume of Iba1 positive cells in neuronal areas or in "fiber" regions. WT macrophages had the same volume regardless of their topography (Fig. 5.4 C). Interestingly, KI macrophages showed larger volume near neurons (440 ± 30 μm^3, $n = 51$) than in the fiber area (320 ± 30 μm^3, $n = 22$, * $p < 0.05$; Fig. 5.4 C).

Hence, propinquity to neurons in R192Q KI mice conferred an activation phenotype to macrophages and seemed crucial for the crosstalk between neurons and surrounding macrophages.

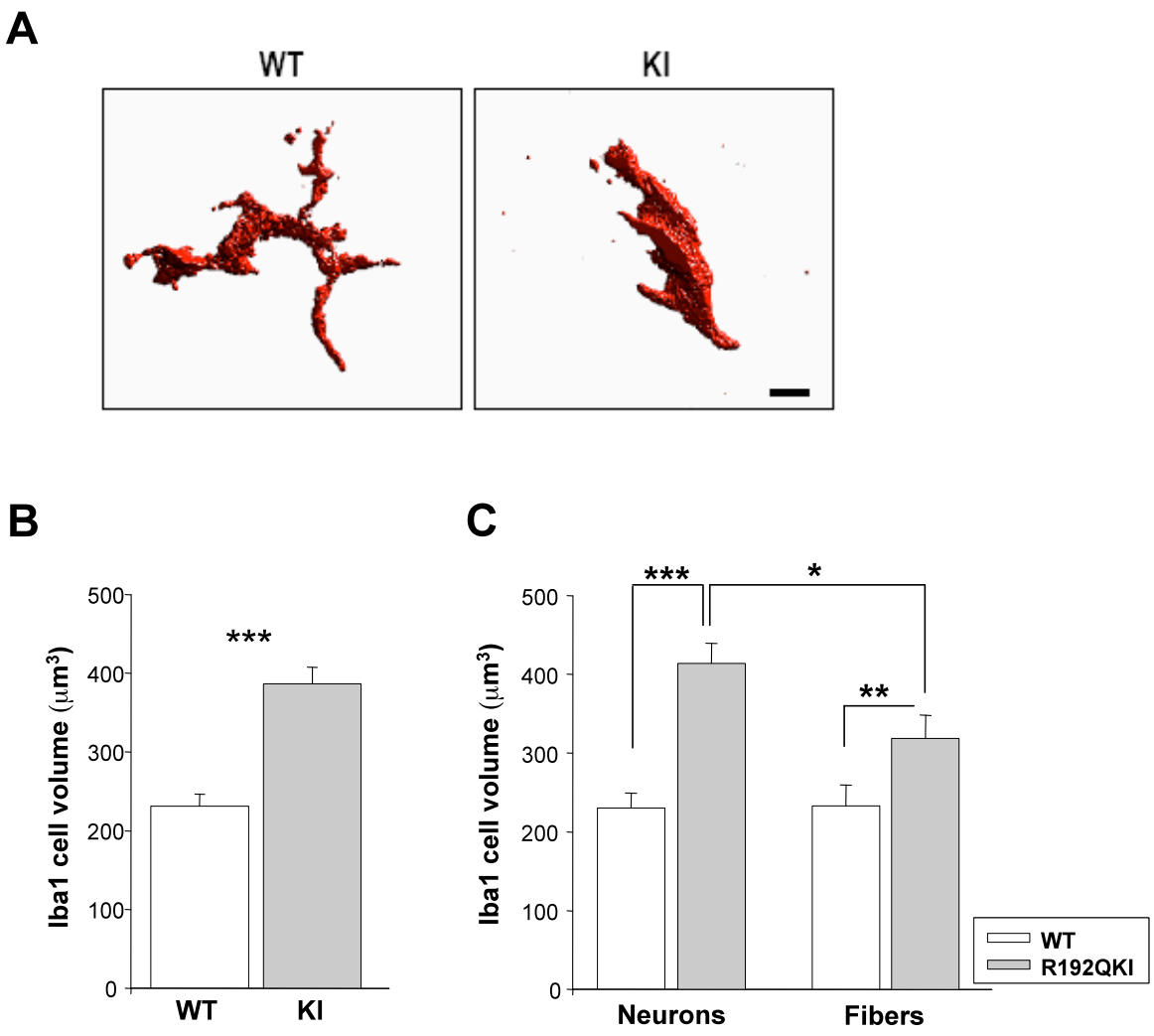


Figure 5.4 - Different Iba1 cell volumes in WT and R192Q KI trigeminal ganglia

A, Examples of 3D reconstruction of Iba1-positive cells (high magnification confocal images from 0.5 μm optical sections) from WT or R192Q KI ganglia. Note large branching of WT cell vs compact, almost process-free, KI cell morphology. Scale bar: 5 μm. *B*, Histograms quantify average volume (μm^3) of Iba1-positive cells in WT and R192Q KI ganglia, obtained from voxel analysis of 3D images. Data were collected from three independent experiments with a total of 83 cells for WT and 70 cells for R192Q KI; * $p < 0.001$. *C,* Histograms quantify the average volume of Iba1-positive cells from subregions (i.e. neurons or fibers) in WT and R192Q KI ganglia; n = 20-50 cells (3 WT and 3 KI mice). * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.

5.1.4 - Characterization of Iba1 positive cells in trigeminal ganglia.

Considering the change in morphology and the small, yet significant increase in Iba1 positivity, we analyzed different macrophage markers to confirm the nature of these macrophages (Austin and Moalem-Taylor, 2010). Fig. 5.5 A compares representative confocal microscopy images from WT or R192Q KI samples of the V3 region immunostained with antibodies against Iba1 and CD11b, an adhesion molecule marker of leukocyte activation (Simon et al., 2000) and up-regulated activated macrophage/microglia (Scholz et al., 2008). On average, in WT ganglia, only 20% Iba1-positive

cells also expressed CD11b without any topographical preference (Fig. 5.5 B). On the other hand, in KI samples, the percentage of Iba1 and CD11b of co-expression was significantly higher (up to 45 % of Iba1-positive cells) in all regions (Fig. 5.5 B).

Similar experiments were performed to evaluate the expression of the macrophage antigen ED1 (CD68), a glycoprotein highly expressed by tissue macrophages and associated with larger phagocytic ability (Holness and Simmons, 1993) (Fig. 5.5 C, D). While in WT tissue, ED1 was expressed in fewer than 15% of Iba1 positive cells, this value significantly grew in R192Q KI ganglia, in particular in the V3 region (Fig. 5.5 D).

Expression of the F4/80 antigen is restricted to most (though not all) mature macrophages and also to quiescent macrophages (Martinez-Pomares et al., 1996; Carson et al., 1998). In WT and R192Q KI ganglia, the F4/80 signal was detected in a minor fraction of Iba1 positive cells and was not significantly different between WT and R192Q KI ganglia (Fig. 5.5 E, F).

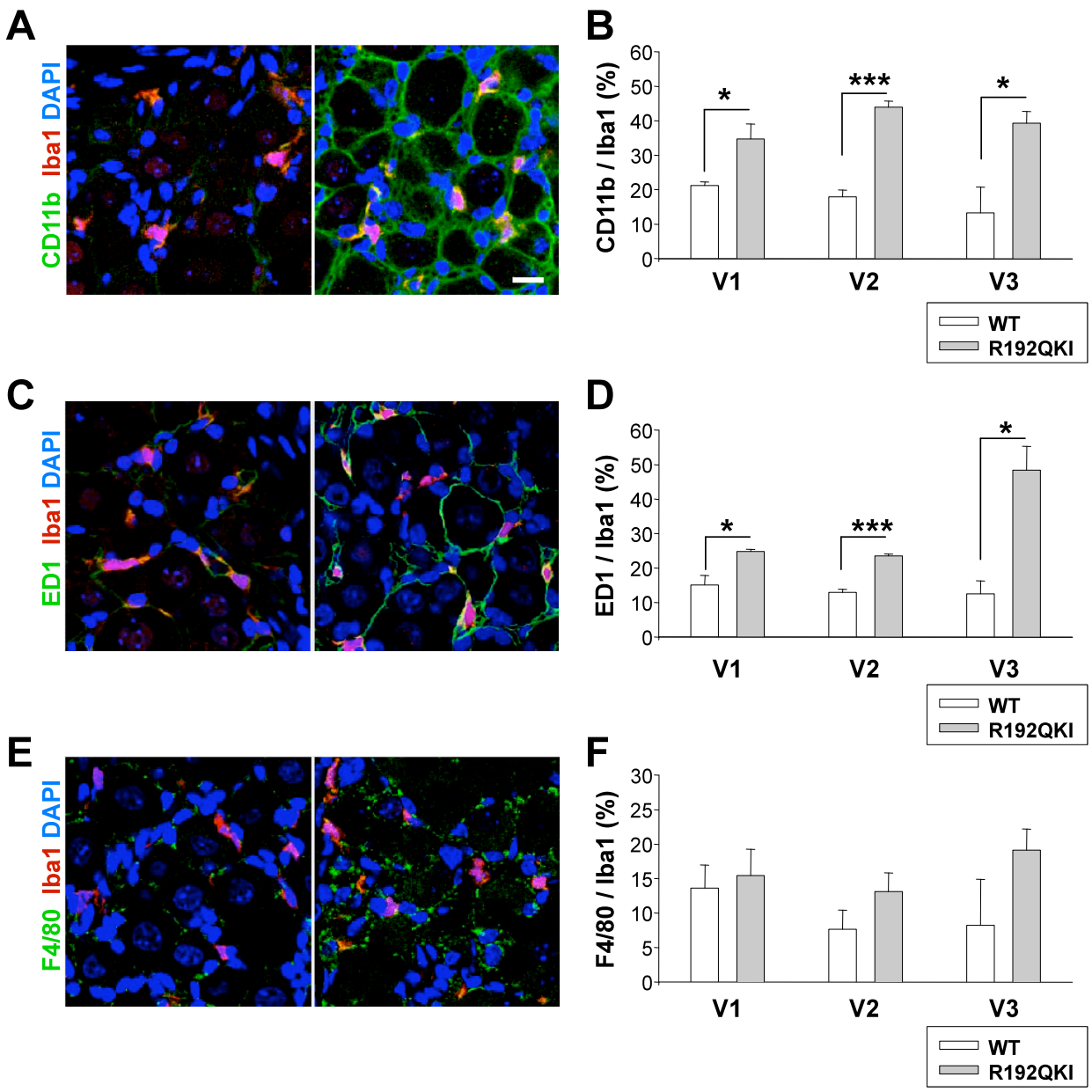


Figure 5.5 - Characterization of Iba1-positive cells in trigeminal ganglia from WT and R192Q KI mice

A, C, D, Representative merged confocal images of WT or R192Q KI trigeminal ganglion sections (V3 region) co-immunostained for Iba1 with CD11b (*A*), ED1 (*C*), or F4/80 (*E*). Immunoreactivity color coding is shown alongside the corresponding panels. Nuclei were labeled with DAPI. Scale bar: 15 μm. *B, D, E*, Histograms quantify the percentage of Iba1-positive cells co-expressing CD11b (*B*), ED1 (*D*) or F4/80 (*F*) in different ganglion regions (V1, V2 or V3); n = 3 WT and 3 KI mice; * $p < 0.05$; ** $p < 0.01$.

These data indicate that R192Q KI ganglion tissue is enriched in macrophages expressing proteins suggestive of a strong activation state.

5.1.5 - Origin of trigeminal ganglion macrophages

The increased number of macrophage in KI ganglia might be due to either proliferation of resident macrophages or increased trafficking of macrophages recruited from the periphery in response to signaling by cytokines, chemokines and other immune mediators (Morgan et al., 2010). To clarify the origin of macrophages in KI TG tissue, first we evaluated the vascular supply of trigeminal ganglia analyzing the presence of vessels with an antibody recognizing the endothelial cells with microscopy experiments (CD31, Jimenez-Andrade et al., 2008; Fig. 5.6 A). There was no significant difference between WT and R192Q KI CD31 signals indicating an apparently comparable extension of vessels in both mice (Fig. 5.6 B). Like for DRG (Jimenez-Andrade et al., 2008), neuron enriched areas were more vascularized than fiber regions (Fig. 5.6 A). Fig. 5.6 C shows a higher magnification of neuronal enriched areas in WT and KI trigeminal ganglion tissue.

The Evans Blue technique was used in order to analyze the potential vascular permeability of TG vessels from WT and R192Q KI mice. These data indicated no difference in blood vessel permeability (Fig. 5.6 D).

Since a crucial role for recruiting of macrophages is played by immune mediators, the most important cytokines and chemokines in trigeminal ganglia were next studied.

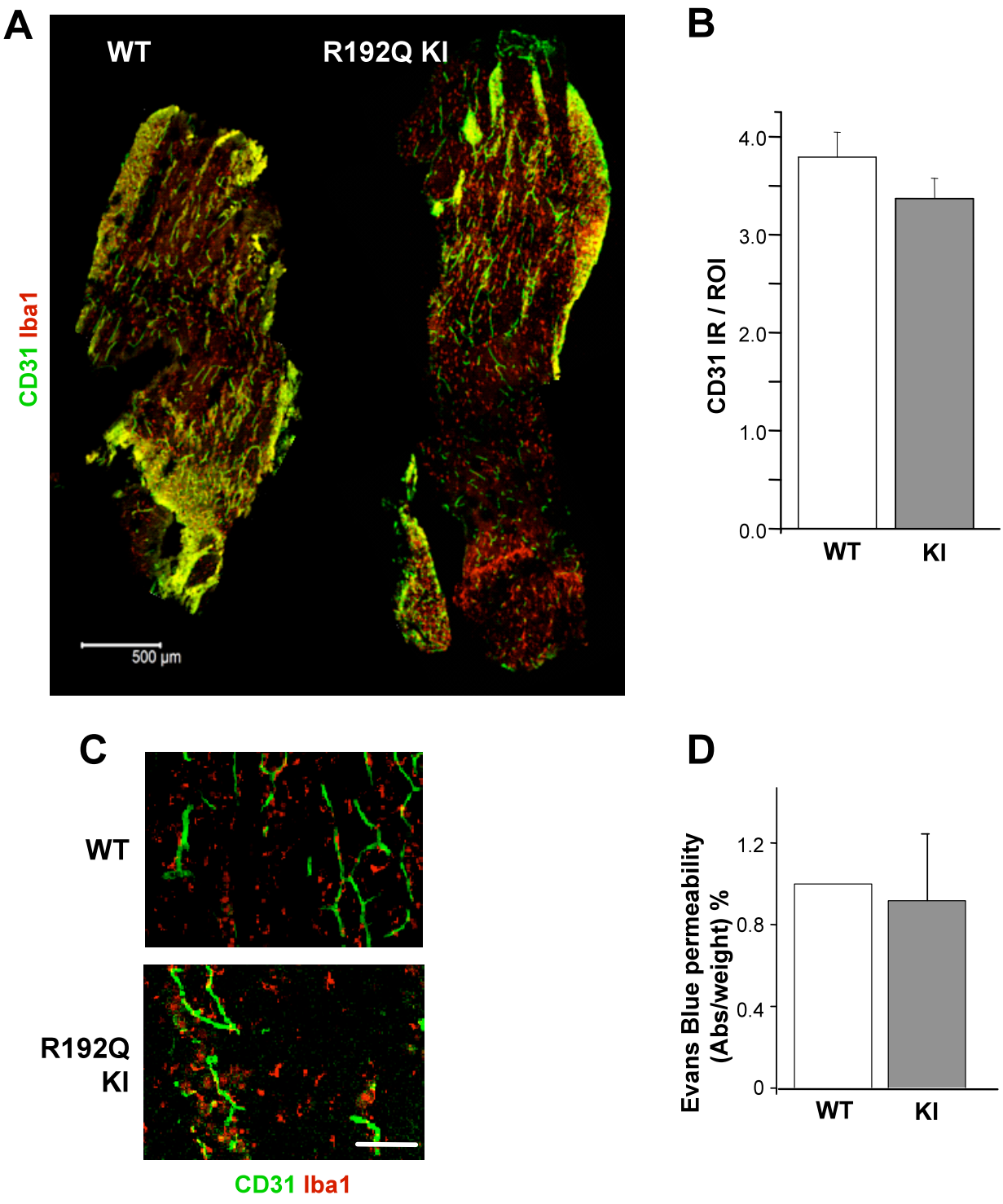


Figure 5.6 – Vascular supply and permeability of WT and R192Q KI trigeminal ganglia

A, Reconstruction of an entire longitudinal section of mouse trigeminal ganglion (from multiple confocal microscopy images taken in a single plane) from WT and R192Q KI trigeminal ganglia immunostained with antibodies against Iba1 protein (red) and CD31 (green). Scale bar: 500 µm. *B*, Histograms quantify CD31 immunoreactivity (expressed as average percent of positive signals per ROI) from WT and KI trigeminal ganglia. $n = 4$. *C*, Representative confocal images from neuron area of mouse trigeminal ganglion from WT and KI mice immunostained with Iba1 (red) and CD31 (green). Scale bar: 100 µm. *D*, Histograms quantify Evans Blue permeability (expressed as Absorbance over wet weight) of TG from WT and KI trigeminal ganglia; $n = 4$.

5.2 Pro-inflammatory profile of R192Q trigeminal ganglia

5.2.1 - Cytokine and chemokine expression in WT and R192Q KI trigeminal ganglia

It is well known that, in chronic pain models, cytokine expression is substantially up-regulated (Donnelly and Popovich, 2008; Milligan and Watkins, 2009): these substances are released by different cell populations, namely, activated macrophages as well as satellite glial cells (Ceruti et al., 2011). Thus, we measured, from whole ganglion extracts, the mRNA and protein expression of a series of cytokines in WT and R192Q KI mice (Fig. 5.7-8). As shown in Fig. 5.7 A and B, we evaluated (with an ELISA kit) in parallel 9 different cytokines in TG tissue lysates.

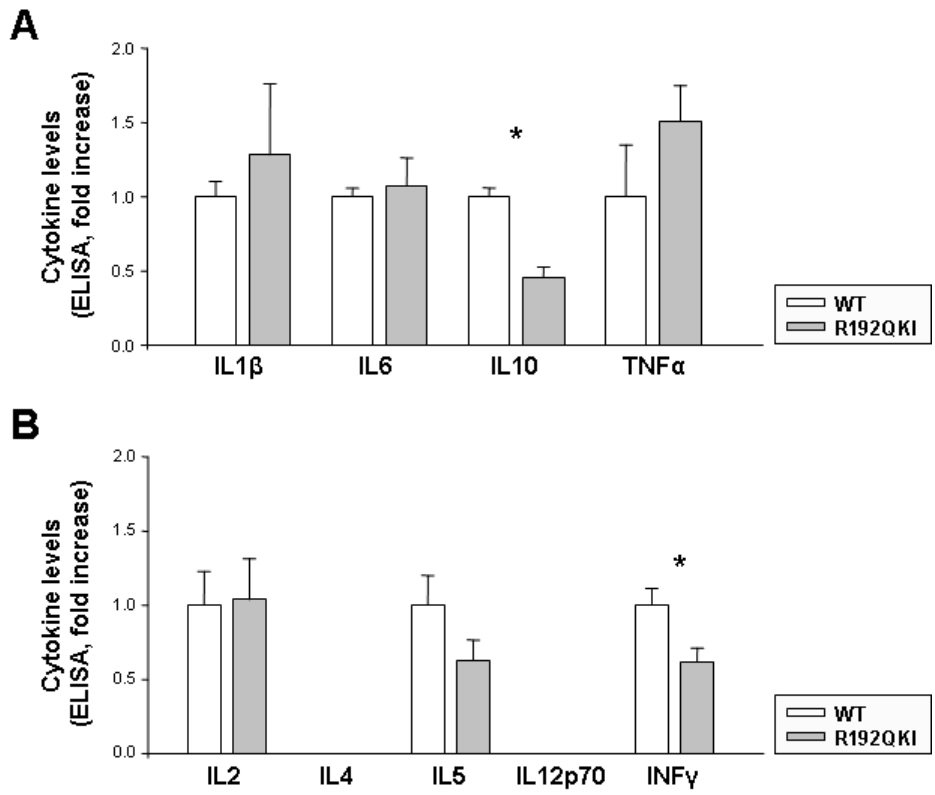


Figure 5.7 - Comparison of inflammatory mediators in WT and R192Q KI trigeminal ganglia under basal conditions

A, *B*, Histograms quantify changes (fold increase with respect to WT) in interleukin- (IL) 1β, IL6, IL10, Tumor necrosis factor-alpha (TNFα) (*A*), IL2, IL4, IL5, IL12p70 and interferon-gamma (IFN-γ) (*B*) cytokine protein levels from WT or R192Q KI ganglia; $n = 5$ WT and 5 KI mice; * $p < 0.05$.

Data demonstrated no significant difference between the protein levels of different cytokines involved in the pro-inflammatory pathways, like IL1β, TNFα or IL6; while a down-regulation of IFN-γ and the anti-inflammatory cytokine IL10 (Fig. 5.7 A, B) was found. IL4 was not detectable (below 3.1 pg/ml) and IL12p70 was either undetectable or low.

Over all, these data did not indicate an on-going inflammatory state in R192Q KI ganglia, confirming the generally-good health condition of these animals.

Interestingly, the pro-inflammatory IL1β and TNFα, IL6 and anti-inflammatory IL10 mRNA levels were significantly higher in R192Q KI than WT ganglia (Fig. 5.8 A), suggesting, thus, that ganglia from KI mice have a larger potential to express inflammatory cytokines.

We evaluated also different mRNAs typically up-regulated by microglia/macrophage-like activated cells, such as Iba1, CD11b (Scholz et al., 2008; Ren and Dubner, 2011), and also the mRNAs of purinoreceptor 4 (P2X4) and 7 (P2X7) expressed in microglia/macrophages and mediating an important role in activation of these cells (Sandhir et al., 2008; Scholz et al., 2008). In R192Q KI trigeminal ganglia all these genes were up-regulated in comparison to WT ones (Fig. 5.8 B) confirming a potential activation state of these immune cells.

These genes could be differently regulated in an age-dependent way. Thus, we evaluated Iba1, CD11b, IL1β TNFα, P2X4 and P2X7 mRNA also in younger mice (P10), but we found no differences (data not shown).

High levels of the chemokine receptor type 2 (CCR2), also called monocyte chemoattractant protein-1 (MCP-1), are associated with meningeal neuroinflammation in migraine and macrophage recruitment (Reuter et al., 2002; White and Wilson, 2008; Ren and Dubner, 2011; Yang et al., 2011). Metalloproteinases are necessary for cell migration through the extracellular matrix and development of immune responses in neuropathic pain (Ram et al., 2006; Shubayev et al., 2006; Kawasaki et al., 2008; Kim et al., 2008; Ren and Dubner, 2011). Previous studies proposed a role for metalloproteinase (MMP-9) in migraine, because this marker is increased in plasma of migraineurs during the migraine attack (Gursoy-Ozdemir et al., 2004; Leira et al., 2007; Imamura et al., 2008). As shown in Fig. 5.8 C, we found significantly higher mRNA levels of these markers in R192Q KI rather than WT ganglia.

These data, together with the macrophage characterization observed in trigeminal ganglia of R192Q KI mice, suggested lack of ongoing inflammation, but a basal inflammatory potential in KI ganglia with respect to the WT ones.

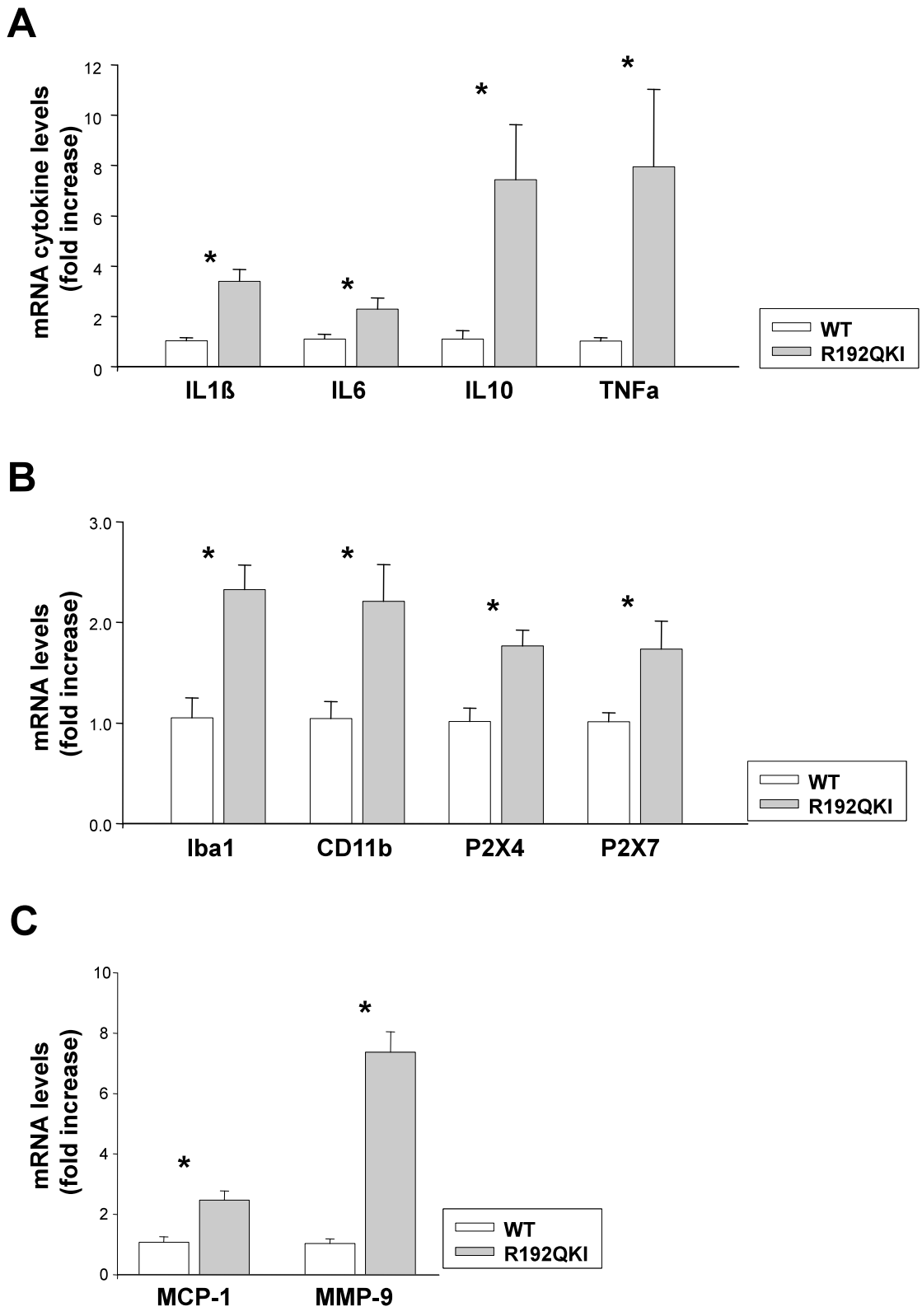


Figure 5.8 – mRNA levels of inflammatory mediators in WT and R192Q KI trigeminal ganglia under basal conditions

A, B, C, Real-time PCR experiments quantify IL1β, IL6, IL10, TNFα (*A*); Iba1, CD11b, P2X4, P2X7 (*B*) and MCP-1, MMP-9 (*C*) mRNA levels in WT or R192Q KI trigeminal ganglia. Data were normalized with respect to the β-Tubulin and GAPDH housekeeping gene; and expressed as fold increase with respect to WT levels; $n = 4$ WT and 4 KI mice; * $p < 0.05$.

5.2.2 - TNFα expression in R192Q KI or WT trigeminal ganglia

It is known that TNFα is mainly produced and released by active macrophages (Cunha et al., 1992; Scholz and Woolf, 2007). Interestingly, it is also produced by satellite cells and neurons after inflammatory stimuli (Schafers et al., 2003; Li et al., 2004). TNFα powerfully sensitizes trigeminal ganglia (Balkowiec-Iskra et al., 2011; Ren and Dubner, 2011; Zhang Z et al., 2011) and stimulates release of CGRP to evoke migraine pain (Bowen et al., 2006).

We investigated the TNFα expression by macrophages in trigeminal ganglia. Fig. 5.9 A compares representative confocal microscopy images from WT or R192Q KI ganglia co-stained with antibodies against Iba1 and TNFα. In all three areas, the percentage of Iba1 positive cells that expressed also TNFα was higher in KI trigeminal ganglia than WT ones (Fig. 5.9 B).

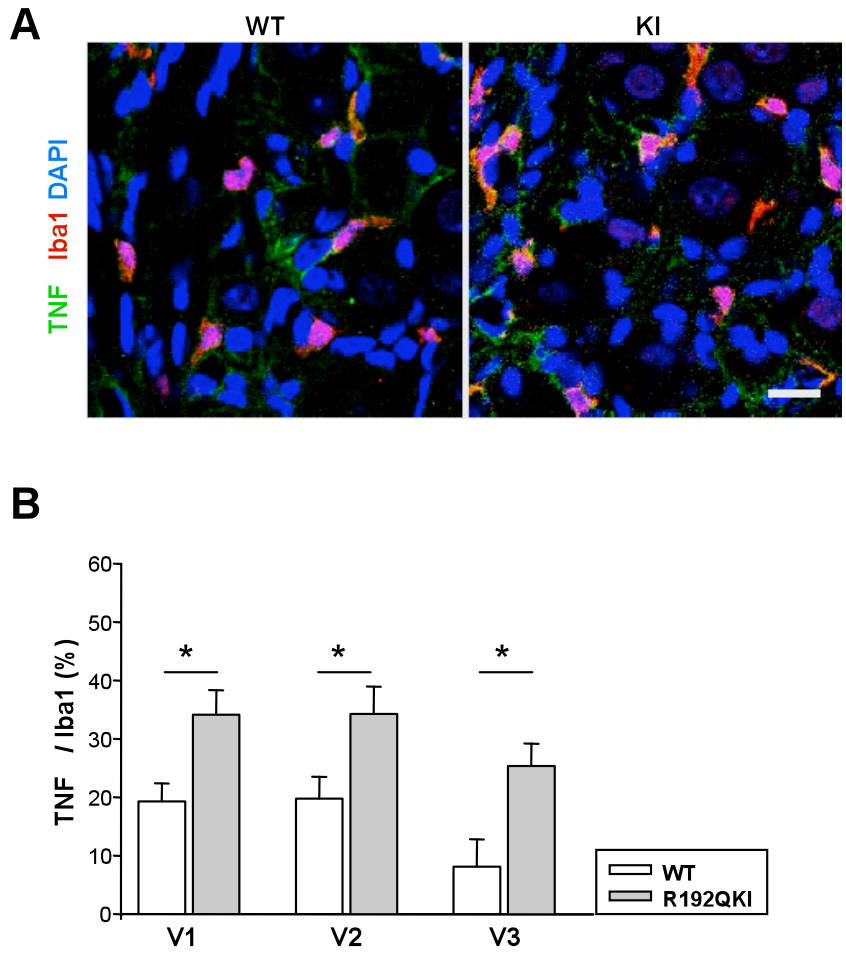


Figure 5.9 - TNFα in WT and R192Q KI ganglion macrophages

A, Representative confocal microscopy images of WT or R192Q KI trigeminal ganglion sections under basal condition immunostained for Iba1 (red) and TNFα (green). Nuclei are visualized with DAPI (blue). Scale bar: 15 μm. *B*, Histograms quantify the percentage of Iba1-positive cells co-expressing TNFα in V1, V2 or V3 regions (ROI of 370 x 370 μm); $n = 4$ WT and 4 KI mice; * $p < 0.05$.

5.2.3 – An inflammatory stimulus strongly enhanced the number of macrophages in trigeminal ganglia

Our data support the hypothesis that the R192Q mutation conferred a particularly pro-inflammatory phenotype to trigeminal ganglia. Could the different background of R192Q KI mice condition the inflammatory response in comparison to WT ones? To check for this possibility, we tested the inflammatory response of WT and R192Q KI mice after an inflammatory challenge like LPS (Tollner et al., 2000; Kobierski et al., 2000; Kovacs et al., 2008). Two different concentration of LPS were used (5 and 10 mg/kg) (Tollner et al., 2000; Kovacs et al., 2008) and the dose-response effects on IL1β and TNFα mRNA levels evaluated after 1 h (Fig. 5.10 A, B) (Haddad, 2002; Ohtori et al., 2004; Scholz and Woolf, 2007). The histograms show higher mRNA levels in R192Q KI trigeminal ganglia than WT ones. Indeed, the effect of LPS on mRNA levels was dose dependent. We also performed some preliminary data (not shown) evaluating the cytokine protein in WT and R192Q KI mice after LPS injection (10mg/kg). Pro/anti-inflammatory cytokines involved in the "classical" macrophage activation were enhanced such as IL1α, IL10, IFN-γ (2-times more that naïve) and IL6 (100-times more than naïve) in both WT and KI trigeminal ganglia (Austin and Moalem-Taylor, 2010; David and Kroner, 2011). Interestingly, there is a different up-regulation in TNFα: doubled in WT, 5-times in R192Q KI.

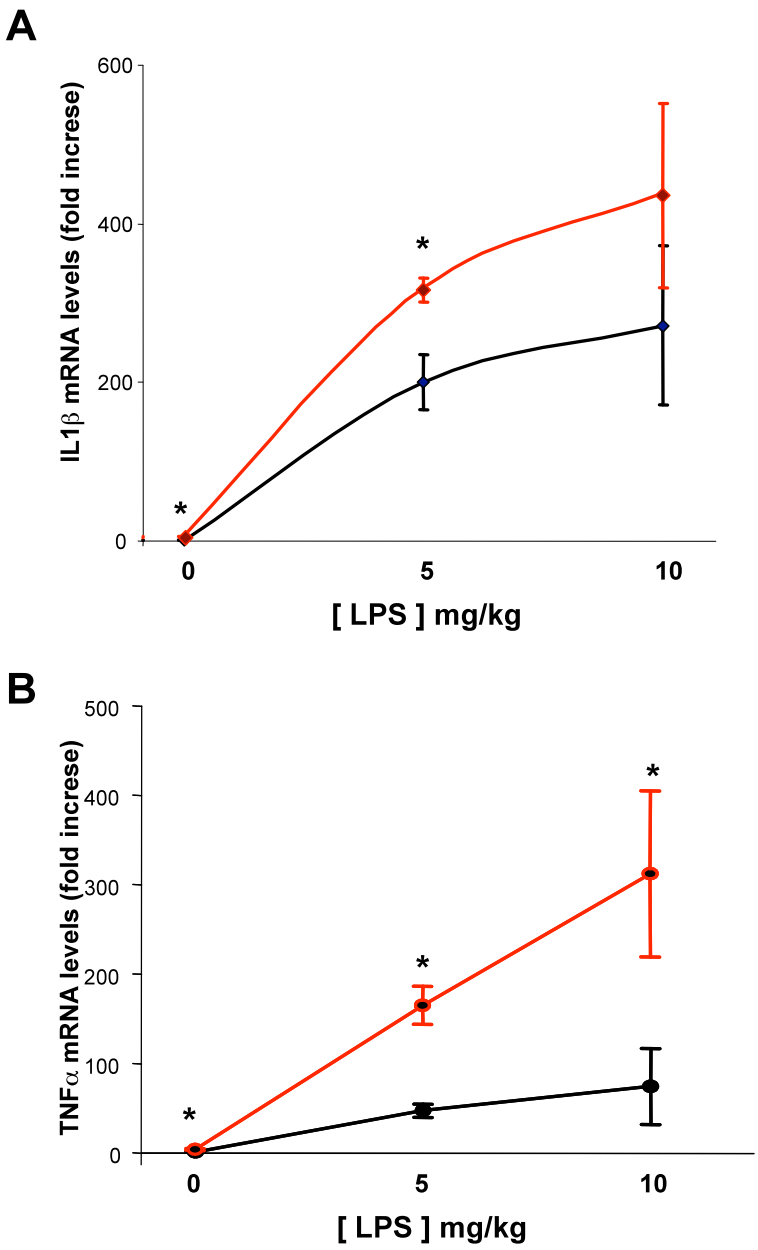
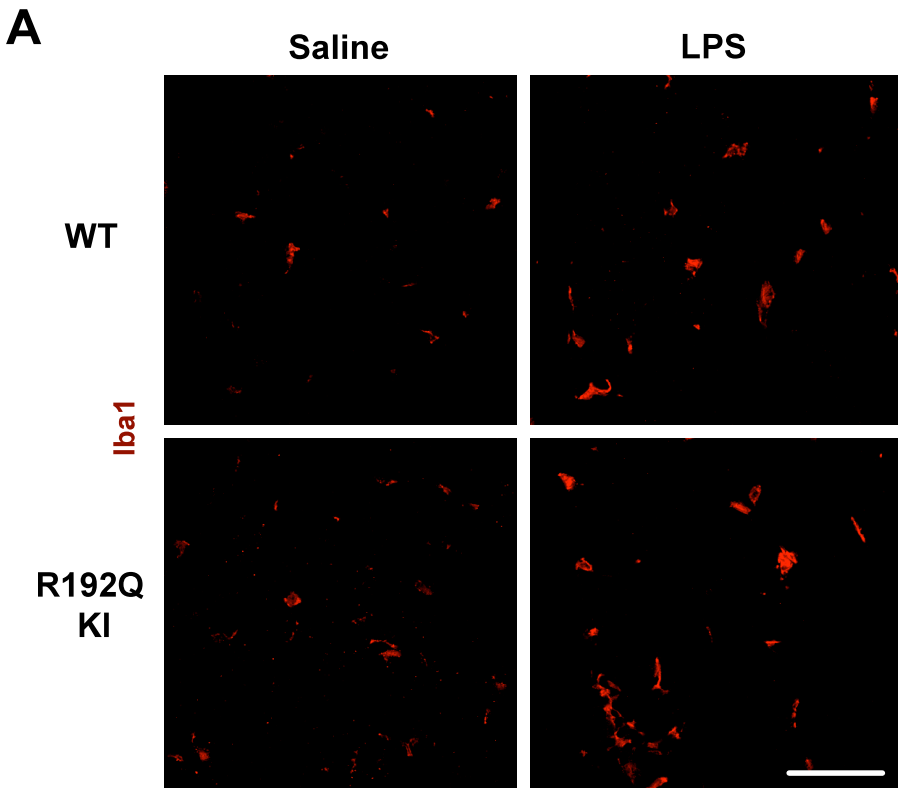


Figure 5.10 - LPS dose-responses of pro-inflammatory cytokines in WT and R192Q KI ganglia

A, B, Real time PCR data indicate IL1β (*A*) and TNFα (*B*) mRNA levels in WT (black line) and R192Q KI (red line) trigeminal ganglia 1 h after i.p. injection of LPS at different doses (5-10mg/kg) normalized over saline injected mice. $n = 3$; * $p < 0.05$.

Further tests were done with 5mg/kg dose because with the higher dose we observed occasional deaths after 1-2 days.

First, we evaluated after 5 h the effect of LPS on the number of Iba1 positive cells (Fig. 5.11 A). While the LPS injection caused a significant increase of Iba1 cells in WT and R192Q KI trigeminal ganglia (Fig. 5.11 B), the difference between WT and KI, was conserved in all areas also after LPS induction.



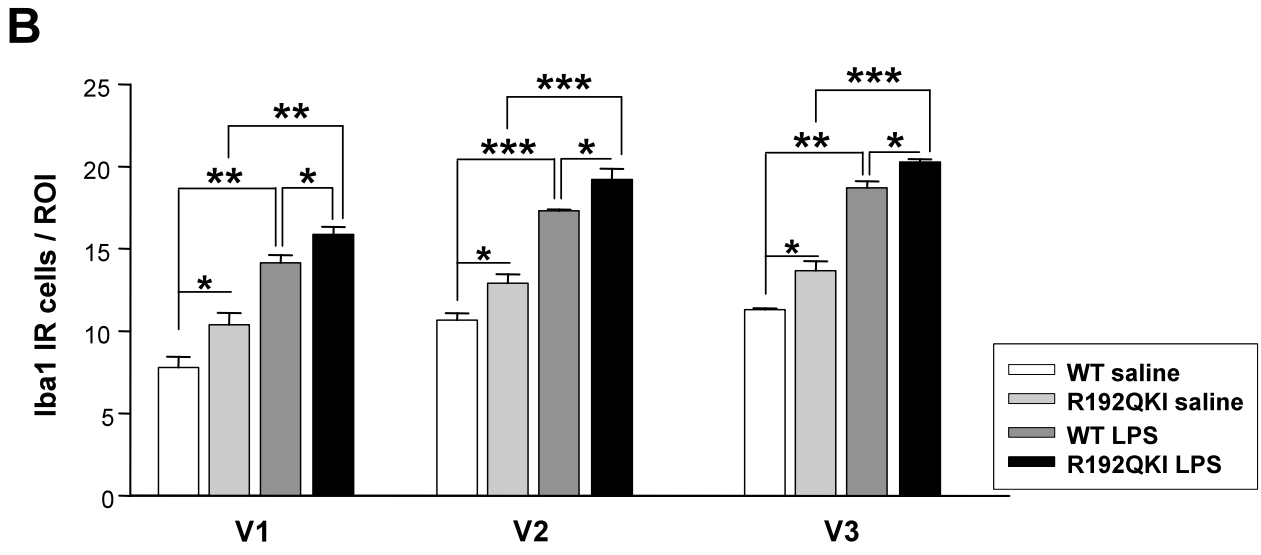


Figure 5.11 - LPS increased Iba1 cells in WT and R192Q KI ganglia

A, Representative confocal microscopy images of WT or R192Q KI trigeminal ganglion sections under basal condition immunostained for Iba1 (red) 5 h after i.p. injection of saline (left column) or LPS (right column, 5 mg/kg). Scale bar: 50 μm. *B*, Histograms quantify the number of Iba1-positive cells per ROI (370 x 370 μm) in V1, V2 or V3 regions; n = 3 WT and 3 KI mice; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.2.4 - Effect of inflammatory stimuli on TNFα expression in WT and R192Q KI trigeminal ganglia

LPS is known to induce, in sensory ganglia, an early rise in TNFα mRNA followed by strong expression of TNFα protein by macrophages a few hours later (Li et al., 2004). In agreement with data from DRG ganglia (Li et al., 2004), we also observed that, at an early phase after LPS injection, there was a very large increment in TNFα mRNA levels in WT and in R192Q KI ganglia (Fig. 5.12 A). As reported in the literature (Li et al., 2004; Austin and Moalem-Taylor, 2011; David and Kroner, 2011; Guillot et al., 2011), in WT trigeminal ganglia there was a higher expression in the early phase of inflammation (1-2 h) and, after that, it started to decrease (Fig. 5.12 A, note the significant reduction of TNFα mRNA levels 5 h after LPS stimulation in comparison with 30 min or 1 h). KI samples showed different profile: there was a very large peak of TNFα after 1 h of LPS i.p. that collapsed after 5 h (Fig. 5.12). We observed that the TNFα mRNA levels at 30 min or 5 h are the same in WT and R192Q KI ganglia. Only after 1 h, there was a difference between them. Hence, we could conclude that the R192Q KI mutation responded with a different TNFα mRNA profile.

As reported by Li et al. (2004), we evaluated the TNFα protein content with ELISA assay in trigeminal ganglion lysates 5 h after LPS injection. After LPS injection, TNFα expression was enhanced in both WT and R192Q KI trigeminal ganglia, interestingly higher TNFα levels were detected in KI than WT ganglia only after inflammatory stimulation (Fig. 5.12 B).

In order to check which cell types produce TNFα, we performed immunostaining experiments on trigeminal ganglia from WT and R192Q KI mice stimulated with LPS for 5 h (Fig. 5.12 C). We observed significantly larger occurrence of co-staining for TNFα and Iba1 in all ganglion regions (Fig. 5.12 C, D). These results, thus, suggested an enhanced response of R192Q KI ganglia to a standard inflammatory stimulus.

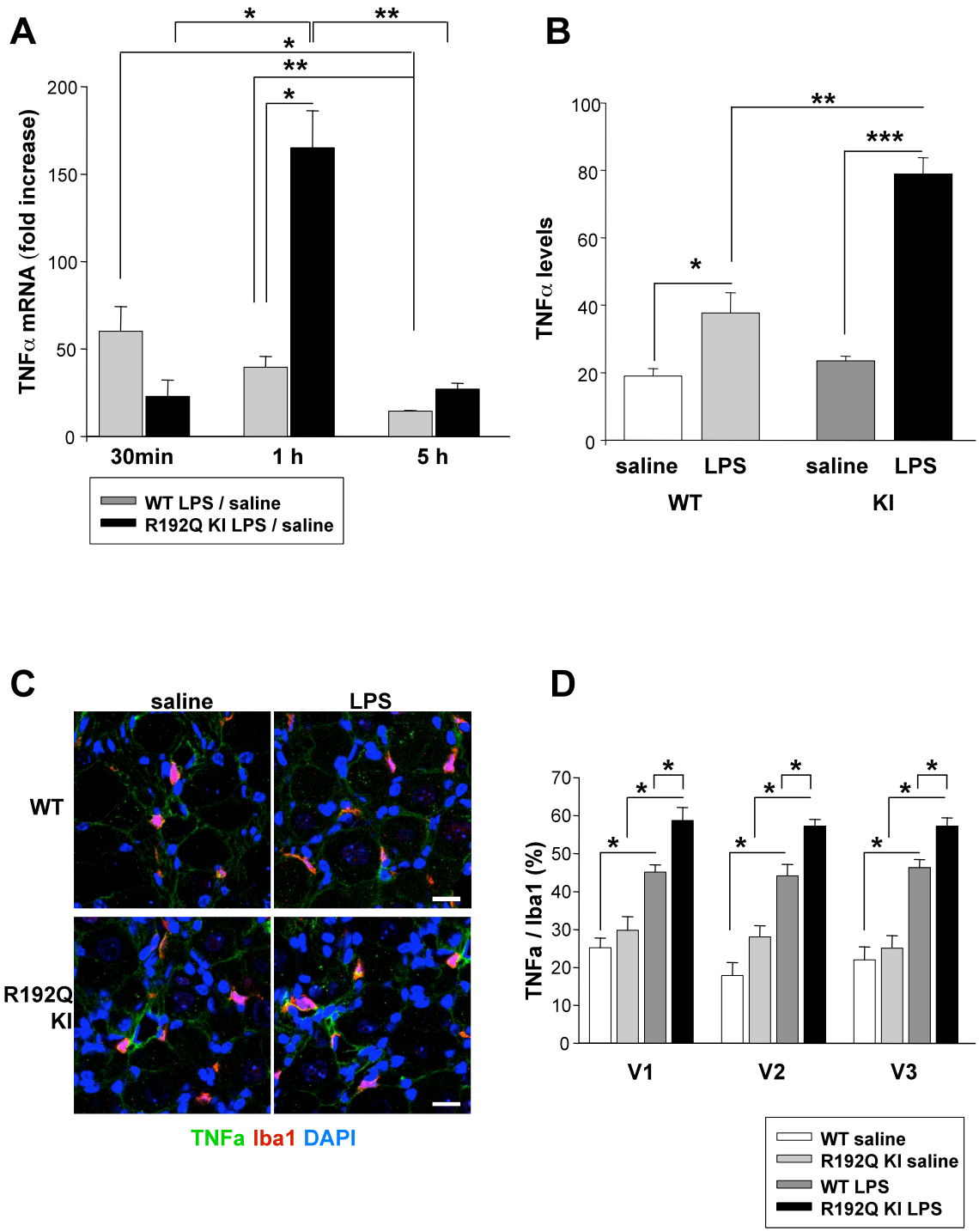


Figure 5.12 - LSP evoked different acute inflammatory response in WT and R192Q KI ganglia

A, Histograms quantify changes (Δ) in TNFα mRNA level in WT or R192Q KI ganglia following LPS-injection (i.p., 0.5 - 1 - 5 h). Data are normalized with respect to results from saline-injected WT and KI mice; n = 3 WT and 3 KI mice; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *B,* TNFα protein levels in whole ganglia (pg/ml / tissue wet weight; ELISA assay) from saline- and LPS-injected WT and R192Q KI mice. n = 3 WT and 3 KI mice; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. *C*, Representative confocal microscopy images of WT or R192Q KI trigeminal ganglion sections under basal condition immunostained for Iba1 (red), TNFα (green) and DAPI (blue) 5 h after i.p. injection of saline (left column) or LPS (right column, 5 mg/kg). Scale bar: 50 μm. I, Histograms quantify the percentage of Iba1 positive cells that were also TNF□ positive per ROI (370 x 370 □m) in V1, V2 or V3 regions; n = 3 WT and 3 KI mice; * $p < 0.05$.

5.3 Primary cultures of trigeminal ganglia: characterization of trigeminal ganglion macrophages in naïve conditions and after inflammatory stimuli

5.3.1 - Primary cultures of WT and KI trigeminal ganglia reflect the basic ganglion characteristics

The ganglion profile that is compatible with an inflammatory pre-activation state prompted further investigation into the possibility of an interaction between macrophages and trigeminal neurons. To this end, we used dissociated trigeminal ganglion cultures as a model to study the cross-talk between neuronal and non-neuronal cells in the migraine mouse model.

Keeping trigeminal ganglion primary cultures for 2 days *in vitro* did not affect the number of neurons (Simonetti et al., 2006) and macrophages (Fig. 5.13 A, B). The difference between WT and R192Q KI macrophage morphology found in trigeminal ganglion tissue (Fig. 5.2 B, C) was maintained in culture (Fig. 5.13 B). R192Q macrophages were larger than WT ones (Fig. 5.13 C, D). Macrophages kept in culture for 2 days had larger area than at 24 h (Fig. 5.13 C, D).

Cultured macrophages preserved the expression of different markers. The percentage of Iba1 positive cells that expressed also CD11b (about 20% in WT and 30% in KI; Fig. 5.13 E, F) was comparable with data found in the tissue (Fig. 5.5 A, B).

The 30-40 % of Iba1 positive cells expressed TNFα *in vitro* (Fig. 5.13 G, H) a similar percentage is found in tissue macrophages (Fig. 5.9 B).

As reported by Li et al. (2004), we found P2X3 neurons immunopositive for TNFα (Fig. 5.12 I, J), in particular in R192Q KI cultures. Apparently, the TNFα up-regulation in neurons was correlated with inflammatory states (Schäfers et al., 2003; Li et al., 2004). Considering the total TNFα positive cells in WT and R192Q KI trigeminal ganglion cultures, around 60% were macrophages and less than 30% were P2X3 neurons.

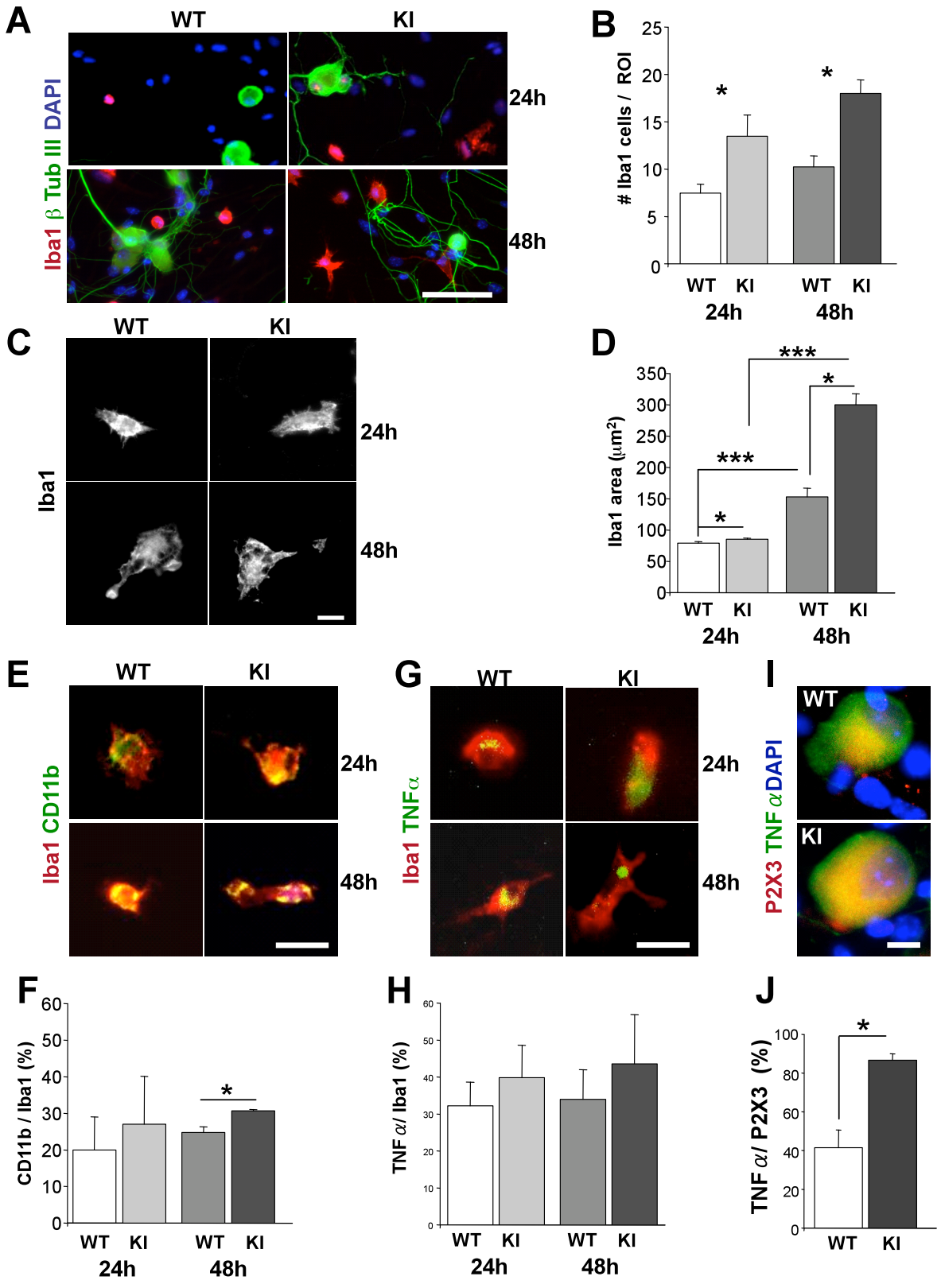


Figure 5.13 - Macrophages in primary cultures from WT or KI trigeminal ganglia

A, Examples of fluorescence microscopy images of cultures (24 – 48 h) from WT or R192Q KI trigeminal ganglia immunostained with antibodies against Iba1 (red), β-Tubulin III (green) and counterstained with DAPI (blue). Scale bar: 50 μm. *B*, Histograms quantify Iba1-positive cells in cultures from WT or KI ganglia. Data are expressed per ROI (320 x 240 μm); $n = 4$ experiments, * $p < 0.05$. *C*, Representative images of WT and R192Q KI Iba1 cells in culture

after 24 - 48 h. Scale bar: 10 μm. *D*, Histograms quantify the area of Iba1-positive cells in WT or KI ganglion cultures after 24 - 48 h. $n = 4$ experiments; * $p < 0.05$; *** $p < 0.001$. *E*, Examples of fluorescence microscopy images from WT or R192Q KI trigeminal ganglion cultures immunostained with antibodies against Iba1 (red), or CD11b (green). Scale bar: 10 μm. *F*, Histograms quantify percentage of Iba1 cells that co-expressed CD11b in WT or R192Q KI ganglion cultures. $n = 3$ experiments, * $p < 0.05$. *G*, Examples of fluorescence microscopy images from WT or R192Q KI trigeminal ganglion cultures immunostained with antibodies against Iba1 (red), or TNFα (green). Scale bar: 10 μm. *H*, Histograms quantify percentage of Iba1 cells that co-expressed TNFα in WT or R192Q KI ganglion cultures. $n = 5$ experiments. *I,* WT or R192Q KI trigeminal ganglion culture microscopy images immunostained with antibodies against P2X3 (red), TNFα (green) and stained for DAPI (blue). Scale bar: 10 μm. *J*, Histograms quantify percentage of P2X3 cells that co-expressed TNFα in WT or R192Q KI ganglion cultures. $n = 3$ experiments, * $p < 0.05$.

5.3.2 - LPS effects on WT and R192Q KI trigeminal ganglion cultures

In analogy with studies investigating the LPS-mediated inflammatory reaction of sensory trigeminal ganglia *in vivo* (Fig. 5.11-12) and *in vitro* (Li et al., 2004), we induced an acute inflammation of WT and R192Q KI trigeminal cultures with LPS (0.5 μg/ml). Fig. 5.14 A compares representative images of trigeminal ganglion cultures from WT and R192Q KI mice in sham conditions or 5 h after LPS application. Although there was no change in the number of Iba1 immunoreactive cells (Fig. 5.14 B), LPS treatment increased the average area of Iba1 positive cells in both culture conditions (Fig. 5.14 C), suggesting a stronger activation state of macrophages (Seifert et al., 2011). These observations indicated that KI Iba1 positive cells had a stronger basal activation state that could be further enhanced by LPS. The number of P2X3 positive neurons was not affected by LPS (Fig. 5.14 D), suggesting that this inflammatory stimulus was not neurotoxic on these cultures.

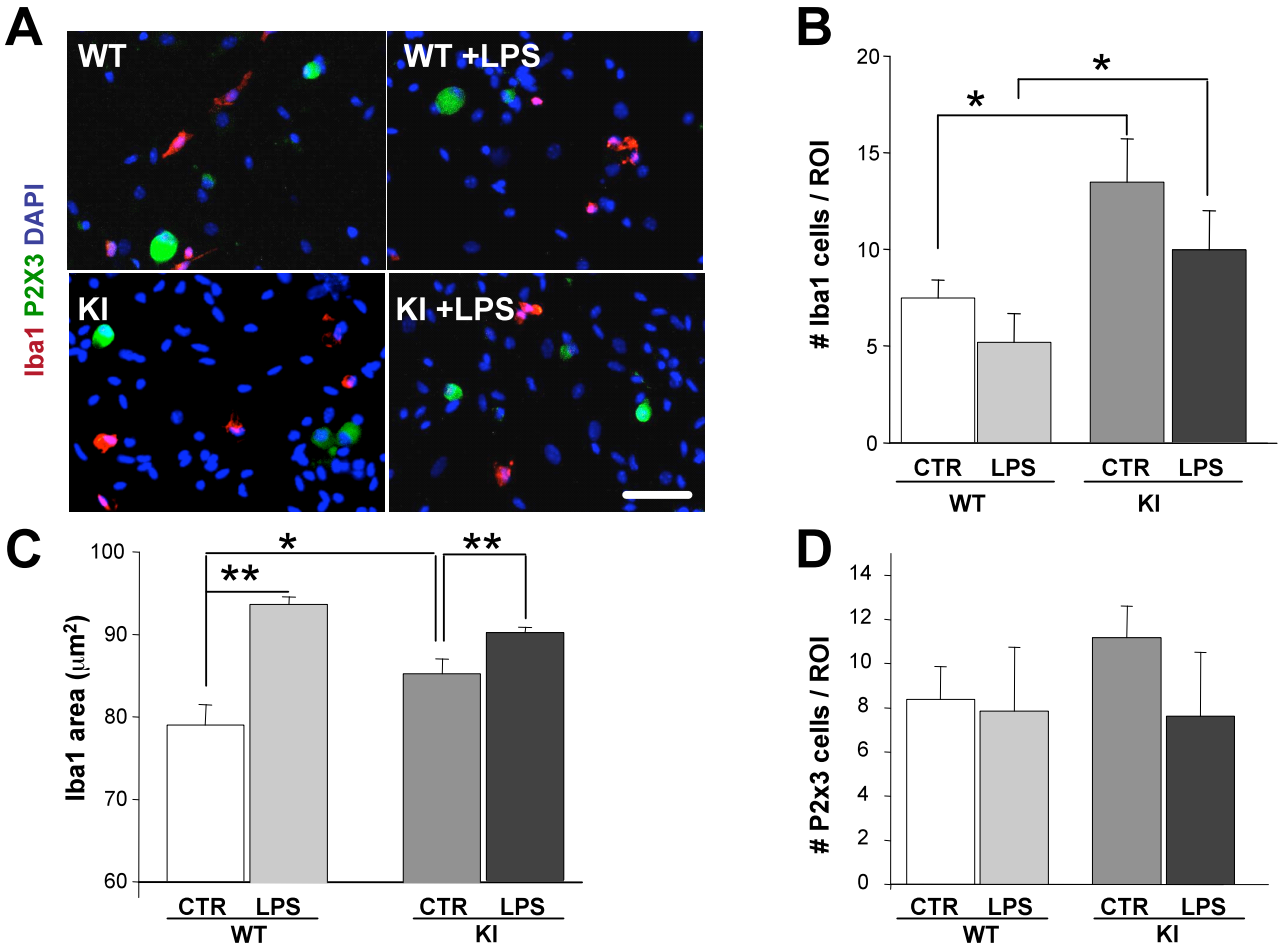


Figure 5.14 - Inflammatory induction with LPS in trigeminal ganglion cultures

A, Representative images of WT and R192Q KI trigeminal ganglion cultures in control condition (sham, left column) or after 5 h of LPS treatment (0.5 µg/ml, right column), immunostained with antibodies against Iba1 (red), P2X3 (green) and counterstained with DAPI (blue). Scale bar: 50 µm. *B*, Histograms quantify the number of Iba1 positive cells per ROI (320 x 240 µm) in WT or KI trigeminal cultures with or without LPS treatment (0.5 µg/ml) for 5 h. $n = 4$ experiments, * $p < 0.05$. *C*, Histograms quantify the area of Iba1 positive cells in cultures from WT or KI ganglia, with or without LPS treatment (0.5 µg/ml) for 5 h. $n = 4$ experiments; * $p < 0.05$; ** $p < 0.01$. *D*, Histograms quantify the number of P2X3 positive cells per ROI (270 x 270 µm) in WT or KI trigeminal cultures with or without LPS treatment (0.5 µg/ml) for 5 h. $n = 5$ experiments.

5.3.3 - Pro-inflammatory cytokine expressions in WT and KI trigeminal cultures stimulated with LPS

The responses evoked by LPS are characterized by an early rise of pro-inflammatory cytokine mRNA levels in vitro (Li et al., 2004; Donnelly and Popovich, 2008; Ochoa-Cortes et al 2010). We tested, with real-time PCR, TNFα mRNA changes as an early inflammatory marker. In both WT and R192Q KI cultures, LPS treatment significantly increased TNFα mRNA levels (Fig. 5.15 A; 1-5-24 h vs CTR). As reported before, in inflammatory conditions, TNFα mRNA levels reached the

peak of expression in the first hours (Li et al., 2004; Austin and Moalem-Taylor, 2011; David and Kroner, 2011; Guillot et al., 2011). In WT cultures LPS addition caused a significant up-regulation of TNFα mRNA levels after 1 h. After 5 or 24 h, TNFα mRNA levels were significantly higher than controls, but lower than after 1 h from LPS stimulation (Fig. 5.15 A). KI cultures showed a TNFα mRNA peak only after 5 h from LPS application (Fig. 5.15 A).

As already observed in entire trigeminal ganglia (Fig. 5.12 A), LPS induced an up-regulation of TNFα mRNAs consistent with data reported in the literature (Li et al., 2004; Austin and Moalem-Taylor, 2011; David and Kroner, 2011; Guillot et al., 2011). Viceversa, in KI trigeminal ganglia *in vivo* and *in vitro* TNFα mRNA expression was slower but stronger than WT ones. In fact, KI TNFα mRNA levels were significantly higher than WT ones 5 or 24 h after LPS stimulation, but there was no difference after 1 h (Fig. 5.15 A).

TNFα protein released into the culture medium after 5 h of LPS treatment was evaluated with ELISA assay (Fig. 5.15 B). In control conditions, we observed a basal difference between WT and KI cultures. In both cultures, however, LPS treatment caused a higher TNFα release (Fig. 5.15 B). In parallel with higher TNFα levels released into the medium, we found an LPS-induced fall in the expression of the TNFα precursor protein in WT and R192Q KI cultures (Fig. 5.15 C, D). Hence, these results suggest that LPS elicited a stronger inflammation state in R192Q KI cultures accompanied by enhanced TNFα release, as found in WT trigeminal ganglion tissue (Fig. 5.12).

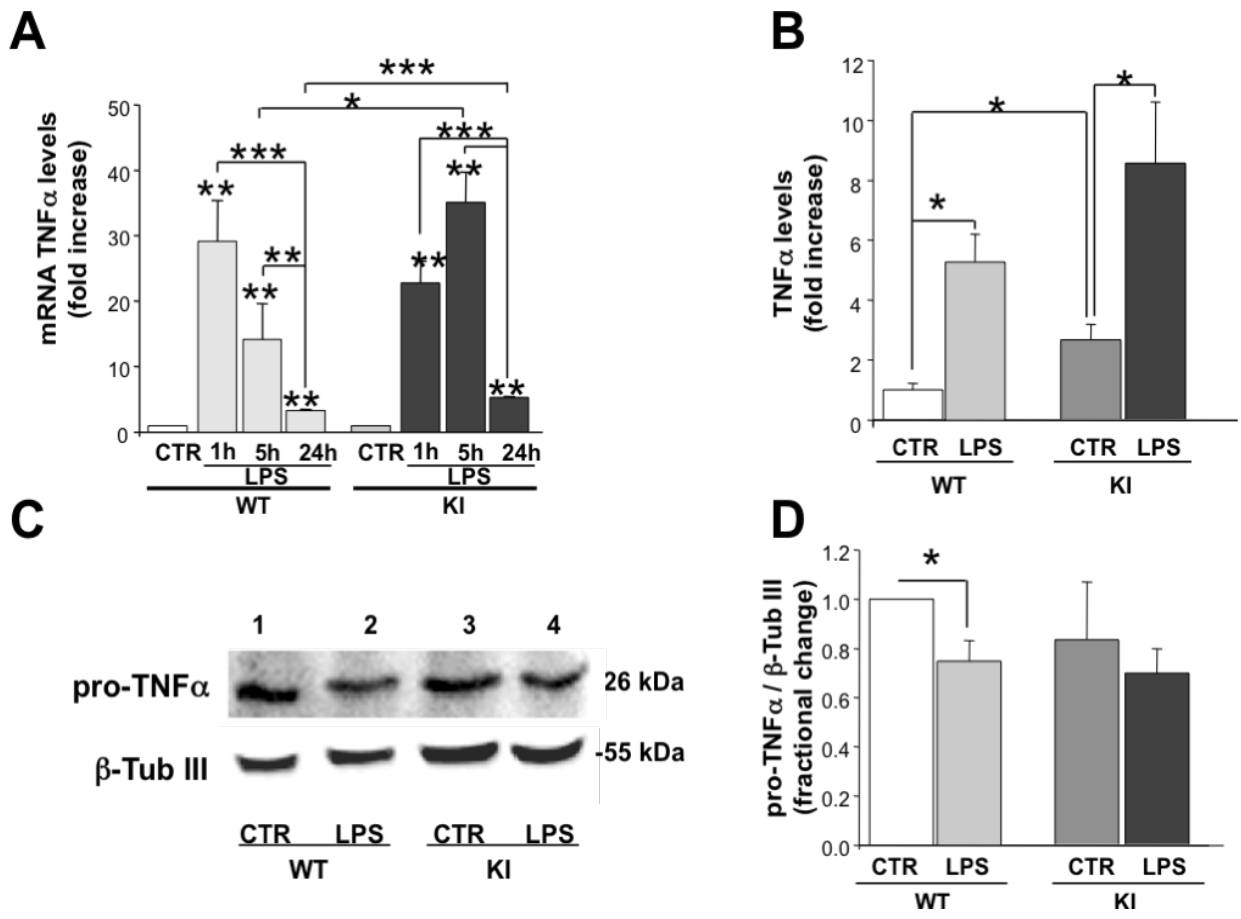


Fig. 5.15 - TNFα levels in WT and R192Q KI trigeminal ganglion cultures after LPS induction

A, Histograms quantify levels of TNFα mRNA of cultures of WT or KI trigeminal ganglia in control (CTR) and after 1 - 5 - 24 h of LPS (0.5 μg/ml) expressed as fold increase in comparison to their controls (WT and KI sham respectively - CTR) and normalized over GAPDH. Note higher TNFα mRNA level in all conditions (WT+LPS and KI +LPS) in comparison to their controls (CTR); $n = 3$, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. *B*, Histograms quantify levels of TNFα released from cultures of WT or KI trigeminal ganglia (24 h) control (CTR) and after 5 h of LPS (0,5 μg/ml) expressed as fold increase in comparison to WT controls. $n = 3$, * $p < 0.05$. *C*, Example of western blots shows different levels of TNFα expression in lysates from WT or KI trigeminal cultures (lanes 1 and 2), and after 5 h of LPS (WT+LPS and KI+LPS, lanes 3 and 4). β-TubulinIII is shown as loading control (bottom panel). *D*, Histograms show mean values (optical density AUs) of pro-TNFα expression normalized with β-Tubulin III expressed asfractional change in comparison to control; $n = 4$; * $p < 0.05$.

Interestingly, IL1β mRNA expression after stimulation with LPS followed a different time profile in WT and KI cultures (Fig. 5.16 A). WT cultures showed steady-state increase mRNA levels, while, viceversa, in KI cultures there was a peak of mRNA IL1β levels after 5 h from LPS application (Fig. 5.16 A).

IL1β released in the medium had the same profile of TNFα (Fig. 5.16 B): basal release of IL1β was larger from KI cultures than WT ones, while after LPS treatment there were an even higher release in both conditions without difference between WT and KI cultures (Fig. 5.16 B).

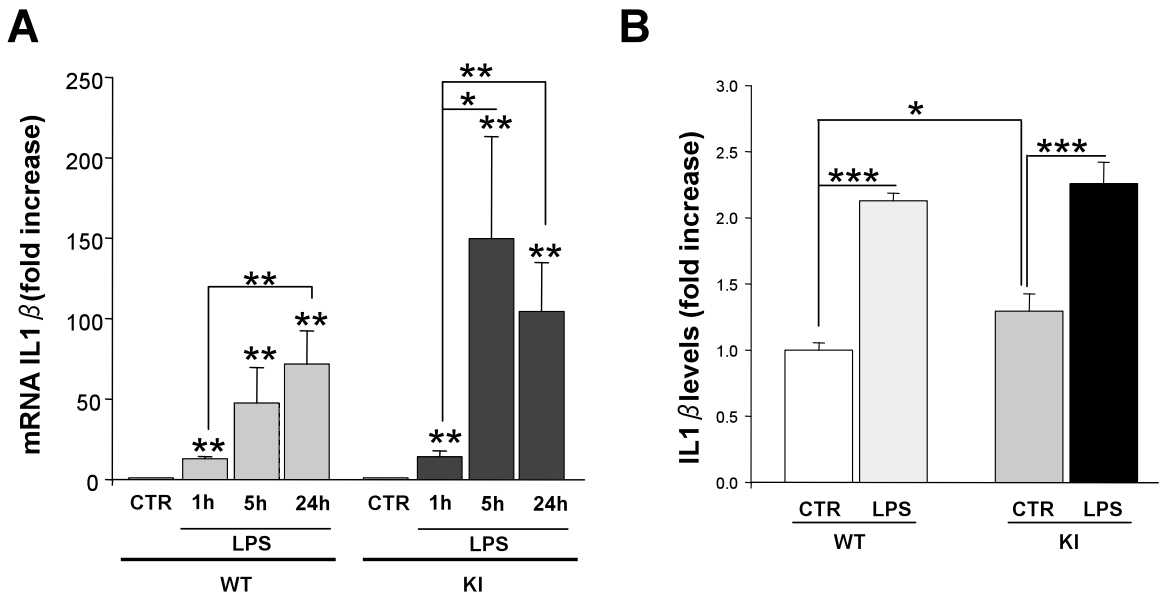


Figure 5.16 - IL1β levels in WT and R192Q KI trigeminal ganglion cultures after LPS application

A, Histograms quantify levels of IL1β mRNA of cultures of WT or KI trigeminal ganglia in control (CTR) and after 1 – 5 - 24 h of LPS (0.5 μg/ml) expressed as fold increase in comparison to their controls (WT and KI sham respectively - CTR) and normalized over GAPDH. Note higher IL1β mRNA level in all conditions (WT+LPS and KI +LPS) in comparison to their controls (CTR), $n = 3$, * $p < 0.05$; ** $p < 0.01$. *B*, Histograms quantify levels of IL1β released by cultures of WT or KI trigeminal ganglia (24 h) control (CTR) and after 5 h of LPS (0.5 μg/ml) expressed as fold increase in comparison to WT controls. $n = 3$, * $p < 0.05$, *** $p < 0.001$.

5.3.4 - Effect of LPS on P2X3 receptor function

Because most trigeminal ganglion neurons express P2X3 receptors (Simonetti et al., 2006; Nair et al., 2010) whose overactivity is implicated in pain transducing mechanisms of migraine models (Burnstock, 2009; Nair et al., 2010), we investigated whether LPS could differentially affect P2X3 receptor expression and function in WT or R192Q KI neurons in culture. We confirmed that the KI mutation did not change the P2X3 protein expression in WT or KI cultures (Fig. 5.17 A, B). Fig. 5.16 C shows patch clamp records of membrane currents induced by a 2 s application of the selective P2X3 receptor agonist α,β-meATP (10 μM; a concentration eliciting near-maximal response; Simonetti et al., 2006) to WT and KI neurons in basal conditions or 5 h after LPS application (0.5 μg/ml). In all cases, α,β-meATP evoked a fast-developing inward current (Fig. 5.17 C) that rapidly decayed because of receptor desensitization. As previously reported (Nair et al., 2010), baseline KI neuronal currents were larger than those recorded from WT neurons (Fig. 5.17 C, D). When WT ganglion cultures were treated with LPS 5 h, significant potentiation of WT P2X3 receptor mediated currents was observed (Fig. 5.17 C, D). Indeed, the increment in WT currents brought their values to the level normally seen in KI neurons. Unexpectedly, P2X3 receptors from

KI neurons were not potentiated by LPS, suggesting a different mechanism of cellular-mediated responses in ganglia from R192Q KI mice.

P2X3 receptor current rise-time (τ_{on}) and desensitization onset (τ_{fast}) were not significantly changed by LPS application; while recovery from desensitization at 30 s interpulse intervals was up-regulated in WT treated with LPS (0.5 µg/ml; 5 h) vs WT control conditions (Fig. 5.17 E).

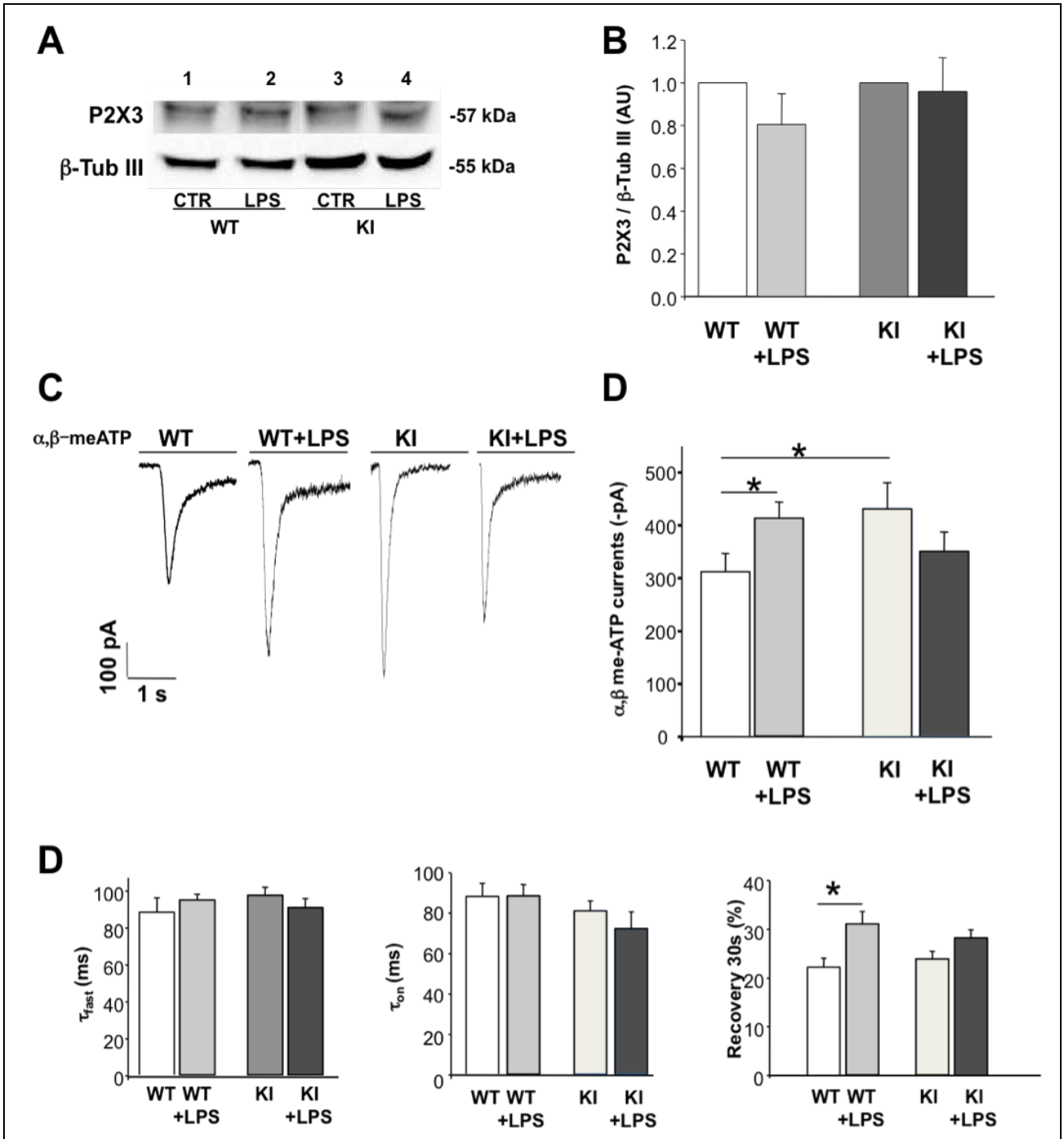


Figure 5.17 - Neuronal P2X3 receptor-mediated responses in control conditions and after LPS

A, Example of western blots shows similar levels of P2X3 receptor expression in lysates from WT or R192Q KI trigeminal cultures (lanes 1 and 3), and in LPS (5 h) treated cultures (WT+LPS and KI+LPS, lanes 2 and 4). β-TubulinIII levels are shown as loading control (bottom panel). *B*, Histograms show mean values (optical density AUs)

of P2X3 subunits obtained in western blot experiments normalized over β-TubulinIII signals; $n = 4$; $p > 0.05$. *C*, *D*, Representative examples of currents induced by application of α,β-meATP (10 μM, 2 s; horizontal bar) to trigeminal neurons cultures from WT (n = 30) or KI (n = 34) ganglia in control conditions (left traces) or when treated for 5 h with LPS (WT+LPS, n = 38; KI+LPS, n = 34). Note that LPS increases P2X3-mediated responses from WT neurons. Average data are plotted in *D*; * $p < 0.05$. *E*, Rise time (left; expressed as τ_{on} calculated on the 10 - 90 % current rise), and desensitization onset (middle; expressed as the first time constant, τ_{fast}, of current decay) of P2X3 receptor currents are $p > 0.05$ for WT, KI and LPS treated neurons (for τ_{on} $n = 10$ for WT, $n = 17$ for WT+LPS, $n = 22$ for KI, and $n = 14$ for KI+LPS. For τ_{fast} $p > 0.05$, $n = 8$ for WT, $n = 18$ for WT+LPS, $n = 35$ for KI, and $n = 20$ for KI+LPS). Recovery from desensitization (right; expressed as % of control amplitude in a paired pulse agonist application. All responses were evoked by α,β-meATP (10 μM, 2 s); * $p < 0.05$; $n = 21$ for WT, $n = 40$ for WT+LPS, $n = 33$ for KI, and $n = 40$ for KI+LPS.

5.4 Cross-talk between neurons and macrophages

5.4.1 – Peritoneal macrophages co-cultured with trigeminal ganglion cultures

Previous data on trigeminal ganglion cultures with addition of LPS demonstrated that inflammatory stimuli could influence P2X3 receptor responses, making them similar to those of R192Q KI neurons. However, it was unclear if P2X3 responses were directly or indirectly mediated by macrophages. Acosta and Davies (2008) have shown that DRG neurons could be directly activated by LPS. Thus, we decided to investigate the P2X3 mediated responses in trigeminal ganglion cultures enriched with peritoneal macrophages (MΦ) purified from WT adult mice (see Methods). Peritoneal macrophages were Iba1 positive (95 ± 4 % Iba1 / DAPI, n = 4) and expressed P2X4 and P2X7 receptors (Fig. 5.18 A).

Comparing standard WT or KI cultures with mixed cultures (Fig. 5.18 B), the number of Iba1 positive cells was doubled in mixed cultures (Fig. 5.18 C) and the difference between WT and KI cultures was maintained (Fig. 5.18 C).

With this approach, we could study if the activation of macrophages could be differently affected by WT or R192Q KI neurons and, viceversa, if P2X3 responses were modulated by macrophages.

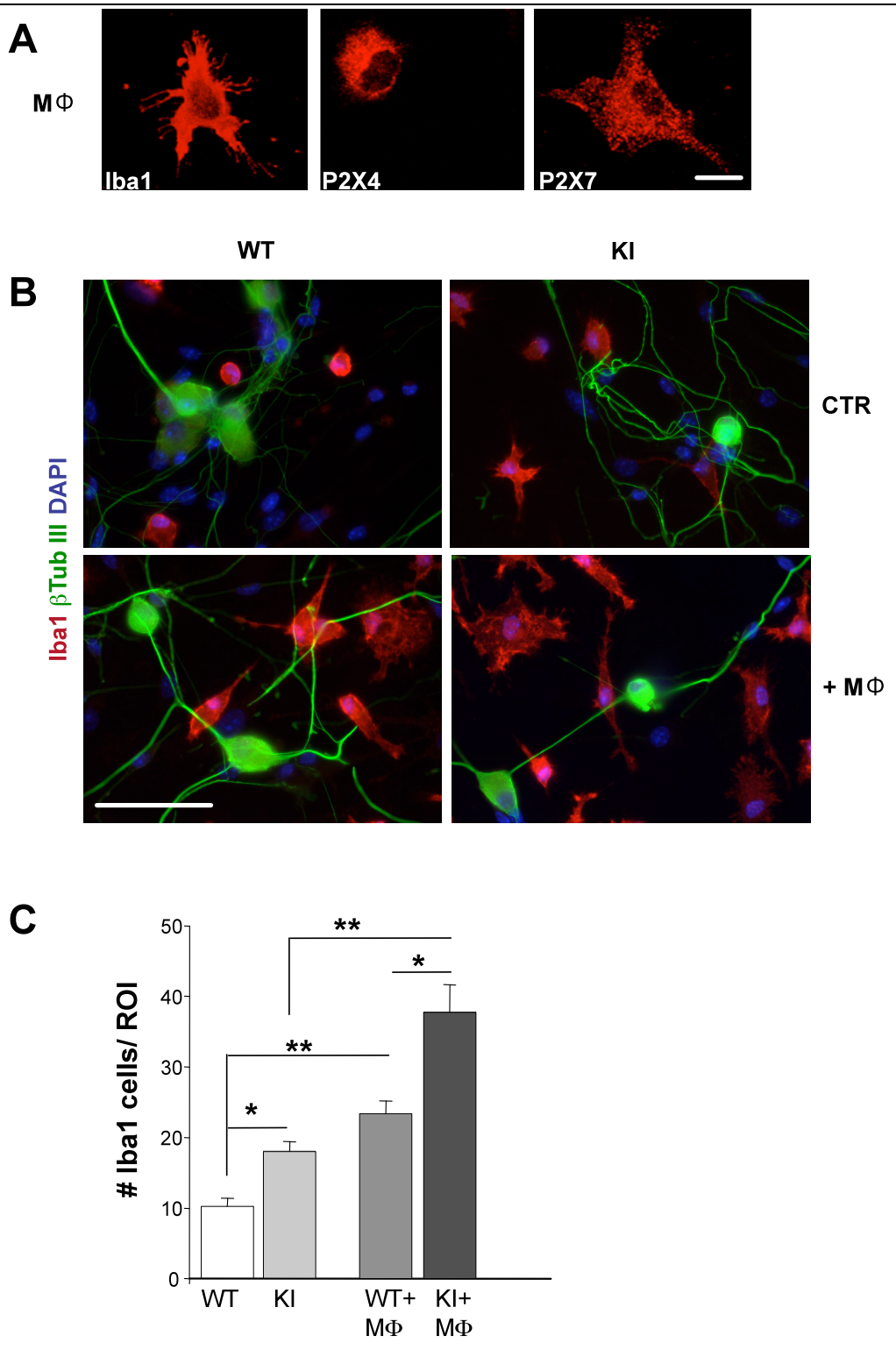


Figure 5.18 - Trigeminal ganglion cultures co-cultured with peritoneal macrophages (MΦ)

A, Examples of fluorescence microscopy images of peritoneal macrophages (MΦ) immunostained with antibodies against Iba1 (left), P2X4 (middle) and P2X7 (right). Scale bar: 10 μm. *B*, Examples of fluorescence microscopy images of macrophages in cultures from WT and KI trigeminal ganglia (top row) and with peritoneal macrophages (bottom row, WT+MΦ and KI +MΦ) immunostained with anti-Iba1 antibody (red), β-Tubulin III (green) and counterstained with DAPI (blue). Scale bar: 50 μm. *C*, Histograms quantify the number of Iba1 positive cells per ROI (320 x 240 μm) in WT or KI trigeminal cultures with or without peritoneal macrophages; $n = 4$ experiments, * $p < 0.05$; ** $p < 0.01$.

5.4.2 – Strong phagocytotic activity of R192Q KI macrophages *in vitro*

The activation state of macrophages was studied with a phagocytosis test (Harrigan et al., 2008), based on the uptake of Zy-FITC added to the culture medium (see Methods). Fig. 5.19 A shows an example of images in which there was higher basal macrophage activation in R192Q KI than WT culture. On average, KI macrophages had significantly larger number (9 ± 1) of Zy-FITC granules/cell than WT ones (5 ± 1; $p < 0.05$) as indicated in the histograms of Fig. 5.19 C, despite the number of Iba1 positive cells taking up Zy-FITC (over the total number of Iba1 positive cells) was not significantly different between WT (49 ± 6 %) and KI (57 ± 6 %) cultures. In accordance with Farber et al (2009), as shown in Fig. 5.19 D, we calculated the phagocytosis index (see Methods) to express the macrophage activity that was 260 ± 70 and 480 ± 50 for WT and KI cultures, respectively ($p < 0.05$). Cumulative probability plots to calculate the occurrence of granule uptake in WT and KI cells are shown in Fig. 5.19 E in which the higher probability of detecting a larger number of granules was clearly assigned to KI cells. These data, therefore, provided a functional validation of higher constitutive macrophage activity in R192Q KI ganglion culture.

5.4.3 - Functional crosstalk between macrophages and trigeminal ganglion cultures

We then enquired if the intrinsic properties of neuronal WT or R192Q KI cultures might differentially change phagocytic activity. To study this issue we used peritoneal macrophages with an average number of 7.0 ± 1.5 granules. Since 75 ± 5 % of such cells had phagocytotic activity, their phagocytosis index was 540 ± 150 ($n = 5$).

Fig. 5.19 C shows that, in co-culture, the average number of granules in macrophages was significantly larger for WT co-cultures vs WT cultures. The same observation was obtained when comparing KI co-cultures with KI cultures (Fig. 5.19 C). Thus, the phagocytosis index of WT co-cultures rose to 830 ± 70, while the index for KI co-cultures became 950 ± 70 (Fig. 5.19 D; $n = 5$). In either case the percentage of Iba1 cells taking up Zy-FITC was in the range 72 - 78 %. Cumulative plots for granule uptake process by host macrophages, and WT and KI co-cultures are shown in Fig. 5.19 F, demonstrating similar probability for detecting analogous granule uptake values in WT and R192Q KI co-cultures. Thus, in co-culturing conditions, the granule uptake by macrophages was strongly enhanced, especially in KI ganglia cultures.

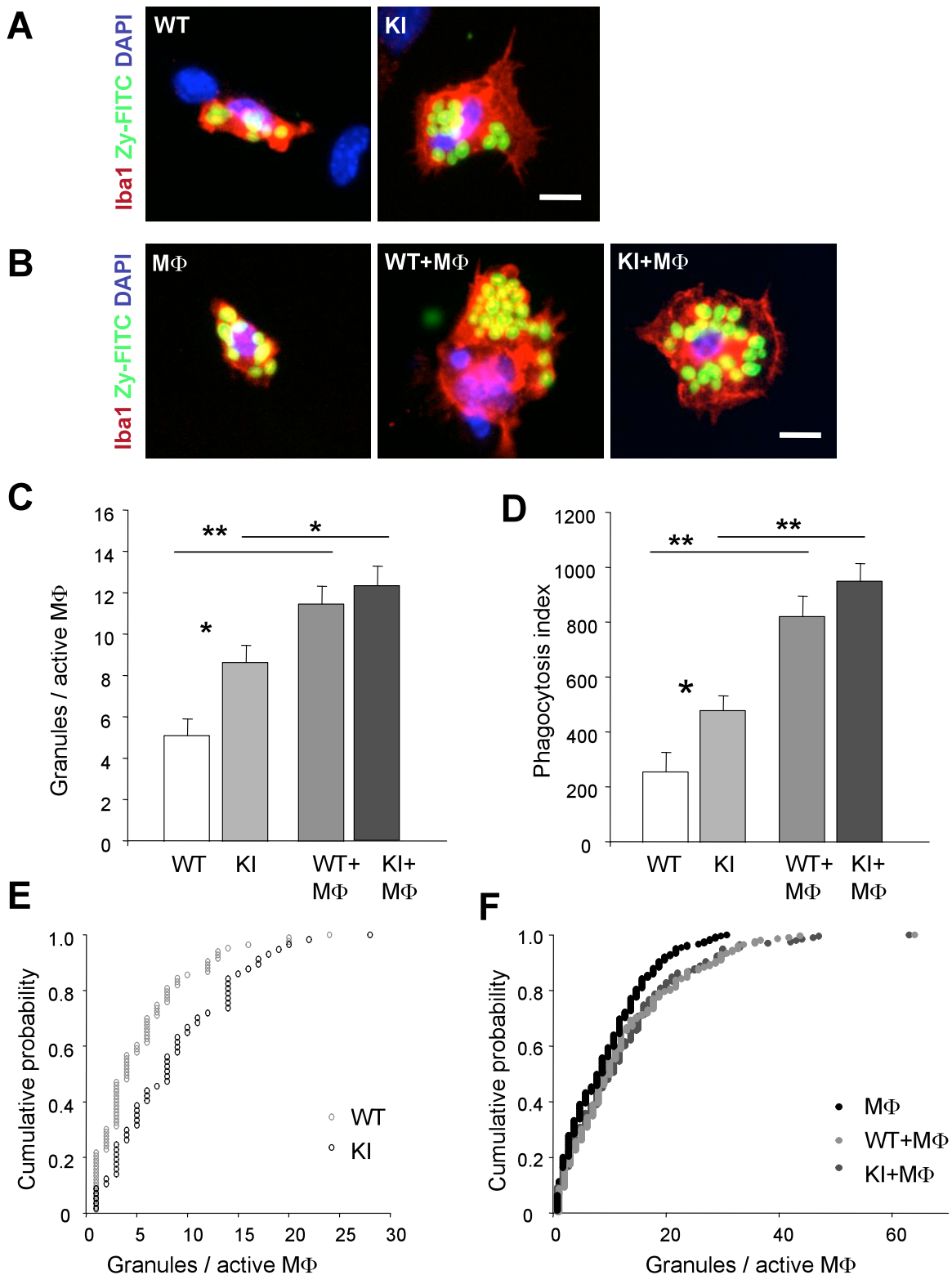


Figure 5.19 - Macrophage activity in culture

A, Examples of fluorescence microscopy images of macrophages in cultures from WT or KI trigeminal ganglia immunostained with anti-Iba1 antibody (red). Active Iba1 immunoreactive macrophages show round-shaped Zy-FITC granules. Cell nuclei are shown in blue. Scale bar = 10 μm. *B*, Images show peritoneal macrophages in culture (MΦ) and macrophages/trigeminal co-cultures (WT+MΦ and KI+MΦ) processed like in *A*. Note that peritoneal macrophages

are labeled by Iba1 and contain granules. Scale bar: 10 μm. *C*, Histograms quantify the number of Zy-FITC granules per active Iba1 immunoreactive cells. $n = 5$ experiments, * $p < 0.05$; ** $p < 0.01$. *D*, Phagocytosis index for WT or R192Q KI cultures. $n = 5$; * $p < 0.05$; ** $p < 0.01$. *E, F*, Cumulative probability plots to calculate the occurrence of granule uptake in Iba1 positive cells in WT (grey open circle, $n = 93$) or R192Q KI cultures (dark grey open circle, $n = 64$; *E*), as well as for peritoneal macrophages (MΦ, black, $n = 241$; *F*) and from co-cultures from WT (WT+MΦ grey circle, $n = 202$) and R192Q KI (KI+MΦ dark grey circle, $n = 234$; *F*). Note that macrophages from R192Q KI trigeminal cultures display larger phagocytosis activity.

5.4.4 - P2X4 and P2X7 in trigeminal ganglion cultures with peritoneal macrophage addition

Different P2X receptors are involved in pain. P2X4 and P2X7 are expressed by macrophage/microglia cells and are crucial in inflammatory transducing signals (Tsuda et al., 2010; Ren and Dubner, 2011). P2X4 and P2X7 receptors are expressed by macrophages (Brone et al., 2007), and differentially regulated with compensatory effects (Weinhold et al., 2010; Ren and Dubner, 2011). They also show a different dose dependence on ATP (Brone et al., 2007). Activation of purinergic P2X7 receptors by extracellular ATP is a key physiological inducer of rapid IL1β release from LPS-primed macrophage (Pelegrin et al., 2008). P2X7 is also involved in TNFα release (Inoue et al, 2006; Brone et al., 2007).

That's why we investigated P2X4 and P2X7 mRNA and protein levels under control condition or after macrophage addition to WT or R192Q KI TG cultures. Data were normalized on total cell content (Real Time PCR with GADPH, and Western blot experiments with actin). There were no changes in mRNA and protein levels in WT and KI cultures for P2X4 and P2X7 (Fig. 5.20 A-D). However, P2X4 mRNA levels were higher in WT+MΦ cultures than WT ones, reflecting the presence of exogenous macrophages; without an increase in protein levels (Fig. 5.20 A, B). In KI+MΦ cultures both mRNA and protein showed only an increased trend (Fig. 5.20 A, B).

These cultures revealed different profiles for P2X7 (Fig. 5.20 C, D). WT+MΦ cultures showed P2X7 mRNA and protein down-regulated vs WT ones. There was no change in mRNA and protein expression in KI+MΦ cultures vs KI ones (Fig. 5.20 C, D).

Even if, P2X7 receptor was down-regulated in WT co-cultures, TNFα and IL1β mRNA levels were up-regulated after macrophage addition to WT and KI trigeminal ganglion cultures (Fig. 5.20 E, F).

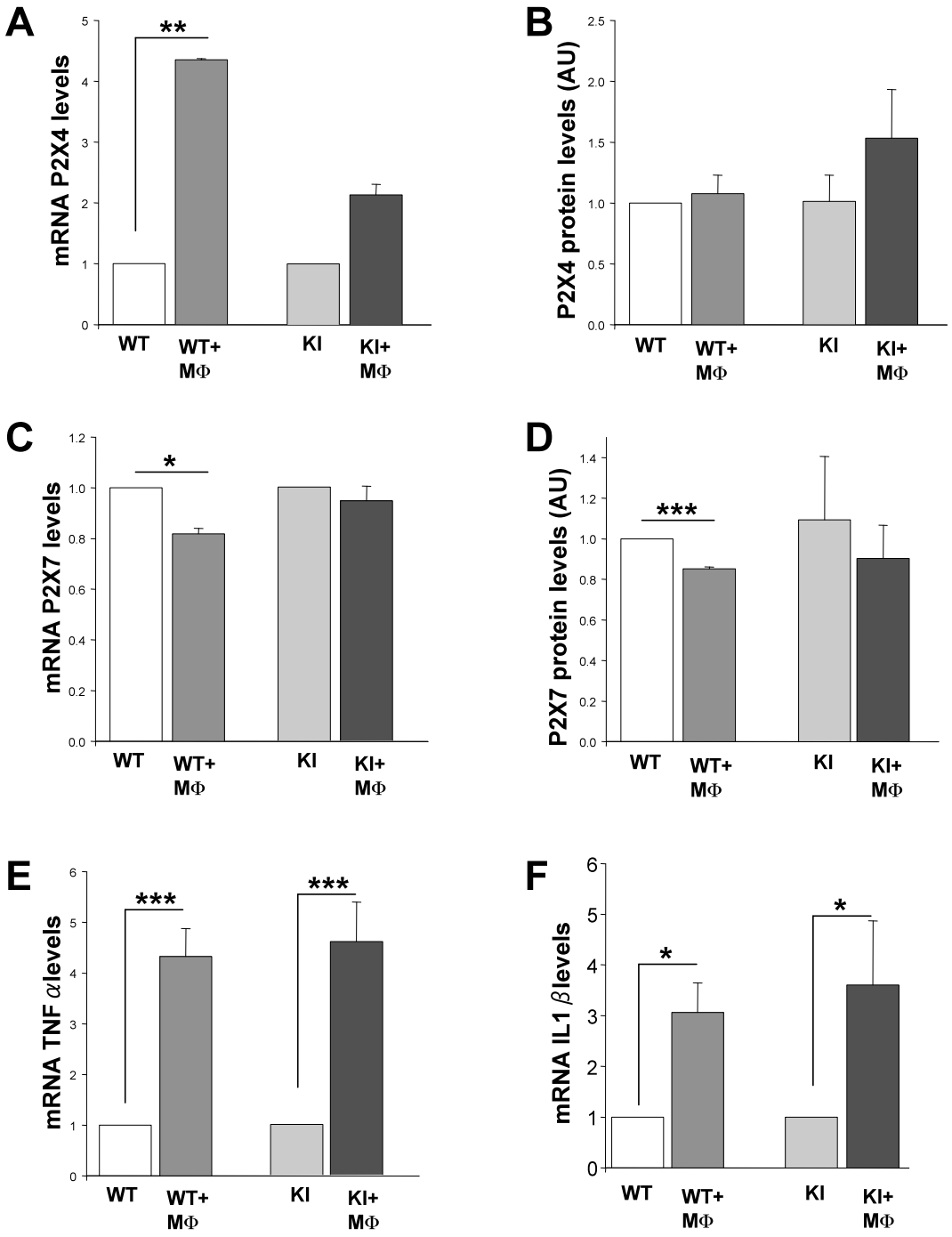


Figure 5.20 – P2X4, P2X7 and pro-inflammatory cytokines in trigeminal ganglion cultures in the presence of host macrophages

A, Histograms showed preliminary data of P2X4 mRNA levels in WT or KI trigeminal ganglion cultures with 120,000 peritoneal macrophages (MΦ). Data normalized on GADPH mRNA content. $n = 2$. *B*, Histograms showed preliminary data of P2X4 protein levels in WT and KI trigeminal ganglion cultures with 120,000 peritoneal macrophages. Data normalized on actin protein content. $n = 2$. *C*, Histograms showed P2X7 mRNA levels in WT or KI trigeminal ganglion cultures with 120,000 peritoneal macrophages. Data normalized on GADPH mRNA content. $n = 3$; * $p < 0.05$. *D*, Histograms showed P2X7 protein levels in WT and KI trigeminal ganglion cultures with 120,000 peritoneal macrophages. Data normalized on actin protein content. $n = 4$; *** $p < 0.001$. *E, F*, Histograms showed TNFα (*E*) and IL1β (*F*) mRNA levels in WT and KI trigeminal ganglion cultures with 120,000 peritoneal macrophages. Data normalized on GADPH mRNA content. $n = 6$; * $p < 0.05$; *** $p < 0.001$.

5.4.5 - P2X3 receptor-mediated responses in the presence of host macrophages

We investigated whether the presence of exogenous macrophages affected P2X3 receptor expression and function. P2X3 receptor expression (protein and mRNA expression) was not significantly altered by co-culturing trigeminal ganglion cells with host macrophages (Fig. 5.21 A-C). Nonetheless, Fig 5.21 D shows examples of current traces induced by a 2-s application of the selective P2X3 receptor agonist α,β-meATP (10 μM) to WT and R192Q KI neurons when cultured in standard conditions or co-cultured with macrophages. As previously reported, R192Q KI neuronal currents were larger than those recorded from WT neurons (Fig. 5.21 E; Nair et al., 2010). When WT ganglia were co-cultured with macrophages, a significant potentiation of P2X3 mediated currents was observed while this effect was not present in KI ones (Fig 5.21 D-E). The increment in WT currents brought their values to the level normally seen in KI neurons. Other parameters of P2X3 receptor function, such as current rise-time (τ_{on}), desensitization onset (τ_{fast}) were not significantly changed by macrophage co-culturing. Interestingly, macrophages changed P2X3 receptors recovery from desensitization that was down-regulated in WT co-cultures (Fig. 5.21 F).

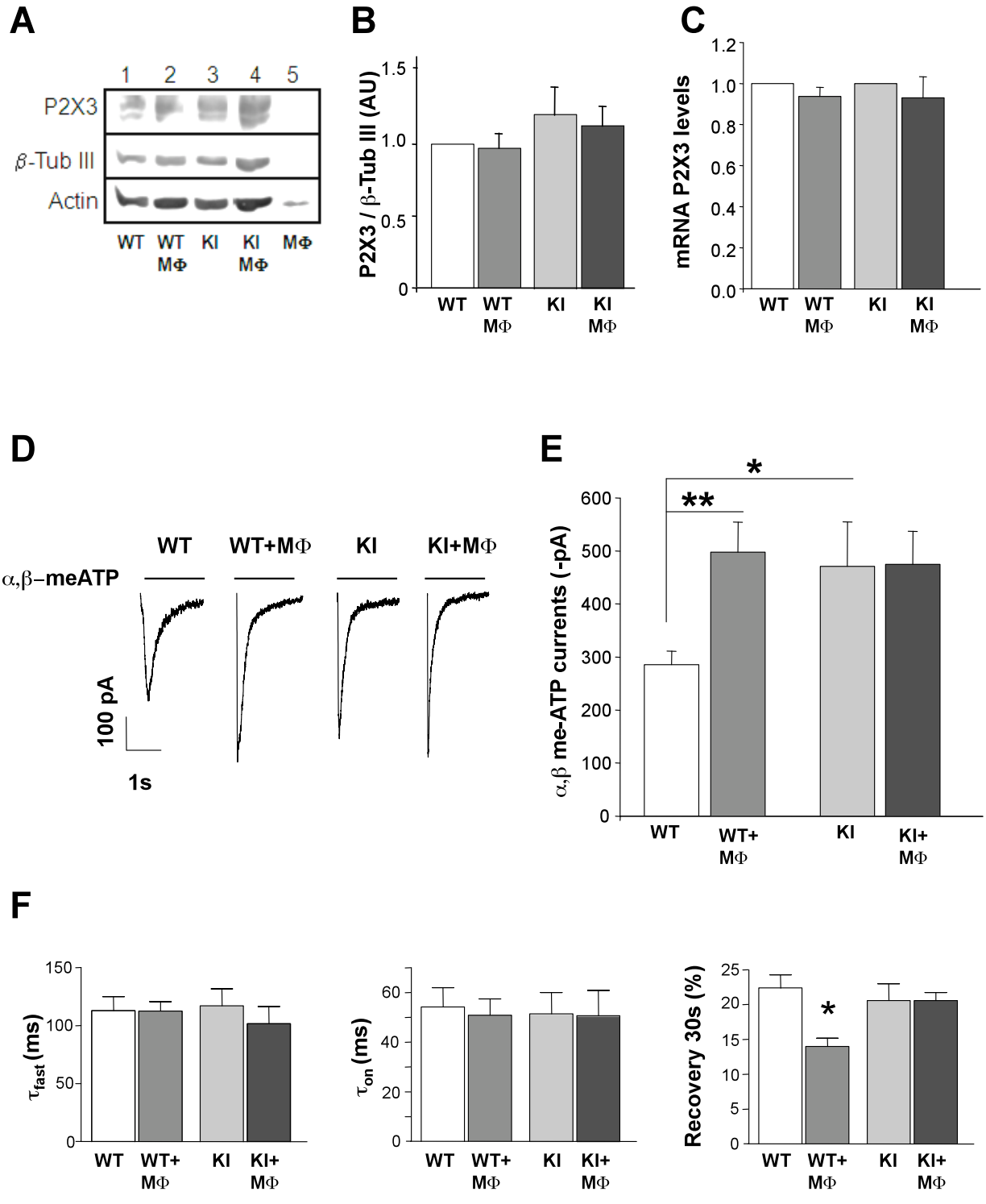


Figure 5.21 - Neuronal P2X3 receptor-mediated responses in trigeminal ganglia cultures in the presence of host macrophages

A, Example of western blots shows similar levels of P2X3 receptor expression in lysates from WT or R192Q KI trigeminal cultures (lanes 1 and 3), and in macrophages/trigeminal co-cultures (WT+MΦ and KI+MΦ, lanes 2 and 4). Lysates from macrophages only (lane 5) show no signal. β-TubulinIII and actin are shown as loading control (bottom panel). *B*, Histograms show mean values (optical density AUs) of P2X3 subunits obtained in western blot experiments normalized over β-TubulinIII signals; $n = 4$; $p > 0.05$. *C*, Histograms quantify P2X3 mRNA levels normalized over β-tubulin mRNA levels; $n = 4$; $p > 0.05$. *D*, Representative examples of currents induced by application of α,β-meATP (10

μM, 2 s; horizontal bar) to trigeminal neurons cultures from WT (n = 15) or R192Q KI (n = 17) ganglia in standard conditions (left traces) or when co-cultured with macrophages (WT+MΦ, n = 24; KI+MΦ, n = 20. Note that macrophage co-culturing increases P2X3-mediated responses from WT neurons. Average data are plotted in *E*. * p < 0.05; ** p < 0.01. *F,* Desensitization onset (left; expressed as the first time constant, τ_{fast}, of current decay) and rise time (middle; expressed as τ_{on} calculated on the 10 - 90 % current rise) of $P2X_3$ receptor currents are similar for WT, KI and co-culture neurons (for τ_{on} p > 0.05, n = 13 for WT, n = 18 for WT+MΦ, n = 17 for KI, and n = 17 for KI+MΦ. For τ_{fast} p > 0.05, n = 9 for WT, n = 22 for WT+MΦ, n = 11 for KI, and n = 19 for KI+MΦ). Recovery from desensitization (right; expressed as % of control amplitude in a paired pulse agonist application) was faster for WT+MΦ vs WT (* p = 0.007, n = 13 for WT, n = 23 for WT+MΦ), while it did not change for KI cultures vs co-cultures (n = 17 for KI, and n = 19 for KI+MΦ. All responses were evoked by α,β-meATP (10 □M, 2 s).

5.5 CGRP effects on trigeminal ganglion cultures

Different soluble substances are involved in neuronal-non-neuronal cell crosstalk during neuroinflammation. Previous studies have shown that CGRP has a key role in inflammation and in migraine pain, despite the fact that the role of CGRP in FHM-1 patients is under debate (Goadsby, 2005; Sarchielli et al., 2004). Previous studies from our group have shown that CGRP upregulates P2X3 neuronal responses in C57-black trigeminal ganglion cultures (Fabbretti et al., 2006). We therefore tested the CGRP effects on R192Q KI neurons in order to see the effect of a specific migraine mediator on WT and R192Q KI trigeminal ganglion cultures.

Fig. 5.22 A shows microscopy images of WT and R192Q KI trigeminal ganglion in basal condition or after 2 h of CGRP application (1μM). The percentage of P2X3 expressing neurons did not change after CGRP application (Fig. 5.22 B). P2X3 mRNA levels were up-regulated after CGRP addition to WT trigeminal ganglion cultures (Fig. 5.22 C), as expected (Simonetti et al., 2008). Interestingly, in KI cultures CGRP did not change P2X3 mRNA expression (Fig. 5.22 C), suggesting also in this case, that R192Q mutation confers a new phenotype resistant to further potentiation of P2X3 mediated responses.

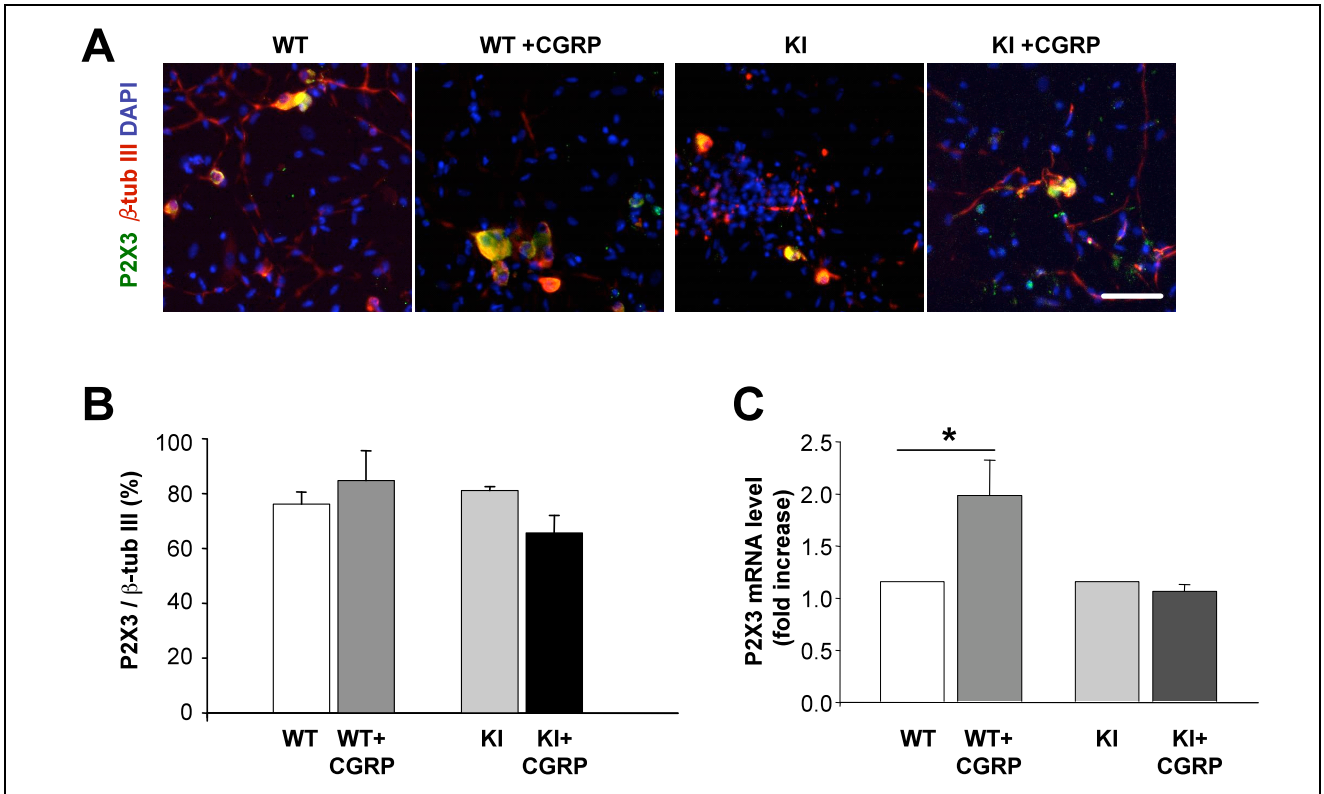


Figure 5.22 – Effect of CGRP on P2X3 receptor in trigeminal ganglion cultures

A, Representative images of WT or R192Q KI cultures in control condition and after CGRP addition (2 h, 1 μM), immunostained for P2X3 (green), β-Tubulin III (red) and DAPI (blue). Scale bar: 50 μm. *B,* Histograms show the

percentage of P2X3 immunoreactive neurons (taking as 100% the β-tubulin III immunoreactive); $n = 3$, $p > 0.05$. *C*, Histograms showed real time PCR data of P2X3 mRNA levels in WT or R192Q KI trigeminal ganglia cultures in control or after CGRP treatment. Data are normalized over β-tubulin III mRNA levels and expressed as fold increase with respect to control; $n = 3$; * $p < 0.05$.

Chapter 6: DISCUSSION

Neuroinflammation and migraine

The pathophysiology of migraine pain and the factors predisposing a patient to frequent acute attacks remain still unclear. The neurogenic inflammatory theory proposes that soluble factors released by neuronal and non-neuronal cells, such as macrophages and mast cells, contribute to trigger hyperactivity of peripheral nociceptors in afferents trigeminal sensory fibers neurons innervating dura mater and to generate a sterile inflammatory condition that supports (and facilitates) pain (Moskowitz, 2007; Moskowitz and Buzzi, 2010). Interestingly, in our laboratory the pain phenotype of R192Q KI mice has been investigated showing a larger sensitivity to α,β-meATP in the trigeminal area (Fabbretti E., personal communications).

The functional cross-talk between non-neuronal cells and pain sensory neurons is an important phenomenon in the pathophysiology of chronic pain (Bartley, 2009; Ren and Dubner, 2010; Robbins and Maides, 2011) The complex interactions occurring and the cellular and molecular mediators involved are not fully characterized, in particular little is known about time course and effects of inflammatory mediators. It would be useful understand whether such inflammatory-like alterations are also present in the trigeminal ganglia, namely the site where integration of the afferent nociceptive signals of sensory neurons occurs to transmit impulses to trigeminal brainstem nuclei, and in particular which are the facilitating and inhibitory mediators of this cross-talk.

Macrophages in trigeminal ganglia of R192Q KI mice show activation state

Macrophages/microglial cells are considered the principal players of neuroinflammatory processes in the PNS and in the CNS (Austin and Moalem-Taylor, 2010; David and Kroner, 2011). At the site of injury, macrophages increase in number and release different types of mediators, such as pro- and anti-inflammatory cytokines and chemokines, considered crucial for the onset, the maintenance and the end of the inflammatory conditions (Bernardino and Malva, 2007; Austin and Moalem-Taylor, 2010; David and Kroner, 2011; Kettenmann et al., 2011). However, the multiple and crucial role of macrophages in chronic pain states, including migraine, is debated (Austin and Moalem-Taylor, 2010; David and Kroner, 2011; Guillot et al., 2011).

The present study shows that endogenous macrophages were present in trigeminal ganglia, and that, with respect to WT, R192Q KI ganglia were characterized by a significantly larger number of such cells, with typical morphology of activation (i.e. “ameboid shape”) (Austin and Moalem-Taylor,

2010; Kettenmann et al., 2011).

Using Iba1, as a canonical marker of macrophages and microglia (Imai et al., 1996; Ito et al., 1998), we observed that Iba1-immunoreactive macrophages in the trigeminal ganglion were a relatively small cell population (around 4 % over the total of nuclei, as found in the CNS and the PNS (Tsuda et al., 2005)), nevertheless significantly up-regulated in R192Q KI.

Furthermore, it was noteworthy that, in R192Q KI ganglia, macrophages were consistently found more numerous, specially, in close association with neurons, outlay their potential role as "sensors" of neuron-derived signaling, thus, part of a neuron-glia interactive process important for chronic pain development (Tsuda et al., 2005; Milligan and Watkins, 2009).

The present study found no difference in distribution of macrophage cells in the three branches of the trigeminal nerve, suggesting that the expression of R192Q mutation in trigeminal ganglion neurons per se caused the increase of macrophages close to neuronal area. Thus, apparently, from this point of view, the entire trigeminal ganglia from R192Q KI mice is characterized by an overall larger activation of inflammatory cells and/or larger expression of inflammatory mediators, contributing to a global alteration in the environments of trigeminal ganglia.

Neurons could be sensitized and functionally modulated by immune mediators released by active macrophages localized in their neighborhood. At the same time, the R192Q mutation presents on KI neurons could modulate the activation and presence of macrophages.

Thus, we characterized macrophages for their expression of different markers in order to classify them. Iba1 is considered per se a marker of macrophage activation especially if its expression is up-regulated (Sasaki et al., 2011), because Iba1 protein is involved in the phagocytosis process and in the reorganization of actin filaments, during the morphological changes that lead from resting to active macrophages (Sasaki et al., 2001). As well as the number of Iba1 cells were more numerous in R192Q KI trigeminal ganglion, even Iba1 intensity and mRNA was up-regulated in R192Q KI trigeminal ganglia.

Other markers up-regulated in inflammatory site or at the site of injury in different chronic pain animal models are CD11b and ED1 (Scholtz et al., 2008). While CD11b is a general index of leukocyte activation, ED1 is a marker for phagocytic macrophages (Scholtz et al., 2008; David and Kroner, 2011). Interestingly, R192Q Iba1 positive cells showed also higher co-expression with these activation markers, especially with CD11b.

We evaluated a marker typically expressed in resident tissue resting macrophages: F4/80. We found a very basal expression of F4/80 in both WT and R192Q KI ganglia, in less then 20% of Iba1 positive cells in accordance with low expression levels of F4/80 in activated macrophages (Lin et al., 2005).

In summary, then, morphological and antigen-expression data suggested that, in R192Q KI ganglia,

a large majority of macrophages was in its active state.

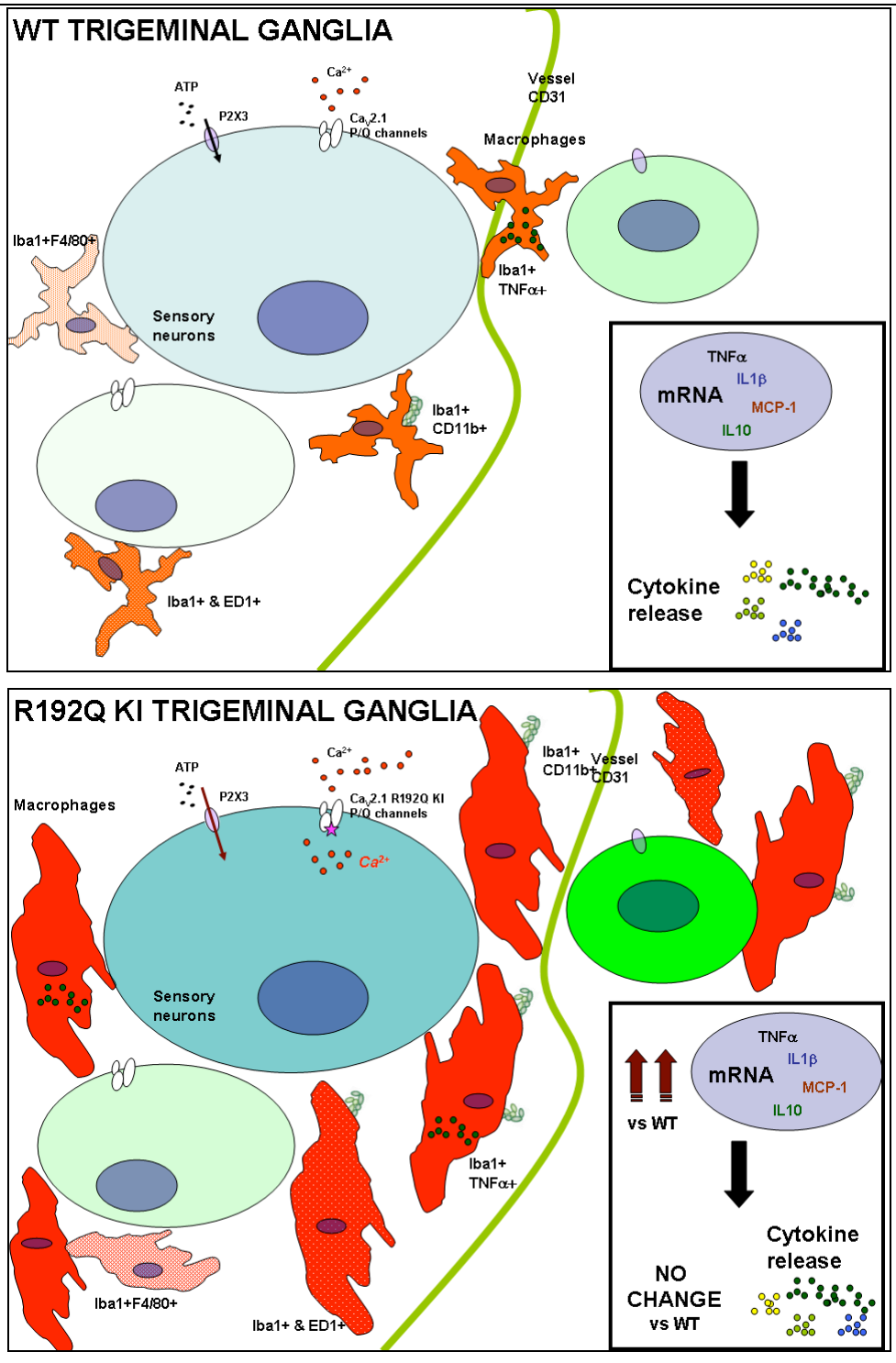


Fig. 6.1 – Schematic representation of WT and R192Q KI trigeminal ganglia in naïve conditions

Representation of trigeminal ganglion neurons and macrophages with macrophage markers. In the small box a summary of mRNA levels and release of cytokines in trigeminal ganglia

Recruitment of macrophages

Larger number of macrophages persistently found at different postnatal ages in R192Q KI ganglia (from P10 to P90) could be attributed to the R192Q mutation that confers a different environment to trigeminal ganglia causing a higher rate of macrophage proliferation, renewal or recruitment from periphery. As observed in DRGs (Jimenez-Andrade et al., 2008), also in mouse trigeminal ganglia the neuronal area is strongly vascularized. This could generate an easier recruitment of macrophages from periphery as well as the uptake of circulating inflammatory mediators from blood.

Despite the fact we found no difference in vascular supply or vascular permeability between WT and R192Q KI ganglia, additional experiments should be planned to confirm the origin of trigeminal macrophages and which classes they belong to (Mosser and Edwards, 2008; David and Kroner, 2011). Since it is known that macrophage recruitment could be enhanced by inflammatory mediators produced by active resident macrophages, we evaluated cytokines and chemokines produced by active macrophages knowing to be involved also in migraine pathophysiology (Bernardino and Malva, 2007; Uçeyler et al., 2009; Austin and Moalem-Taylot, 2010; Guillot et al., 2011). In particular, the up-regulation of MCP-1 and MMP-9 in association with an up-regulation of TNFα founded in R192Q KI trigeminal ganglia could be correlated with active macrophage recruitment (Shubayev et al., 2005).

Cytokine profile of R192Q KI ganglia

In neuroinflammatory processes, immune cell activity is associated with larger expression of chemokines and cytokines believed to be one of the key contributors to chronic pain (Scholz and Woolf, 2007; Donnelly and Popovich, 2008; David and Kroner, 2011; Guillot et al., 2011). In particular, during inflammatory conditions, glial cells and resident tissue macrophages, once activated, can release pro-inflammatory cytokines such as TNFα and IL1β, promoting the onset of inflammation and further activation of neuronal and non-neuronal cells (Haddad, 2002; Hanish, 2002; Bernardino and Malva, 2007; Donnelly and Popovich, 2008; Austin and Moalem-Taylor, 2010). The chronic phase of inflammation is sustained by both pro-inflammatory cytokines (including IL6, active in this phase) and chemokines, recruiting immune cells at the inflammation site (Bernardino and Malva, 2007; Donnelly and Popovich, 2008; Austin and Moalem-Taylor, 2010).

We evaluated the pro-inflammatory cytokines profile in intact trigeminal ganglia from WT and R192Q KI mice with Real Time PCR experiments and with an ELISA-based platform that allow to measure multiple cytokines in the same sample. Interestingly, both IL4 and IL12p70 cytokines, responsible for B and T cells activation, were rarely detectable in the trigeminal ganglia, while we

found discrete levels of pro/anti-inflammatory cytokines known to be involve in the signaling pathways between primary sensory neurons, Schwann cells and immune cells (Scholz and Woolf, 2007; David and Kroner, 2011; Guillot et al., 2011).

Considering mRNA expression levels of intact naïve ganglia, we noticed up-regulation of different pro-inflammatory cytokines (such as TNFα, IL1β and IL6) in R192Q KI ganglia with respect to WT, without, however, higher protein levels. It has been shown that maturation and effective cytokine release in inflammation are the result of a complex integration of multiple signaling, suggesting the primed state of KI ganglia, not associated with an active protein expression and an ongoing inflammation.

Interestingly, lower IL10 cytokine levels in R192Q KI trigeminal ganglia, were associated to an increased IL10 mRNA expression. It is known that IL10 shows this profile in chronic pain models, since IL10 protein levels decrease in the injured nerve within 1 day with a prolonged recovery period, in spite of increased gene expression or increased turnover (Austin and Moalem-Taylor, 2010).

Surprisingly, we observed a down-regulation in Interferon γ (IFN-γ) expression in R192Q KI ganglia. Hanisch and Kettenmann (2007) described the role of IFN-γ as pro-inflammatory mediator in association with IL4, and, in particular, IFN-γ showed beneficial effect only when it is present at low concentration at the site of inflammation/injury.

Globally, the discrepancy between mRNA and protein expression of different cytokines showed us a complex scenario in KI trigeminal ganglia that possibly involve new and different type of cytokine-expressing cells than in WT. Our hypothesis is that R192Q KI mice showed a pro-inflammatory profile not actually transduced in ongoing inflammation, a fact that require multiple stimuli. Hence, our results suggest that R192Q KI mutation determines a new background molecular state, different than the one found in WT, that could be readily convert into an active neuroinflammatory condition under specific migraine triggers.

Acute inflammatory stimuli strongly enhanced TNFα protein and mRNA production and further macrophage activation

LPS-stimulated mice showed, as expected (Qin et al., 2007), a higher number of Iba1-immunoreactive cells in WT as well as in R192Q KI trigeminal ganglia. Iba1 positive cells in trigeminal ganglia from WT and R192Q KI mice in control condition or after LPS stimulation also showed different morphology (ameboid and larger) suggesting highly reactivity of these cells under inflammation stimuli (Kettenmann et al., 2011).

LPS evoked a standard M1 macrophage activation (Austin and Moalem-Taylor, 2010; David and

Kroner, 2011) in both WT and R192Q KI mice, inducing the release in ganglia of IL1β, IL6, IFN-γ and IL10.

In particular, it is known that TNFα contributes to peripheral sensitization of nociceptor neurons, it is a gene associated to migraine susceptibility (Li et al., 2004; Shaible et al., 2010; Balkowiec-Iskra et al., 2011) and increased concentrations of TNFα have been reported in the jugular blood of patients 2 h after the onset a migraine attack (Sarchielli et al., 2006; Wessmann et al., 2007; Yilmaz et al., 2010).

Thus, we explored the TNFα expression in WT and R192Q KI mice after LPS stimulation (5mg/kg). In addition to the actual rise in Iba1 expression after LPS injection, in KI ganglia a substantially larger number of such cells was observed to co-express TNFα, alluding to hyper-reactivity of R192Q KI ganglia to inflammatory stimuli. This notion, however, does not rule out the contribution of other non-neuronal cells to synthesis and release of TNFα.

Interestingly, while TNFα was doubled in WT after LPS injection, in R192Q KI, TNFα increased 5-times. A different profile between WT and R192Q KI was also observed in mRNA levels: while WT mice expressed a "classical" TNFα mRNA profile (higher levels, 50-times more than saline in the first hours and later decay) as described in other model of inflammation, R192Q KI trigeminal ganglia showed a slower but stronger (150-times more than saline after 5 h of LPS) TNFα mRNA enhancement after LPS.

We suppose that, at ganglion level, the strong rise in TNFα synthesis and production by KI ganglia after LPS injection might become a contributor to the cross-talk between neurons and glia which is proposed to be an important factor of chronic pain.

Future studies are necessary to fully understand the molecular mechanisms that may induce TNFα neosynthesis and translate it into nociceptor sensitisation of trigeminal sensory neurons observed in the R192Q KI mouse.

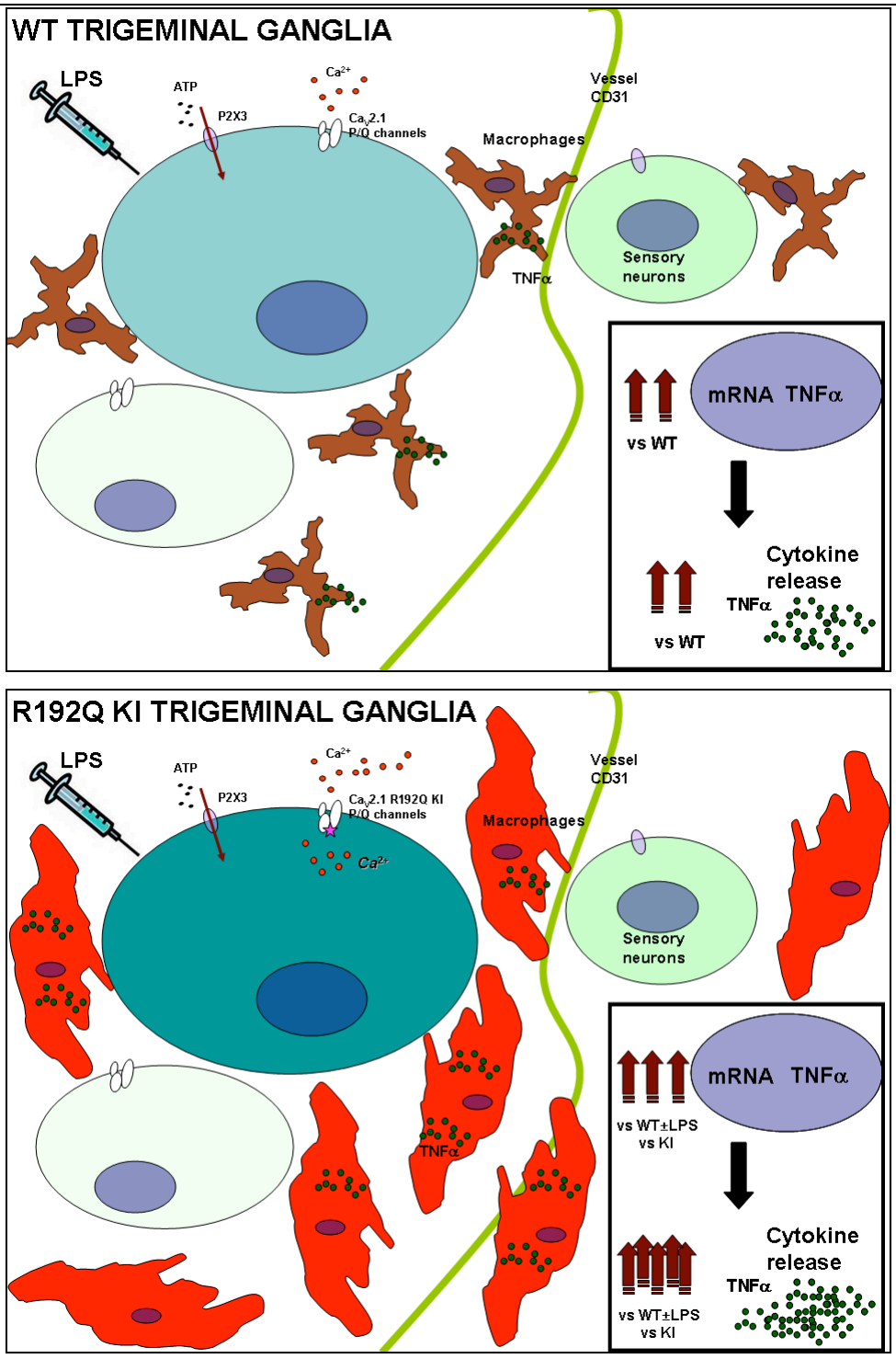


Fig. 6.2 – Schematic representation of WT and R192Q KI trigeminal ganglia after LPS application

Representation of trigeminal ganglion neurons and macrophages. In the small box a summary of mRNA levels and release of cytokines in trigeminal ganglia.

KI trigeminal cultures retain a background neuroinflammatory profile

To clarify the potential interaction between sensory neurons and macrophages, we resorted to primary cultures of trigeminal ganglia as these are an advantageous model to investigate the molecular mechanisms underlying nociceptor sensitisation important to migraine pain (Simonetti et al., 2006; Nair et al., 2010).

In trigeminal ganglion cultures, the proportion of Iba1 positive cells over total number of cells in culture was similar to the one found in intact trigeminal ganglion (around 4%), suggesting that the mixed culture was providing a sufficient background for the culture of Iba1 positive cells *in vitro*. The difference in number and morphology of macrophages found in WT and R192Q KI intact trigeminal ganglia was observed also in cultures. Cultures preserved also the CD11b and TNFα macrophage expressions, and we could also detect some TNFα expressing neurons, as already found in DRGs (Li et al, 2004; Austin and Moalem-Taylor, 2010).

To quantify the different state of macrophage activation *in vitro* (Farber et al., 2009), we performed the phagocytosis test in WT and R192Q KI trigeminal ganglion cultures. Compared to WT, R192Q KI Iba1 positive cell had different state of activation: higher number of granules uptake and higher phagocytosis index.

These data confirmed that, despite the intrinsic injury provoked by the dissociation, WT and R192Q KI trigeminal ganglion cultures preserved the different phenotypes found in intact ganglia. Using this model, we tested the functional activity of sensory neurons and macrophages. In particular, as it is know from previous studies, gain-of-function of R192Q-mutated $Ca_V2.1$ channels is translated into stronger P2X3 receptor-mediated responses, proposed to be important for inducing headache (Nair et al., 2010).

Inflammatory stimuli in trigeminal ganglion cultures enhanced immune cells and neurons activities

In analogy with *in vivo* experiments, we stimulated WT and R192Q KI trigeminal cultures with LPS (0.5 μg/ml) for 5 h. LPS application did not increase the number of Iba1 positive cells in trigeminal ganglion cultures, but it enhanced their state of activation. As previously found in trigeminal ganglion tissue, LPS caused a higher TNFα release in the culture medium accompanied with a reduction of pro-TNFα proteins inside the cells. The enhanced TNFα released was more pronounced in R192Q KI cultures not only in stimulated cultures, but also in control conditions. In cultures, similarly to what found *in vivo*, a different time course of the TNFα mRNA expression was detected between WT and R192Q KI: TNFα mRNA levels in WT cultures were enhanced in

the first hours and later reduced, while in R192Q KI trigeminal ganglion cultures the TNFα mRNA peak was later and more robust.

In order to mimic an inflammation-like state in culture, we evaluated the P2X3 receptor expression and function in control and in LPS-stimulated trigeminal ganglia cultures from WT and R192Q KI mice. P2X3 receptors are expressed by a large subpopulation of trigeminal nociceptive neurons (Simonetti et al., 2006; Nair et al., 2010). Their activity, therefore, represents a useful index to evaluate the ability of trigeminal ganglia to sense noxious stimuli and transduce them to the brainstem (Simonetti et al., 2006). Although KI neurons express similar amounts of P2X3 receptors like WT ones, the R192Q KI P2X3 receptors generate larger responses when activated by the selective agonist α,β-meATP (Nair et al., 2010).

After LPS application, P2X3 protein levels did not change in both WT and R192Q KI cultures, but a significant potentiation of WT P2X3 currents were found, thus mimicking the effect of more active macrophages found in KI. As this phenomenon was absent in KI neurons after LPS application, it suggests that it was not possible to further potentiate the hyperfunctional P2X3 receptors of KI trigeminal ganglion neurons.

Data obtained from tissue and culture, in basal condition and after LPS stimulation, raised the hypothesis that R192Q mutation conferred not only different presence and activation of macrophages, but also altered the cross-talk between neurons and macrophages modulating inflammation and pain transducing signals.

Thus, we explored if that inflammatory phenotype was due by a neuronal deregulation consequence of $Ca_V2.1$ R192Q mutation and P2X3 hyperfunctionality, or if the activation of immune cells in the ganglia contributes or determines this phenomenon. To clarify these points and better explore functional cross-talk between neurons and macrophages, we enriched trigeminal culture with peritoneal macrophages.

Peritoneal macrophages when co-cultured with trigeminal ganglia were indistinguishable from endogenous trigeminal ganglion ones: they expressed canonical macrophage markers (Iba1, P2X4, P2X7). In pure peritoneal macrophage cultures, these cells had the same phagocytic activity rate of the WT trigeminal ganglion endogenous macrophages (evaluated with Zy-FITC test; Färber et al., 2009).

Neuron-to-macrophage modulation

We evaluated the phagocytic activity of endogenous macrophages from WT and R192Q KI trigeminal ganglion cultures in order to evaluate the neuronal R192Q $Ca_V2.1$ contribution on modulation of macrophage activity. We noticed enhanced activation state in R192Q KI macrophages *in vitro* than in WT cultures.

Addition of peritoneal macrophages to WT or R192Q KI trigeminal ganglion cultures enhanced the overall macrophage activity. In part this phenomenon could be addressed to a macrophage self-activation due by the increased number of macrophages in cultures. This was consistent with the literature: the recruitment of macrophages from the periphery caused a sort of second wave of inflammation with release of cytokines and chemokines (Austin and Moalem-Taylor, 2010; David and Kroner, 2011).

It is possible that in our conditions, mediators released by neurons could be the soluble factors priming macrophages. Thus, the most parsimonious interpretation of the present data is that adding macrophages to trigeminal ganglion cultures generated a microenvironment in which activation of macrophages was facilitated perhaps by soluble factors like CGRP, NGF and transmitters such as ATP (D'Arco et al., 2007; Giniatullin et al., 2008; Burnstock, 2009). While the precise identification of these substances requires further investigation, this notion is consistent with previous studies indicating that high amounts of ATP are released at the site of neuroinflammation (Inoue, 2008), thus making this substance one candidate to mediate an analogous phenomenon in trigeminal ganglia.

Functional studies of macrophages and trigeminal sensory neurons

Macrophages enhance P2X3 receptor responses of WT trigeminal sensory neurons, with no differences in P2X3 receptor expression levels. Nonetheless, this protocol significantly increased the responses of WT receptors that generated currents equivalent in amplitude to those of KI receptors. Furthermore, P2X3 receptors recovered from desensitization significantly faster, a phenomenon previously observed after application of CGRP (Fabbretti et al., 2006). Unlike CGRP, LPS did not cause a direct increase in P2X3 mRNA levels.

These data support the idea that the inflammatory background state is important for P2X3 receptor modulation and that both neuronal and immune mediators are sufficient to potentiate the WT P2X3 responses up to levels found in R192Q KI mice.

Purinergic contribution to inflammation

As mentioned above, ATP is proposed to play a crucial role in macrophage and neurons cross-talk, suggesting that other purinergic receptors, apart from P2X3 ones, should be considered in this

complex scenario. P2X4 and P2X7 receptors are expressed by macrophage/microglia cells and other immune cells, and become very important in inflammatory transducing signals (Tsuda et al., 2005; Tsuda et al., 2010; Ren and Dubner, 2011). Recent studies on these receptors revealed compensatory effects that reciprocally modulate these two (Weinhold et al., 2010; Ren and Dubner, 2011).

Preliminary experiments were conducted in order to explore P2X4 and P2X7 receptor expression. In adult mice, mRNA levels of these two receptors were up-regulate in R192Q KI trigeminal ganglia with respect to WT ones. In cultures, their mRNA or protein levels did not show significant different of expression in WT and KI. However, we observed in WT co-cultures, and not in KI ones, a significant down-regulation of mRNA and protein P2X7 receptor levels. It is known that the P2X7 receptor has been studied for its possible role in inflammatory and pain modulation, and that is substantially modulated also through alternative splicing (Donnelly-Roberts et al., 2008; Ren and Dubner, 2010; Tsuda et al., 2010; Fumagalli et al., 2011). It is also known that P2X7 receptor-activation has a role in IL1β maturation and release (Tsuda et al., 2010; Fumagalli et al., 2011). In WT and R192Q KI co-culture, the IL1β or TNFα pro-inflammatory mRNA levels were up-regulate, due by the increasing of activated macrophages.

We cannot exclude the possibility that P2X7 and P2X3 receptors could be mutually regulated. In fact, recent evidence indicates that P2X7 modulates the P2X3 receptors in DRGs, where P2X7 inhibits P2X3 receptor expression in neurons by activation of neuronal P2Y1 receptors, which prevents the development of inflammatory pain (Ren and Dubner, 2010, Chen et al., 2012). Studies on P2Y role in migraine was performed on R192Q KI trigeminal ganglia (Ceruti et al., 2011) proposing an important role of satellite cell P2Y receptors.

In WT co-cultures the enhanced function of P2X3 receptor was accompanied by lower expression of P2X7 receptor protein, indicating a reverse type of interaction unlike data reported in the literature (Ren and Dubner, 2010, Chen et al., 2012). Future studies will be necessary to clarify the mechanisms underlying this phenomenon, which might be viewed as an adaptive reaction to a system tilted towards sensitization of nociceptors.

Chapter 7: CONCLUSIONS

In summary, regardless the origin of a migraine attack, the involvement of the trigeminal vascular system is likely to be a prerequisite for sustained pain. We posit that the activation of trigeminal ganglia with specific cellular and molecular changes may play a key role in relapse and chronicity of migraine attacks. In the FHM-1 mouse model, the genetic R192Q mutation not only conferred a sensitized P2X3 receptor phenotype to neurons (Nair et al., 2010), but also induced a change in the trigeminal ganglion microenvironment that may predispose to triggering a migraine attack (Fig. 7.1).

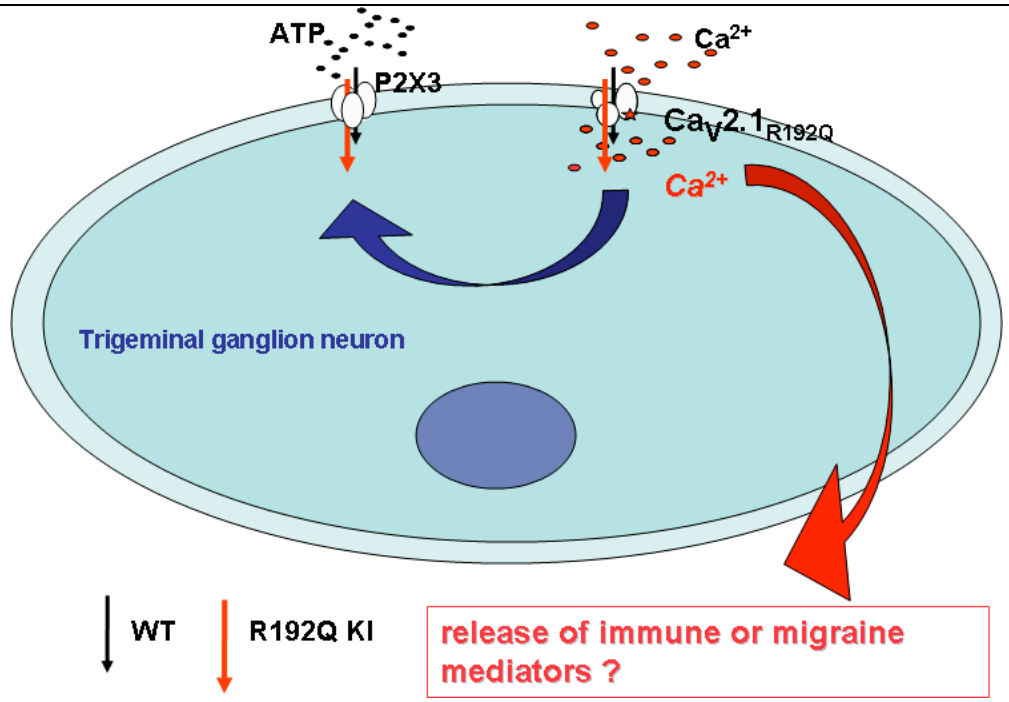


Fig. 7.1 – Cartoon of trigeminal ganglion neuron, with P2X3 and $Ca_V2.1$.
Different arrows indicating the different activities of channels in WT or R192Q KI trigeminal ganglion neurons.

Further investigation will be necessary to identify the molecular mechanisms linking the gain-of-function of mutated $Ca_V2.1$ channels in R192Q sensory neurons to the inflammatory profile of the ganglion tissue. Notwithstanding the resolution of this issue, it is tempting to speculate that the enhanced inflammatory potential of the R192Q KI phenotype, perhaps due to the increased Ca^{2+}-dependent neuronal release of neuropeptides, like CGRP and bradykinin, acting on non-neuronal cells to release soluble factors, including TNFα in particular, providing a further sustained activation of inflammatory cells.

Activated macrophages and the pro-inflammatory profile that characterize R192Q KI mice, represent an important background phenotype ready to be activated by migraine and immune

mediators, that should be taken into consideration for treatment of migraine patients.

The present i*n vitro* studies used the function of P2X3 receptors as readout to understand the immune cell population and mediators involved in migraine pain. In addition to the well established effect of peptides like NGF and CGRP (Fabbretti et al., 2006; D'Arco et al., 2007), we found that macrophages contributed via TNFα to the mechanism of potentiation of P2X3 receptor function. Inflammatory stimuli, like LPS, and activated inflammatory cells are sufficient to modulate P2X3 responses in WT cultures (Fig. 7.2). A different scenario occurs in R192Q KI ganglia. It is known that $Ca_V2.1$ R192Q gain-of function mutation in cortex is per se sufficient to stimulate larger glutamate release (Pietrobon, 2007; Pietrobon, 2010b) and that KI ganglia express higher CGRP release (Ceruti et al., 2011). We think that P2X3 receptors hyperactivation in R192Q KI neurons is due to a complex integration of intracellular (molecular) and extracellular (inflammatory) stimuli, that render them essentially insensitive to further potentiation.

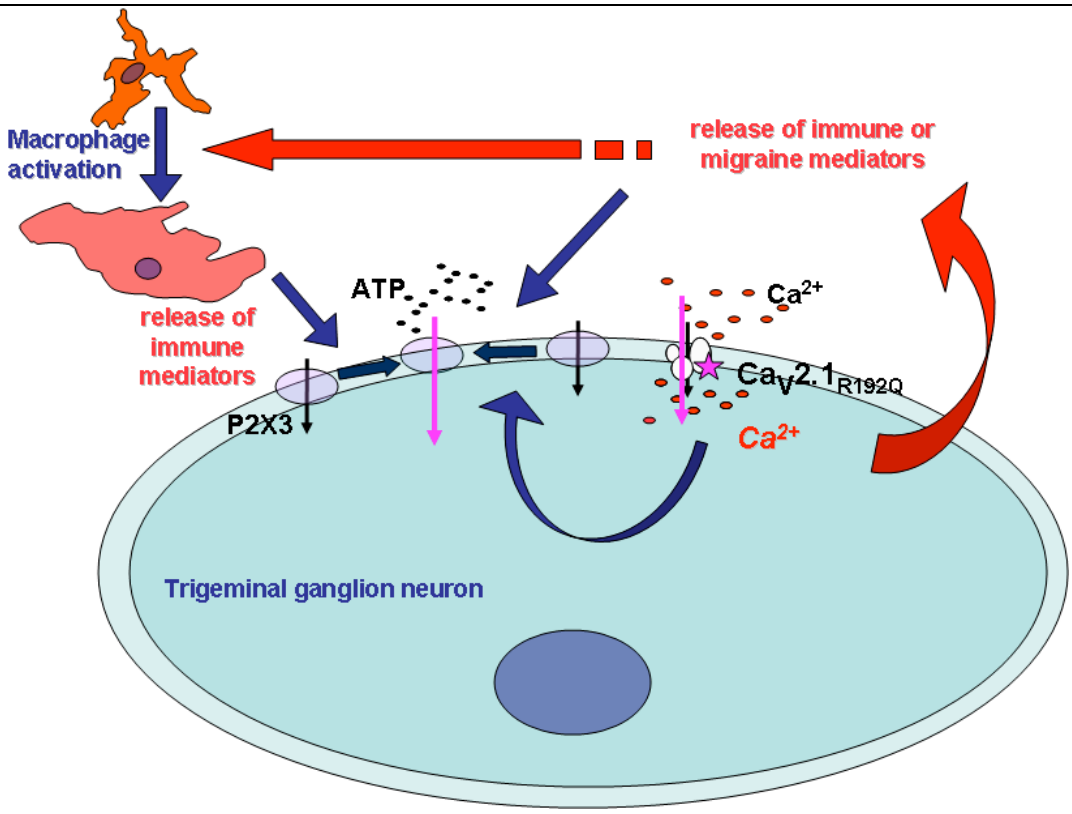


Fig. 7.2 - Mechanism involved in P2X3 fuctional activation

Our data, suggest that the individual background of effective crosstalk between macrophages and neurons might be an important contributor to the sensitization of sensory trigeminal neurons, thus predisposing to migraine susceptibility. Of course, this theory will require in vivo testing for its final validation.

Chapter 8: FUTURE PERSPECTIVES

In order to fully validate our theory, some new approaches are required.

First of all, we have to confirm the role of neuroinflammation in migraine pathophysiology in patients. Since inflammatory genes are considered to be related to migraine pathophysiology (Yilmaz et al., 2010), it would be very important to elucidate the presence of inflammatory markers in autoptic tissue from migraine patients, in particular FHM-1 ones.

Another interesting point to elucidate in the future is the identification of the gene/protein pathways involved in migraine pathophysiology in relation to inflammation. To this end, we could perform a microarray analysis on *Cacna1a* animal models in comparison to WT to explore the complete scenario of gene disregulation that R192Q mutation causes. Indeed, this study could be integrated with the characterization of DRG following nerve injury obtained with microarray experiments (Vega-Avelaira et al 2009). Interestingly, these authors have found a differential regulation of immune responses and macrophage/neuron interactions in this pain model.

The current results prompt various questions to be addressed in a future project extension:

1. The origin of active macrophages in R192Q KI ganglia? Do they come from peripheral tissues or are they due to cell proliferation *in situ*?
2. Is it possible to reverse the R192Q KI phenotype to the WT one by administration of anti-migraine drugs or NSAIDs like indomethacin.
3. The broad role of purinergic signaling in the neuroinflammatory process with particular attention to P2X7 and P2X4 receptors known to be modulated by TNFα and other cytokines (Di Virgilio and Lamaire, 2006; Inoue et al., 2006; Brone et al., 2007; Tsuda et al., 2010). The final goal of these experiments will be to obtain a full scenario of purinergic receptor and cytokine interplay in trigeminal ganglia, that could contribute to elucidate the role of sterile inflammation in migraine pathophysiology.

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E' assurdo, dice la ragione
E' quel che è, dice l'amore
E' infelicità, dice il calcolo
Non è altro che dolore, dice la paura
E' vano, dice il giudizio
E' quel che è, dice l'amore
E' ridicolo, dice l'orgoglio
E' avventato, dice la prudenza
E' impossibile, dice l'esperienza
E' quel che è, dice l'amore.
(Erich Fried)