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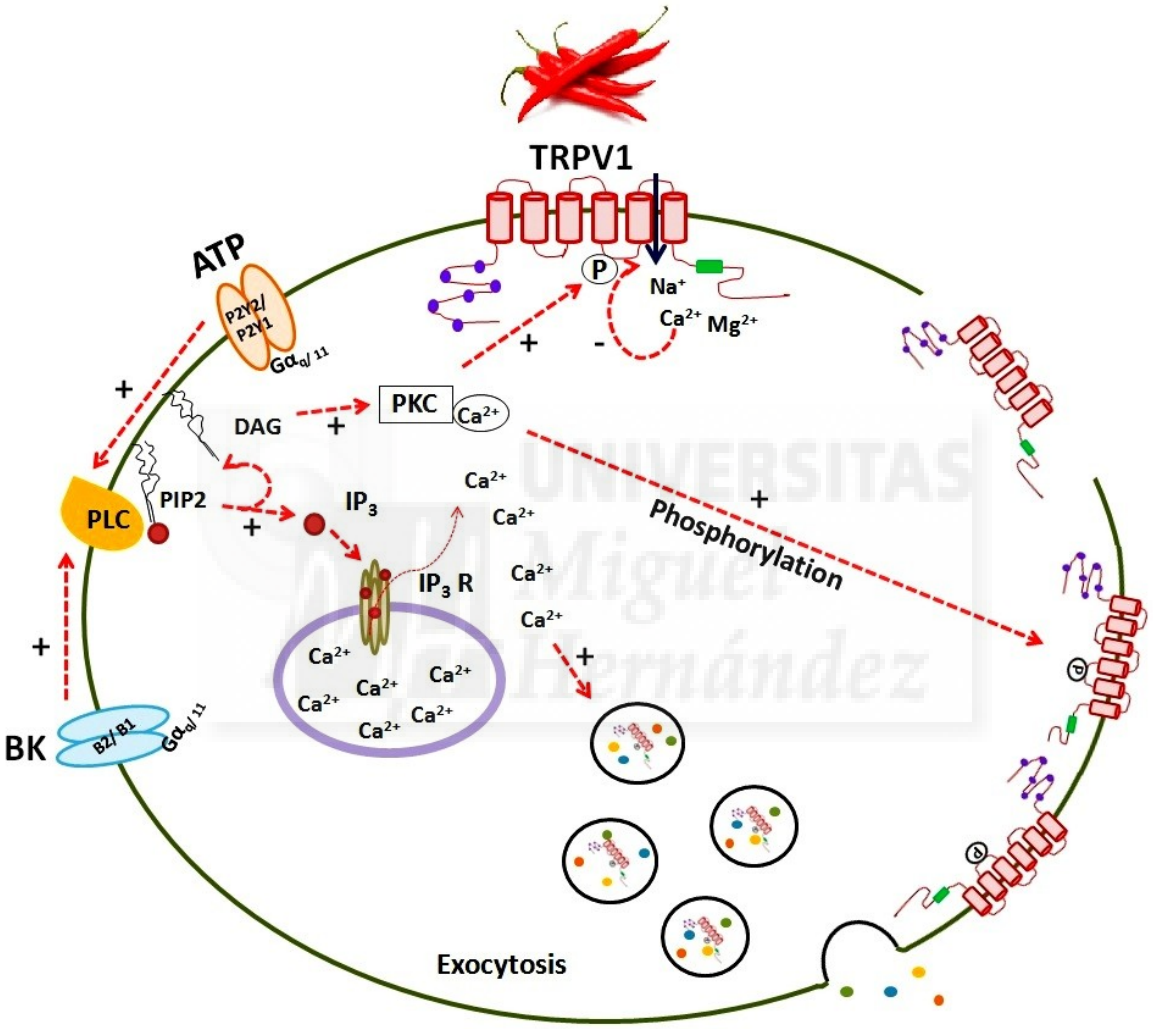
Differential mechanism of TRPV1 sensitization in peptidergic and nonpeptidergic nociceptors



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Doctoral Thesis

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Dr. Antonio Ferrer Montiel, Catedrático y Director del Instituto de Biología Molecular y Celular de la Universidad Miguel Hernández de Elche.

DA SU CONFORMIDAD a la lectura de tesis doctoral titulada: "Differential mechanism of TRPV1 sensitization in peptidergic and nonpeptidergic nociceptors", presentada por D. Sakthikumar Mathivanan

Elche, Abril 2016

Fdo: Dr. Antonio Ferrer Montiel

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Dr. Antonio Ferrer Montiel, Catedrático de la Universidad Miguel Hernández de Elche,

CERTIFICA que el trabajo de investigación que lleva por título "Differential mechanism of TRPV1 sensitization in peptidergic and nonpeptidergic nociceptors", presentado por D. Sakthikumar Mathivanan para optar al grado de Doctor, ha sido realizado bajo su dirección en el Instituto de Biología Molecular y Celular de la Universidad Miguel Hernández de Elche. Considerando que la presente tesis se halla concluida, **AUTORIZA** su presentación para que pueda ser juzgada por el tribunal correspondiente.

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El presente trabajo ha sido realizado en el Instituto de Biología Molecular y Celular (IBMC), de la Universidad Miguel Hernández de Elche.

Sakthikumar Mathivanan ha sido beneficiario de una beca predoctoral del programa Santiago Grisolia financiado por la Generalitat Valenciana, GRISOLIA/2010/058

Este trabajo se ha desarrollado gracias a la financiación procedente del Ministerio de Economía y competitividad y de la Generalitat Valenciana.

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INDEX OF CONTENT

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INDEX OF ABBREVIATIONS

2- APB	2-Aminoethoxydiphenyl borate
4- AP	4 amino pyridine
5- HT	5- Hydroxytryptamine
a.a	Aminoacids
AHP	After hyperpolarization
AITC	Allyl isothiocyanate
AKAP	A kinase anchoring protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Ano 1	Anoctamin 1
AP	Action potential
ARD	Ankyrin repeat domain
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
BIM	Bisindolylmaleimide
BK	Bradykinin
BoNT- A	Botulinum neurotoxin A
CACC	Calcium activated chloride channel
$CaCl_2$	Calcium chloride
CaM	Calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CCL2	Chemokine ligand
CDK 5	Cyclin dependent kinase 5

CFA	Complete freund's adjuvant
CGRP	Calcitonin gene related peptide
Cps	Capsaicin
CPZ	Capsazepine
CsA- CyP	Cyclosporin A- Cyclophilin D
CSP	Constitutive secretory pathway
CT	Current threshold
DAG	Diacylglycerol
DKO	Double knockout
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglion
EC_{50}	Effective concentration
Erk	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GABA	Gamma-Aminobutyric acid
GABARAP	Gamma-aminobutyric acid type A (GABAA) receptor-associated protein
GDNF	Glial cell line derived neurotrophic factor
GPCR	G protein coupled receptor
GTP	Guanosine-5'-triphosphate
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HCN	Hyperpolarization-activated cyclic nucleotide-gated (HCN) channel

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV/ AIDS	Human immunodeficiency virus infection and acquired immune deficiency syndrome
HPETE	Hydroperoxyeicosatetraenoic acid
IASP	International association for the study of pain
IB4	Isolectin B4
IGF- 1	Insulin growth factor 1
IL- 1β	Interleukin- 1β
IP_3	Inositol triphosphate
J (pA/pF)	Current density
KCl	Potassium chloride
KIF13B	Kinesin family member 13B
KOH	Potassium hydroxide
LDCV	Large dense core vesicle
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MEA	Micro electrode array
MES	2-(*N*-morpholino) ethanesulfonic acid
$MgCl_2$	Magnesium chloride
mGluR	Metabotropic glutamate receptor
ml	Milliliter
mM	Millimolar
Mrg	Mas related g protein coupled receptor

ms	Millisecond
mV	Millivolt
MYCBP2	Myc-binding protein 2
NaCl	Sodium chloride
NADA	N-arachidonoyl-dopamine
NaOH	Sodium hydroxide
ng	Nano gram
NGF	Nerve growth factor
NGS	Normal goat serum
NK	Neurokinin
nM	Nano molar
NMDA	N-methyl-D-aspartate receptor
OLDA	N-oleoyldopamine
P2	Purinergic receptor family
pA	picoampere
PAR2	Protease-activated receptor 2
PBS	Phosphate buffered saline
pF	picoFarad
PGE2	Prostaglandin E2
PGI_2	Prostaglandin I2
PI3 kinase	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PKA	Protein kinase A

PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
RMP	Resting membrane potential
RSP	Regulated secretory pathway
RT	Room temperature
RTX	Resiniferatoxin
SAP	Saporin
SEM	Standard error mean
siRNA	small interference ribonucleic acid
SNAP 25	Synaptosomal associated protein - 25
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SP	Substance P
Syt IX	Synaptotagmin IX
TG	Trigeminal ganglion
TGF- β	Transforming growth factor β
TM	Transmembrane
TNF- α	Tumor necrosis factor- α
TP	Threshold potential
TrkA	Receptor tyrosine kinase A
TRPA	Transient receptor potential ankyrin
TRPC	Transient receptor potential canonical
TRPM	Transient receptor potential melastatin

TRPML	Transient receptor potential mucolipin
TRPP	Transient receptor potential polycystic
TRPV	Transient receptor potential vanilloid
TTX	Tetrodotoxin
w/v	weight/ volume
µm	Micrometer
µM	Micromolar
µV	Microvolt

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SUMMARY

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SUMMARY

TRPV1 is a polymodal, non selective cation channel which acts as a major integrator of painful stimuli in nociceptors. During inflammation, the release of inflammatory mediators act on TRPV1 leading to enhanced nociceptor excitability and thermal hyperalgesia. Acute inflammatory sensitization of TRPV1 involves both the modification of channel gating properties by phosphorylation and recruitment of new channels to the neuronal surface. Mobilization of TRPV1 channel to the plasma membrane by some pro- inflammatory mediators occurs through SNARE-dependent exocytosis, but the exact mechanism involved remains to be elucidated. We hypothesize that the inflammatory recruitment of channels occurs in the neuronal subpopulation which contains neuropeptides substance P (SP) and calcitonin gene related peptide (CGRP), also called peptidergic nociceptors.

Therefore, we have investigated the underlying mechanism of pro- inflammatory mediators Adenosine triphosphate (ATP) and Bradykinin (BK) induced inflammatory sensitization of TRPV1 in cultured nociceptors containing both peptidergic and nonpeptidergic subpopulations. We have performed functional analysis using patch clamp electrophysiology and micro electrode array (MEA) technique. We found that the inhibition of neuronal exocytosis results in decreased inflammatory sensitization of TRPV1 induced by both ATP and Bradykinin in peptidergic nociceptors where membrane recruitment of the channel is essential. In addition, knocking out of αCGRP leads to the reduction of inflammatory sensitization of TRPV1.

Hence, this study reveals that both ATP and Bradykinin induces regulated exocytosis of TRPV1 in peptidergic nociceptors where αCGRP plays a significant role. Furthermore, our result validates the therapeutic potential of DD04107 on lessening inflammatory pain through modulation of regulated exocytosis of TRPV1.

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RESUMEN

El canal TRPV1 es un receptor polimodal, no selectivo a cationes, el cual actúa como principal integrador del estímulo doloroso en nociceptores. Durante la inflamación, los mediadores inflamatorios liberados actúan sobre TRPV1 provocando una excitabilidad aumentada del nociceptor y una hiperalgesia térmica. La sensibilización inflamatoria aguda de TRPV1 conlleva tanto la modificación de las propiedades de apertura del canal ("gating") por fosforilación como al reclutamiento de nuevos canales a la membrana neuronal. La movilización del canal TRPV1 a la membrana plasmática provocada por algunos mediadores pro-inflamatorios tiene lugar a través de exocitosis dependiente de SNARE, aunque el mecanismo exacto no ha sido todavía esclarecido.

Con el fin de elucidar dicho mecanismo de exocitosis, nos planteamos la hipótesis de que el reclutamiento inflamatorio del canal TRPV1 ocurría únicamente en la subpoblación neuronal que contiene los neuropéptidos sustancia P (SP) y el péptido relacionado con el gen calcitonina (CGRP), también denominada nociceptores peptidérgicos. Así pues, se ha investigado la sensibilización inflamatoria del canal TRPV1 inducida por los agentes pro-inflamatorios Trifosfato de adenosina (ATP) y Bradiquinina (BK) en cultivos de nociceptores tanto peptidérgicos como no peptidérgicos. Para ello, se ha llevado a cabo el análisis funcional del canal TRPV1 empleando técnicas de electrofisiología patch clamp y MEA (matriz de microelectrodos). Nuestros resultados muestran que la inhibición de la exocitosis neuronal por parte del péptido DD04107 provoca una disminución en la sensibilización de TRPV1 inducida por los agentes pro- inflamatorios ATP y BK únicamente en la subpoblación peptidérgica. Además, la eliminación de la expresión de αCGRP también conduce a la reducción de la sensibilización inflamatoria de TRPV1. Así pues, este estudio revela que tanto ATP como BK inducen la exocitosis regulada de TRPV1 en nociceptores peptidérgicos donde αCGRP juega un papel significativo. Además, nuestros resultados validan el potencial terapéutico del péptido DD04107 en la disminución del dolor inflamatorio a través de la modulación de la exocitosis regulada de TRPV1.

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INTRODUCTION

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PAIN, AN INTRICATE MECHANISM

Pain is the major problem of the health community that affects up to 20 % of the global population. Pain management and treatment are largely unmet which creates enormous emotional and economical burden to the patients. Extensive studies from peripheral nociceptors towards their neural circuits in the brain have been carried out to elucidate the mechanism of pain sensation and its transition from acute to chronic state (1). Perception of pain is a multidimensional subjective experience which encompasses cognitive and emotional components (2). Physiologically, pain sensation acts as an alarming system to protect ourselves from potentially damaging stimuli which consists of adaptive (nociceptive, inflammatory pain) and maladaptive (neuropathic and functional pain) responses. Multiple mechanisms induce pain sensation which includes nociception, peripheral sensitization, phenotypic switches, central sensitization, ectopic excitability, structural reorganization and decreased inhibition. Studies conducted on human brain imaging technique have shown the prominent role of brain circuits in pain perception. Besides environmental factor playing a predominant role in pain sensation, differences in genetic factors with the existence of single nucleotide polymorphisms also plays a key role in pain perception (3).

Definition

Pain, as a multi component complex syndrome, principally consists of unpleasant sensorial, physiological, cognitive and behavioural modules, where noxious stimuli are experienced by the sensory component and transmitted causing acute or potential tissue damage to the brain (4), (5).

Classification of pain

Pain can be classified depending on its duration or pathophysiology (6). Based on the duration of pain, it can be either acute or chronic (7). **Acute pain** is the short lasting pain sensation due to tissue injury which stimulates nociceptors and usually disappears when the injury heals (< 6 months). In contrast, **chronic pain** generally begins as acute pain and persists for longer periods (> 6 months), persists beyond the expected time of healing.

According to the pathological condition, pain can be classified into: nociceptive, inflammatory and neuropathic **(figure- 1)**. **Nociceptive pain** occurs when nociceptors are activated and they are sensitive to potentially damaging noxious stimuli (heat, cold, vibration and stretch stimuli). This type of pain can be somatic or visceral or both. **Inflammatory pain** occurs upon tissue damage that releases different inflammatory mediators and will act on peripheral nociceptors, sensitizing the non noxious or mildly noxious stimuli to become painful. **Neuropathic pain** occurs due to a structural damage and nerve cell dysfunction in the peripheral or central nervous system that enhances pain sensation (8-12).

It is plausible that patients may suffer from **mixed pain**. For instance, neuropathic pain may coexist with nociceptive pain in conditions of trauma, burns and cancer (13). **Functional pain**, that is not common, probably occurs due to an abnormal responsiveness of the nervous system where neurologic defect or abnormality cannot be detected. Pain can also be associated with other diseases like HIV/ AIDS (14, 15), cancer (16-18) and sickle cell disease (19).

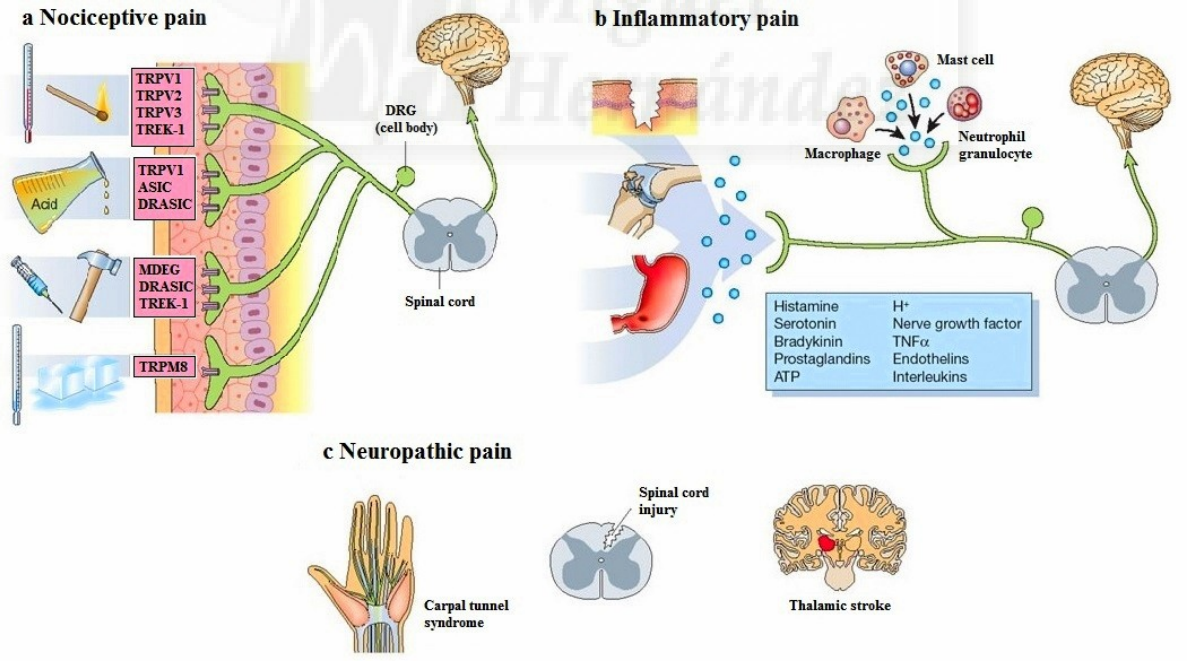

Figure- 1: **Primary types of pain**: A) Nociceptive pain, B) Inflammatory pain, C) Neuropathic pain. Modified from (6).

The sensory dysfunctions mostly observed during pain sensation are: **allodynia** where patients feel pain due to a stimulus that normally does not provoke pain (20-22) and

hyperalgesia where an increased pain response to a normal painful stimulus is observed (23-25). Frequently, allodynia and hyperalgesia occurs during inflammatory and neuropathic conditions (26, 27). In addition, paraesthesia and dysesthesia conditions are also observed as a common symptom of pain where patients feel an abnormal sensation to a stimulus that is normally not unpleasant like tingling or numbness, burning, skin crawling, or itching. These symptoms could be spontaneous or evoked (28-31). Normally, paraesthesia occurs during stroke and transient ischemic attacks (mini-strokes), multiple sclerosis, transverse myelitis and encephalitis, while dysesthesia occurs mainly due to chronic pain and frightening diseased conditions.

Pain Signalling

The process of pain perception involves both peripheral and central nervous system. Peripheral pain machinery comprises **pseudounipolar sensory neurons** where one end of the afferent fibers project as free nerve endings to the skin, and the other end projects to different laminae of the spinal cord. Sensory neurons make connections with dorsal horn neurons of spinal cord through monosynaptic or multiple interneurons (excitatory or inhibitory) thereby facilitating pain transmission. Pain perception is processed from dorsal horn neurons to somatosensory cortex of the brain through the spinothalamic tract **(figure- 2).**

The processes involved in pain signalling are

Transduction: conversion of noxious (chemical, thermal and mechanical) stimuli into electrical activity in the peripheral terminals of nociceptive sensory fibers mediated by specific ion channels expressed in nociceptors.

Conduction: the passage of action potentials from the peripheral terminal along axons to the central terminal of nociceptor in the central nervous system (afferent) or to the peripheral terminal (efferent).

Transmission: synaptic transfer and modulation of input from one neuron to another.

Perception: transfer of sensory inflow to specific areas in cortex.

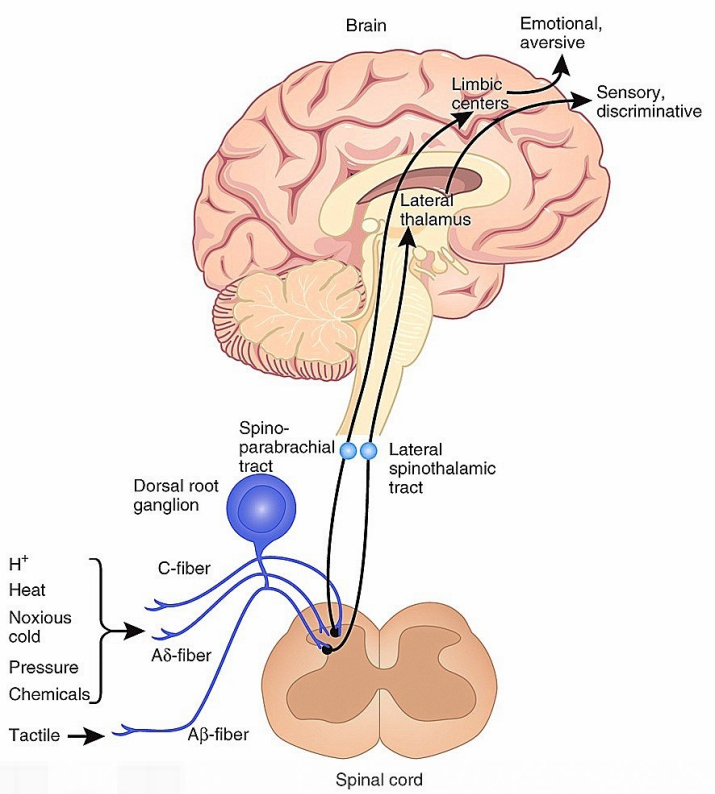

Figure- 2: Schematic view of main circuits mediating physiological pain. Pain sensing neurons in the peripheral nervous system have their soma located in the dorsal root ganglia (DRG). These neurons have a peripheral axon innervating the distal territories (skin, viscera, etc.) where they detect painful stimuli leading to an action potential that travels along the fibers up to the DRG and then to the first relay in the dorsal spinal cord. Sensory neurons within the DRGs are diverse. The sensory information is processed locally in neuronal circuitry within the dorsal horn of the spinal cord before being sent to the thalamus to convey nociceptive information. Following thalamic filtering, the information is sent to the cortical structures of the pain matrix (32).

Recognition of nociceptors

In 1903, Charles Scott Sherrington, a renowned neurophysiologist, after extensive research on neurons, stated that skin is provided with a set of free nerve endings and they can be provoked by noxious stimuli. He coined the term **nocicipient** (recipient of noxious stimuli). Later, he phrased nociceptive reaction as these free nerve endings capable of detecting noxious stimuli (thermal, mechanical and chemical) and tissue damage with the capability to provoke a reflex action, an autonomic response and pain. The apparatus responsible to perceive noxious stimulus was identified as **nociceptors** (33, 34). These nociceptors are electrically silent, transmit all or none action potentials when stimulated and belong to subpopulations of primary sensory neurons.

Primary sensory neurons, have their cell bodies located in the **trigeminal ganglia (TG)** at the base of the skull whose sensory endings covers head and face, or in the **dorsal**

root ganglia (DRGs) outside the spinal cord, with their sensory endings covering the rest of the body (35, 36). Neurons from DRGs and TGs are classified into three main types **(figure-3)**:

- Large diameter, myelinated, rapidly conducting **Aα** and **Aβ** fibers which detect innocuous stimuli from skin, muscle and joints (37).
- Medium diameter, thinly myelinated, more rapidly conducting **Aδ** fibers.
- Small diameter, unmyelinated, slowly conducting **C** fibers (35, 38).

The medium and small diameter sensory neurons are considered as nociceptors, as they respond to noxious stimuli or become responsive after inflammatory sensitization (39).

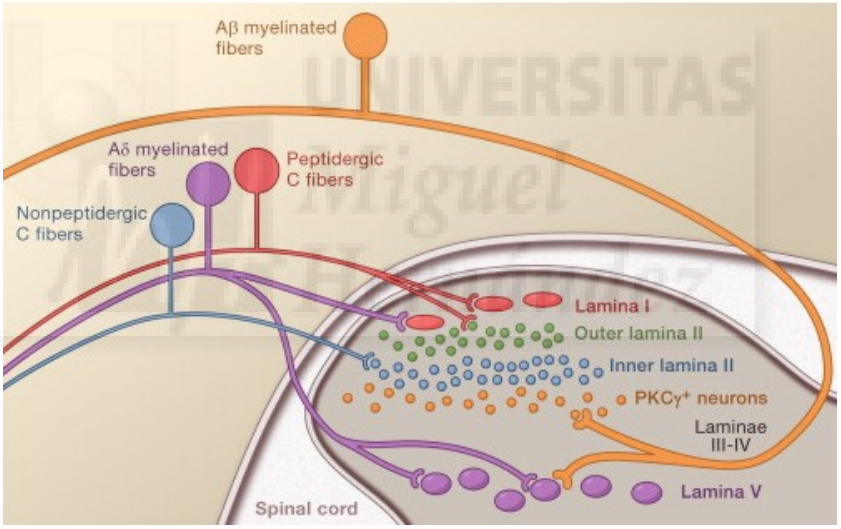

Figure- 3: **Primary Sensory Neurons and their synaptic connections in spinal cord**. The unmyelinated, peptidergic C (red) and myelinated Aδ nociceptors (purple) terminate in lamina I and outer lamina II. The unmyelinated, nonpeptidergic nociceptors (blue) terminate in the inner part of lamina II. Innocuous input carried by myelinated Aβ fibers (orange) terminates on PKCγ expressing interneurons in the ventral half of the inner lamina II. Lamina V (purple) receives convergent input from Aδ and Aβ fibers (35).

Aδ fibers carry the first pain response to injury. They consist of type I and type II fibers, both responding to mechanical stimuli. The difference between these two fibers relies on their responsiveness to intense heat where type I fiber sense temperature at 53 ^{o}C and type II fiber sense temperature at 43 ^{o}C.

Unmyelinated C fibers are widely considered as nociceptors and they are heterogeneous. They carry out the second pain responses to injury. Most of them are polymodal responding to noxious thermal, chemical and mechanical stimuli. Characterisation of C fiber nociceptors based on neuroanatomical, histochemical and molecular analysis further classifies them into two subsets based on their dependency on growth factor and the presence of neuropeptides. These are peptidergic and nonpeptidergic subpopulations (40). Genetic transneuronal tracing studies proved that peptidergic and nonpeptidergic nociceptors process nociceptive information through parallel, perhaps independent pathways (41). These unmyelinated neurons, project their axons distinctly on both peripheral and central targets.

The first subset is a **peptidergic population of C fibers** which contains the neuropeptides SP and CGRP, expresses TrkA neurotropin receptor and responds to nerve growth factor (NGF) (42, 43). Almost all small C fiber nociceptors require NGF for their survival during prenatal and postnatal life (44, 45). The peripheral axons of peptidergic nociceptors terminates at the stratum spinosum of glabrous skin, and its central axons terminates at lamina I and outerpart of lamina II of the dorsal horn of the spinal cord. The second subset is a **nonpeptidergic population of C fibers** which expresses c-Ret neurotropin receptor, is supported by glial derived neurotropic factor (GDNF), contains G protein-coupled receptors of the Mas related genes (Mrg) family (46) and can be selectively labelled with the α- D- galactosyl- binding lectin IB4 ($IB4^+$). They also express purinergic receptor subtype $P2X_3$, a specific subtype of ATP gated ion channel. During early postnatal life a subpopulation of C fiber nociceptors undergoes a shift in growth factor dependence, where neurons which does not express the TrkA receptor and bind to IB4 becomes GDNF-dependent for their survival (47). Nonpeptidergic peripheral axons terminate in superficial stratum granulosum of the epidermis and its central axons terminate in the inner lamina II of the dorsal horn of the spinal cord (48).

$IB4^-$ (peptidergic) and $IB4^+$ (nonpeptidergic) neurons are electrically distinct

In primary sensory neurons (nociceptors) different ion channels are expressed and transmit pain signals to both peripheral and central targets. Hence, they are considered as a potential target for pain pharmacotherapy. Ion channels including classical voltage gated Na^+, K^+, Ca^{2+} channels, transient receptor potential (TRP), purinergic (P2), acid- gated ion channel (ASIC) at the periphery and excitatory (Glutamergic), inhibitory (GABAergic)

neurotransmitter channels at the central connections and are primarily involved in pain signalling. Experimental pain studies have shown that few ion channels and their isoforms are involved in stimulus detection, action potential initiation, propagation in nociceptors and synaptic transmission in the dorsal horn neurons which are being marked as a therapeutic target to impede pain signalling mechanisms.

Ion channels contributing to maintain the resting membrane potential of nociceptors are 4- amino pyridine (4-AP) sensitive Voltage gated Kv channels, Kv7 (KCNQ), 2-pore leak K^+ channels (K2P), Na^+- activated K^+ channels, hyperpolarization activated cyclic nucleotide gated (HCN) channels, low voltage- activated T-type Ca^{2+} channels and Voltage- gated Na^+ channels (49). In general, changes in the membrane potential result from resting membrane potential (E_{rest}) which is due to the steady state interaction of a number of membrane conductances, mostly represented by ion channels with a small contribution by electrogenic pumps (50, 51). During tissue injury or inflammation the cell bodies of injured C fibers and surrounding non injured C fibers can become hyper excitable and generate spontaneous action potentials (AP) thereby transmitting pain signals from the periphery to the central targets (52, 53). An increase in excitability is mainly initiated through activation of Nav channels expressed in primary sensory neurons. Molecular study confirms that the DRG neurons express multiple sodium channel subtypes. Furthermore, electrophysiological studies have proved that one or more tetrodotoxin resistant (TTX-R) sodium channels can support action potential conduction in the unmyelinated C fibers. Two prominent TTX- R sodium channel subtypes Nav 1.8 and 1.9 are found to be expressed in C fibers, where Nav 1.8 is responsible for producing the majority of the current underlying the depolarization phase of action potential and is critically important for the production of multiple APs (54, 55).

Functional characterisation of IB4 binding neurons revealed that $IB4^-$ and $IB4^+$ neurons exhibit different electrical properties. *In vitro* studies found that $IB4^+$ neurons had longer duration action potentials and larger TTX resistant currents compared to $IB4^-$ neurons (56). Consistently, *in vivo* studies from rat DRGs- C fiber nociceptors revealed that $IB4^+$ compared to $IB4^-$ neurons had longer somatic action potential durations, slower conduction velocities, and more negative membrane potentials (57). Generation of multiple APs differs from $IB4^-$ to $IB4^+$ due to the slower inactivation of Nav 1.8 channel in $IB4^-$ compared to $IB4^+$ neurons (58). Negative membrane potential of $IB4^+$ neurons are primarily due to the selective expression of TREK2 channel. Knockdown experiments using siRNA for TREK2

depolarized the resting membrane potential by approximately 10 mV, suggesting that TREK2 is responsible for maintaining hyperpolarizing membrane potential in $IB4^+$ neurons (59).

Inflammatory Sensitization

Although nociceptors functions as a protective system from potentially damaging noxious stimuli, during inflammatory conditions they exhibit enhanced responses and become more sensitive to normal sensory input. Tissue injury or inflammation results in changes in the chemical environment of the nociceptors. Cells surrounding the damaged tissues mediate the release of different inflammatory mediators that act on the peripheral terminals and sensitize nociceptors, a process termed as **peripheral sensitization** (60). Like peripheral neurons, dorsal horn neurons in the spinal cord can also be sensitized upon inflammation, a process termed as **central sensitization** in which synaptic transfer from the nociceptor central terminal to the dorsal horn neurons is amplified and facilitated by an increase in membrane excitability, synaptic efficacy, or a reduced inhibition (61).

Peripheral Sensitization

Tissue injury triggers the release of inflammatory mediators from activated nociceptors and surrounding non neuronal cells like keratinocytes**,** fibroblasts, mast cells, monocytes, macrophages, neutrophils, lymphocytes, basophils, platelets, endothelial cells and satellite glial cells (62-75).

The cocktail of inflammatory soup produced during tissue injury consists of ATP, protons (75), BK (76), Histamine (77, 78), Serotonin (79), Prostaglandin E2 (PGE2) (80), glutamate, NGF (81), SP, CGRP and pro inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukins (82). These inflammatory mediators can act directly on ion channels/ receptors (activators) or modulate the function of ion channels (sensitizers) by either reducing the activation threshold or increasing the responsiveness through phosphorylation mechanism **(figure- 4)**.

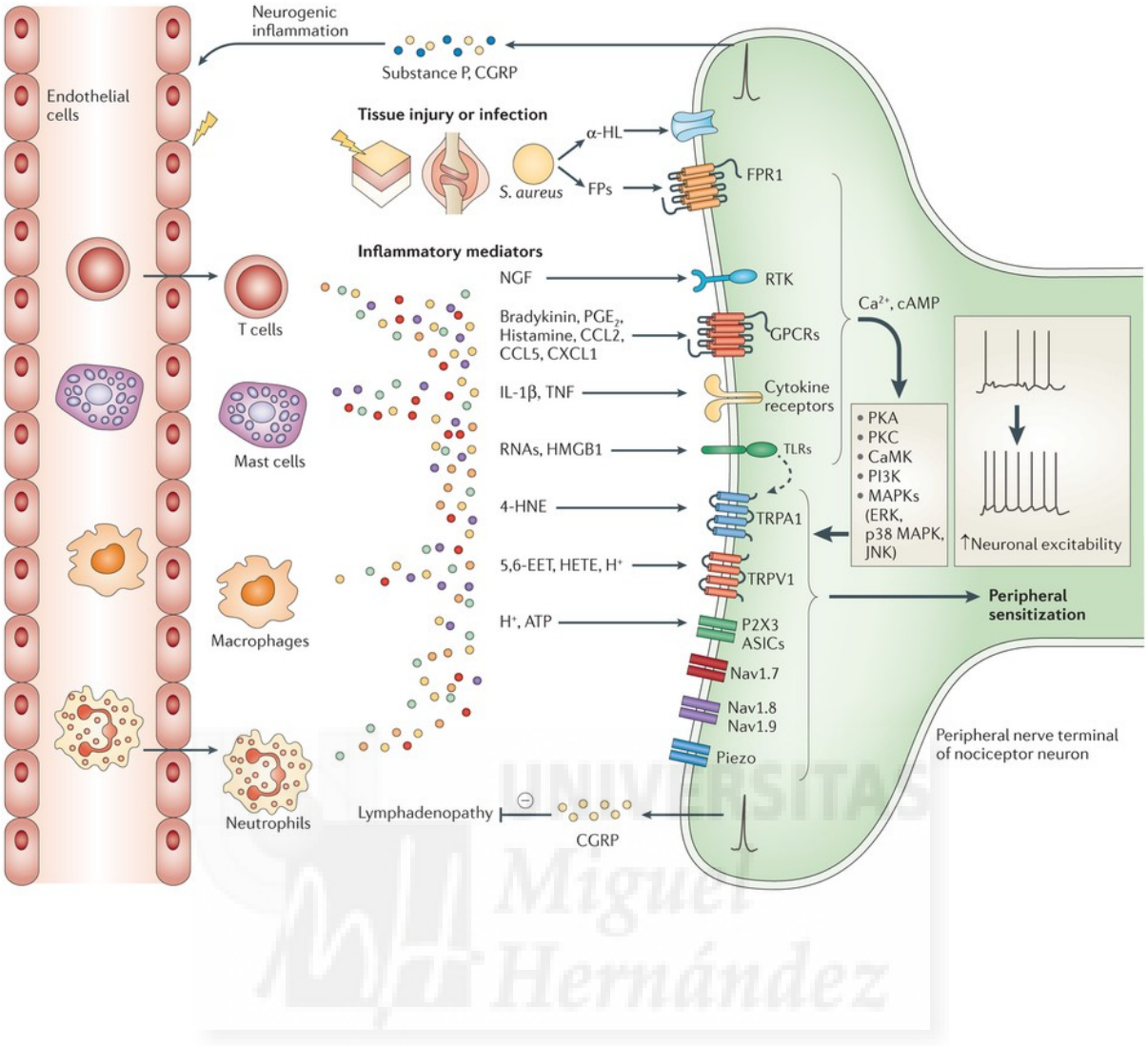

Figure- 4: **Tissue injury and infection cause inflammation via plasma extravasation and infiltration of immune cells into the damaged tissue**. The infiltrated immune cells and resident cells, including mast cells, macrophages and keratinocytes, release several inflammatory mediators, such as bradykinin, prostaglandins, H^+, ATP, NGF, pro-inflammatory cytokines (TNFα), interleukin-1β (IL-1β) and IL-6) and pro-inflammatory chemokines (CC-chemokine ligand 2 (CCL2), CXC-chemokine ligand 1 (CXCL1) and CXCL5). These released inflammatory mediators either directly activates or modulates different ion channels expressed in nociceptors, leading to enhanced neuronal excitability (peripheral sensitization). Activation of nociceptors also releases SP and CGRP, which are involved in the generation of neurogenic inflammation (60).

Central Sensitization

Sensitization of central neurons also involves phosphorylation of ion channels/ receptors through activation of intracellular kinases similar to the peripheral sensitization process (61). There are both excitatory (glutamatergic) and inhibitory (GABAergic) interneurons within the spinal cord, which influence the pain transmission **(figure- 5)**.

Glutamate is an excitatory amino acid neurotransmitter which is released from sensory afferents along with CGRP and SP in response to acute or persistent noxious stimuli. Glutamate exerts an excitatory effect on post synaptic spinal neurons acting through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, N-methyl-D-aspartate (NMDA) receptor and metabotropic glutamate receptor (mGluR) and numerous studies have been reported the role of glutamate in regulating spinal nociception process (83, 84).

ϒ- aminobutyric acid (GABA) is an inhibitory neurotransmitter released by interneurons within the spinal cord, where it controls and limits the post synaptic transmission of sensory input. GABA acts through $GABA_A$ and $GABA_B$ receptors in the spinal dorsal horn. Inhibition of GABA increases pain hypersensitivity. Furthermore, pathologic loss of inhibition (disinhibition) can also lead to increased excitability and pain (85). Studies have reported the role of $GABA_A$ and $GABA_B$ receptors on mediating inhibitory effect of GABA on different pain conditions (86-88).

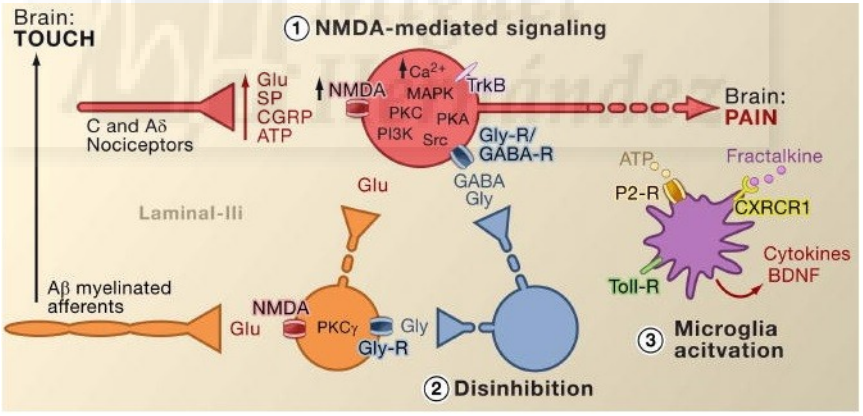

Figure- 5: Spinal cord (central) sensitization, steps involved in inflammation induced central sensitization is as follows, 1. Glutamate/NMDA receptor-mediated sensitization, where normally silent NMDA glutamate receptors will be activated leading to signalling of cascade of events that will increase the excitability of the output neuron and facilitate the transmission of pain messages to the brain. 2. Disinhibition, inflammation induces disinhibition of GABA, resulting in hyperalgesia. 3. Microglial activation. Peripheral nerve injury promotes release of ATP and the chemokine fractalkine that will stimulate microglial cells. Hence activation of microglial cells releases numerous soluble factors which promote increased excitability and enhanced pain in response to both noxious and innocuous stimulation (that is, hyperalgesia and allodynia) (35).

Ion channels involved in pain sensation

Primary peripheral ion channels expressed in DRG neurons and involved in inflammatory pain signalling are voltage gated Na^+ channels, which are responsible for the AP depolarization. Of nine different Nav, four isoforms have been extremely involved in pain sensation. They are Na_V 1.3 (89, 90), Nav 1.7 (91), Na_V 1.8 (55, 92, 93) and Na_V 1.9 (92, 94).

Potassium channels are important regulators of resting membrane potential and action potential repolarization in DRG neurons. DRG neurons expresses multiple types of K_V channels (K_V 1, 2, 3, 4, 7, 9) (95) that are involved in different pain models (96, 97). Kv channels are the key determinants of neuronal firing frequency and spike duration. Defect in the activity of Kv channels leads to hyperexcitability of the neurons. Another subfamily of potassium channels are two pore potassium channels (K2P), which contributes to maintain the hyperpolarized resting membrane potential. DRG neurons express several K2P channel subtypes (TRESK, TRAAK, TASK, TREK, and THIK) (98-100). Ca^{2+} activated K^+ (K_{Ca}) channels and Na^+ - activated K^+ (K_{Na}) channels were found as important determinants of after hyperpolarization following an action potential. K_{Ca} channels include large (BK), intermediate (IK) and small (SK) conductance channels (101, 102). The role of these channels in afferent pain signalling is yet to be explored, though few studies shows that they interact with other channels in regulating the excitability pattern (103, 104).

Na^+ and K^+ ions plays a prominent role in pain signalling mechanism; similarly Ca^{2+} ions also plays an important role in pain signalling through the activation of Cav channels. Primary afferent neurons express multiple types of Cav channels like L, T, N, R, P/ Q- types with specific sub cellular expression pattern and functions (105-107). This suggests a significant role of Cav channels in pain processing. Likewise, Ca^{2+} activated chloride channels expression in DRGs and their conductance contributes to after depolarization suggests its possible role in regulating the excitability of the afferent pain pathway (108, 109). For instance, expression of Ca^{2+} activated chloride current is altered in response to nerve injury, due to increased expression in large and medium sized DRGs. Another example is the Ca^{2+} activated Cl^- channel Ano1 (TMEM16A) known to be a key player in heat sensitivity (110).

Hyperpolarization- activated cyclic nucleotide- gated (HCN) channels are cation channels that open at negative membrane potential, contributing to neuronal excitability. All the four subtypes (HCN1, HCN2, HCN3 and HCN4) (111) are expressed in DRG neurons. The role of HCN channels in pain signalling have been explored in inflammatory and neuropathic pain models (112, 113).

Purinergic (P2X) receptor also contributes to pain signalling. P2X is a ligand gated ion channel family ($P2X_{1-7}$), activated by extracellular ATP. Tissue damage induces the release of ATP from neuronal and surrounding non neuronal cell, acts on P2X receptor specifically P2X3, and evokes pain sensation (75). P2X3 is expressed in DRG neurons (114). Numerous studies have reported the pathologic role of P2X3 on pain sensation (115-118).

Tissue acidosis is associated with inflammation leading to drop in extracellular pH. Proton plays a significant role in pain sensation where decrease in pH (<6) activates nociceptors gated through acid sensing ion channel (ASIC). ASIC are Na^+ channels belonging to degenerin/ ENaC superfamily. Different subtype expression of ASIC has been found on DRG cell bodies and sensory terminals, playing a vital role in nociception and mechanosensation (119-121) of which ASIC 3 is widely expressed in nociceptors (122, 123).

In recent years, immense studies on subfamilies of Transient Receptor Potential (TRP) channels have explored their prominent role in inflammatory pain signalling. Different inflammatory mediators sensitize or modulate the function of TRPV1 and TRPA1 in primary sensory neurons leading to inflammatory hyperalgesia. Both TRPV1 and TRPA1 are polymodal ion channels that are expressed in medium and small diameter sensory neurons. TRPV1 and TRPA1 exhibit higher selectivity for Ca^{2+} rather than Na^+ ions. TRPV1 plays a significant role in inflammatory sensitization where some inflammatory mediators directly activate TRPV1 and some inflammatory mediators modulate the gating of TRPV1 through phosphorylation mechanism or increase the number of TRPV1 channels available to be activated on the nociceptor surface membrane (124-126). Similarly, TRPA1 can also be sensitized during inflammatory conditions. Different inflammatory mediators like PAR2, TNF-α, bacterial endotoxins like lipopolysaccharide activate and sensitize TRPA1 mediated inflammatory signalling (127-130). Furthermore, inflammation is also initiated by activation of sensory neurons and the subsequent release of proinflammatory neuropeptides like SP and CGRP, defined as neurogenic inflammation.

TRP CHANNELS AS SENSORY RECEPTORS

Transient **R**eceptor **P**otential (**TRP)** channels were first identified in Drosophila photoreceptor cells by Cosens & Manning (131, 132). TRP channels are a superfamily of ion channels with 28 mammalian members classified into six related subfamilies namely, TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPA (Ankyrin), TRPP (Polycystin) and TRPML (Mucolipin) **(figure- 6)** (133-137).

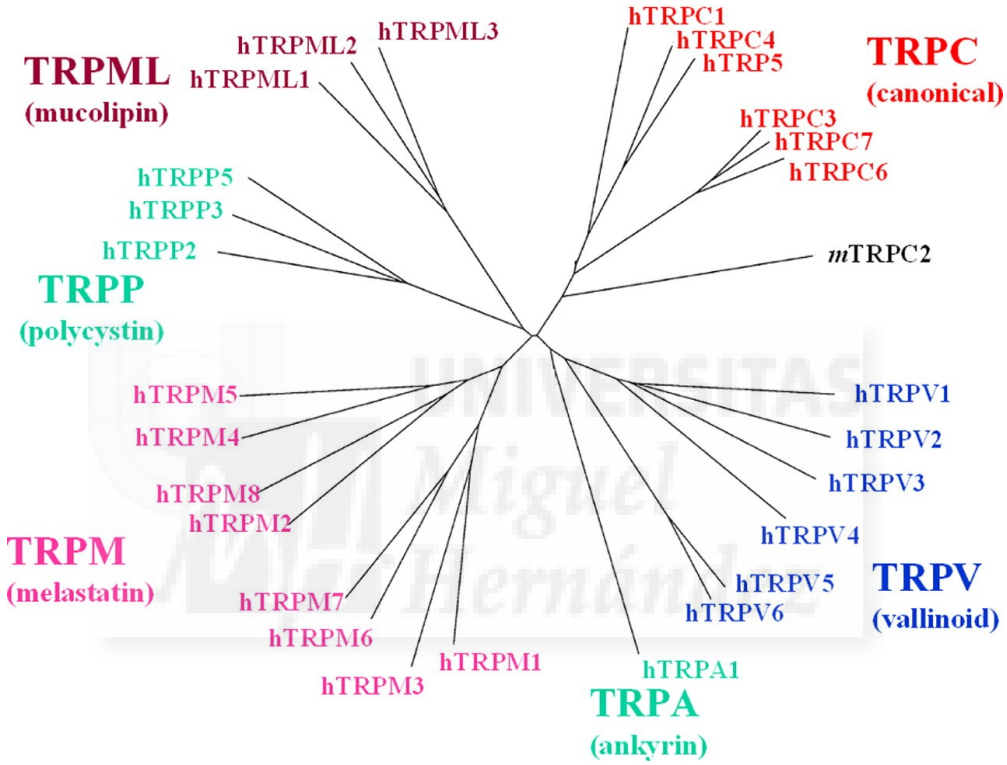

Figure- 6: Phylogenetic tree of the mammalian transient receptor potential (TRP) channel superfamily. TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucolipin) are the only identified sub families in mammals (138).

The membrane topology of TRP channels resembles to the superfamily of voltage gated channels. Mammalian TRP channels exist as homo or heterotetramers of four individual subunits containing six transmembrane segments (TMs) with the putative pore loop between TM5- TM6 for ion permeation (139-143). The amino and carboxy terminal regions are located intracellularly, containing recognised domains and motifs that are involved in differential functions of the channel. TRP channels present limited selectivity to cations, hence termed as ligand gated cationic channels (144). Most TRP channels are highly selective for Ca^{2+} and Mg^{2+} ions (145).

Ten TRP subfamilies have been reported to be expressed in primary sensory neurons. They function as noxious stimuli sensors and have significant role in pain transmission. They are TRPV1, TRPV2, TRPV3, TRPV4, TRPA1, TRPM2, TRPM3, TRPM8, TRPC1, and TRPC6 (133, 146, 147).

Differential expression of TRP channels in DRG and TG neurons

Of these TRP channels expressed in primary sensory neurons, TRPV1, TRPV4, TRPA1 and TRPM8 are expressed predominantly in C-fiber small diameter neurons **(figure-7)**, which express the nociceptive cell markers CGRP or Isolectin B4 (148-153), whereas TRPV2 and TRPC1 were found to be expressed in Aβ and Aδ fibers (154, 155). Furthermore, expression of TRPM3 in $TRPV1^{+}$ neurons suggests that TRPM3 might also express in CGRP and IB4 binding neurons (156). Besides the varied expression of individual TRP channels, extensive co-expression of TRP channels is observed in primary sensory neurons. TRPV1 has been found to be co expressed more with TRPA1 (157), also with TRPV4 (152) and TRPM3 (156). Co expression of TRPV2 with TRPV1 is limited (158) and very little co-expression of TRPM8 with TRPV1 has been observed (159).

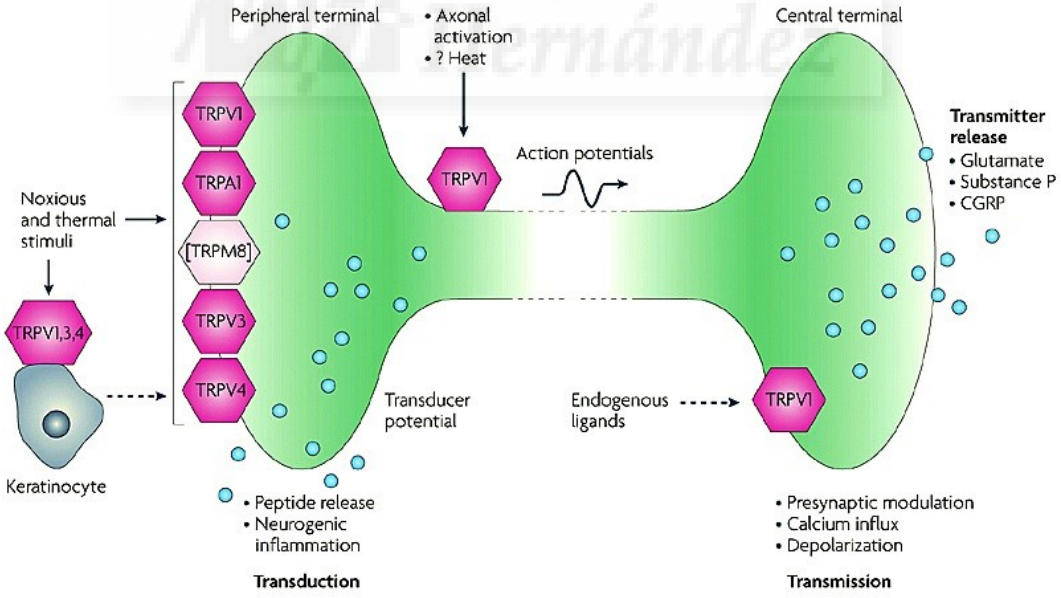

Figure- 7: Noxious and thermal stimuli act directly on the peripheral terminals of nociceptors to activate sensory fibers. Many of the transduction channels that convert thermal, mechanical or chemical stimuli into electrical activity are transient receptor potential (TRP) channels. They are TRPV1, TRPA1, TRPM8, TRPV3, TRPV4. Some TRP channels are expressed on keratinocytes and these cells may respond to noxious thermal stimuli by releasing ATP that then acts on the nociceptor (160).

Thermo TRPs

Thermo sensory channels also termed as **thermoTRPs**, define a subfamily of TRP channels that are activated by changes in the environmental temperature, from noxious cold (<15 ^{o}C) to heat (>52 ^{o}C) **(figure- 8)**. They are polymodal and act as transducers of noxious temperatures like heat and cold, mechanical and chemical stimuli. Numerous studies have reported the potential role of thermoTRP channels in nociceptive and neuropathic pain and their functional modulation by proinflammatory mediators leading to enhanced pain sensation (105, 161, 162).

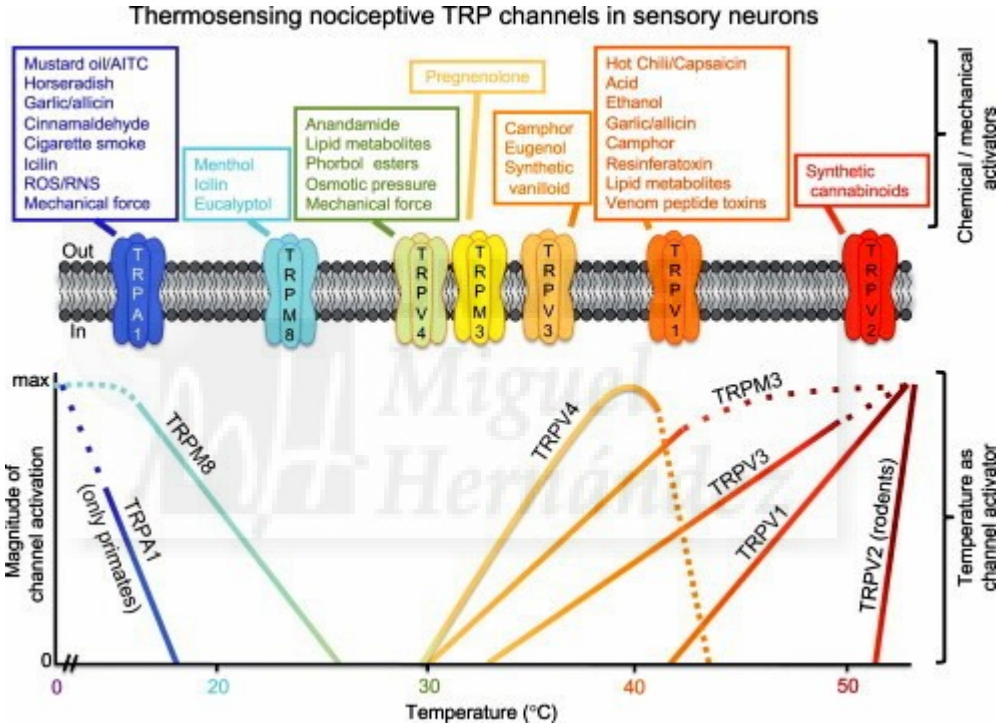

Figure- 8: Classification of thermosensing nociceptive TRP channels in mammalian sensory neurons. The upper row of individual boxes denotes chemical/ mechanical activators of marked TRP channels. The lower panel depicts the magnitude of channel activity upon activation by temperature of the independent TRP channels shown (163).

TRPA subfamily

Transient Receptor Potential Ankyrin 1 (TRPA1) is the only identified member of TRPA subfamily in mammals. TRPA1 (1119 aa), is a non selective cation permeable ion channel (Ca^{2+}, Na^{+}, K^{+}) activated by temperature less than 17 ^{o}C (164). TRPA1 can be directly activated by mustard oil (allyl isothiocynate- AITC), cinnamaldehyde, acrolein, allicin, endogenous ligand like hydrogen peroxide (H_2O_2) and indirectly by bradykinin

through phospholipase C (PLC) - Ca^{2+} pathway (157, 165-171). TRPA1 is expressed in both medium sized Aδ fibers and small sized C fibers (172). Many studies have reported the role of TRPA1 in inflammatory pain and mechanical hyperalgesia (173-177).

TRPM subfamily

Melastatin- related transient receptor potential (TRPM) cation channel family consists of eight mammalian members (TRPM1- 8) with different physiological functions (178, 179). Of these both TRPM8 and TRPM3 have a significant role in nociception.

TRPM8- Transient Receptor Potential Melastatin 8 (1104 aa) is a cold sensitive, non selective cation permeable ion channel (Ca^{2+}, Ba^{2+}, Na^{+}, K^{+}), activated by temperature range between 17 ^{o}C- 25 ^{o}C (180, 181). TRPM8 can be activated by cooling compounds like menthol, eucalyptol, illicin, camphor, WS-12 (182-186). TRPM8 is expressed in both medium sized Aδ and small sized C fibers of DRG and TG sensory neurons. TRPM8 does not coexpress with TRPV1 and TRPA1 (172, 187). TRPM8 mediates analgesia in inflammatory and neuropathic pain models (188, 189). Recently TRPM8 was discovered as neuronal osmosensor for regulating normal eye blinking in mice (190).

TRPM3- Transient Receptor Potential Melastatin 3 (1732 aa) is a recently identified heat sensitive, non selective cation permeable ion channel (Ca^{2+}, Na^{+}, K^{+}) activated by temperature >30 ^{o}C (191). TRPM3 can be activated by nifedipine, neurosteroid pregnenolone sulphate (PS), membrane depolarization, clotrimazole, CIM0216 (156, 191, 192). TRPM3 is expressed in DRG and TG sensory neurons (156). TRPM3 frequently coexpress with TRPV1 and TRPA1. TRPM3 activation leads to neuropeptide release from peptidergic subpopulations similar to TRPV1 and TRPA1 activation (192).

TRPV subfamily

Based on the structure and function, mammalian TRPV channels are subdivided into six types, TRPV1, TRPV2, TRPV3, TRPV4, TRPV5 and TRPV6 (193). TRPV1, 2, 3, and 4 are non selective cation channels activated by diverse stimuli, whereas TRPV5 and TRPV6 are selective for Ca^{2+} ions and are strictly regulated by intracellular Ca^{2+} concentration. All

TRPV channels are blocked by ruthenium red. Of these six subtypes of TRPV family TRPV1, TRPV2, TRPV3 and TRPV4 belong to a subset of thermo sensory channels or thermo- TRPs.

Structurally all TRPV channels are similar to most of the TRP channel family members, with six transmembrane segments (TM1- TM6) forming a pore loop between TM5 and TM6 region (139). The N and C termini are located in the cytoplasmic region and the channel exists as homo or heterotetramer.

TRPV1- Transient Receptor Potential Vanilloid 1 (838 aa) is a non selective cation channel activated by temperature above 43 ^{o}C (194). TRPV1 is also activated by capsaicin, protons (194) and other endovanilloids like anandamide (195), N-Arachidonoyl dopamine (NADA) (196), N- Oleoyldopamine (OLDA) (197). TRPV1 is expressed in both medium sized Aδ fibers and small sized C fibers including peptidergic and nonpeptidergic subpopulations in rats (198) whereas in adult mice TRPV1 is primarily restricted to peptidergic sub populations (199). Numerous studies have reported the role of TRPV1 in inflammatory pain (124, 200-202). TRPV1 knockout animals exhibited impaired inflammatory thermal hyperalgesia (124).

TRPV2- Transient Receptor Potential Vanilloid 2 (764 aa) is a cation channel activated by temperature above 52 ^{o}C (148) and also by mechanical stretch (203). Chemically, murine and rat but not human TRPV2 is activated by 2- amino ethoxydiphenyl borate (2-APB) (204). TRPV2 expression is concentrated in a subset of medium to large sized DRG neurons and is independent of TRPV1 expression (155, 205, 206). An up regulation of TRPV2 channel is also observed after inflammation (206, 207).

TRPV3- Transient Receptor Potential Vanilloid 3 (790 aa) is a cation channel activated by temperature >39 ^{o}C (208). TRPV3 is chemically activated by 2- amino ethoxydiphenyl borate (2- APB**)** (209), Camphor (210) and Drofenine (211). TRPV3 is found to be strongly immunoreactive in large diameter DRG neurons (208). An upregulation of TRPV3 channel is observed after traumatic and diabetic neuropathy (212).

TRPV4- Transient Receptor Potential Vanilloid 4 (871 aa) is a cation channel activated by temperature with moderate heat of around 24 ^{o}C, cell swelling (213-215) and

mechanical stimuli (216). Chemically TRPV4 can be activated by 4α-phorbol 12, 13-didecanoate (4α- PDD) (217, 218), anandamide and arachadonic acid (217). TRPV4 channel is expressed in large diameter sensory neurons (149). Numerous studies have found that the expression of TRPV4 is upregulated after inflammation or nerve injury (147, 152, 219-222).

TRPV1 AND PAIN

The pungent compound capsaicin provokes intense burning sensation and pain when applied on the skin. Furthermore, studies on primary sensory neurons found that exposure to capsaicin induced an inward current leading to depolarization of the nociceptor membrane suggesting its possible role in pain (223-225). Later, capsaicin has been recognized as a sole activator of TRPV1, a primary nocisensor in nociceptors. Since then many studies were performed to understand the role of TRPV1 and its molecular mechanism in pain sensation. During inflammatory conditions, the released inflammatory mediators at the injured site sensitize TRPV1 through a reduction in the temperature and protons activation thresholds, leading to thermal hyperalgesia. Experimental evidences showed that the mice lacking TRPV1 ($TRPV1^{-/-}$) exhibited impaired thermal nociception and inflammatory hyperalgesia (124, 126, 226, 227). Furthermore, transgenic mice studies showed that knockdown of TRPV1 using short hairpin RNA displayed lack of capsaicin induced nocifensive behaviour and reduced sensitivity to noxious heat similar to TRPV1 knockout mice (228).

In order to treat TRPV1 mediated pain, in earlier day's capsaicin was used due to its ability to desensitize TRPV1. The mechanism of desensitization involves both channel desensitization and cellular toxicity due to prolonged calcium influx. Capsaicin was also proved to be effective for treating pain caused by osteoarthritis, rheumatoid arthritis and peripheral neuropathy such as diabetic neuropathy. But the wide spread of this treatment has been reduced due to its higher burning sensation and erythema. This led to the identification of small molecules either agonists or antagonists of TRPV1 to treat different pathophysiological pain conditions like inflammatory pain, migraine, osteoarthritis pain, dental pain, HIV neuropathy associated pain, cluster headache, neuropathic pain and bone cancer pain (229-235). Many TRPV1 target drugs are currently in clinical phase.

TRPV1 – A POTENTIAL NOXIOUS SENSOR

Transient Receptor Potential Vanilloid type 1, the founding member of TRP channels, is a polymodal thermoTRP channel, non selective for cations (Ca^{2+}, Na^{+}, Mg^{2+}) (194, 236). TRPV1 is considered as a major integrator of nociceptive signals in primary sensory neurons. The channel was first identified as a capsaicin and heat sensitive ion channel in nociceptive sensory neurons (76, 237) and later confirmed by using expression cloning strategy (194). Noxious heat directly gates TRPV1 channel at ~43 ^{o}C (238). Capsaicin, a pungent chemical found in chilli peppers solely activates TRPV1 of TRP family through an intramembrane binding site (239, 240). Other exogenous stimuli include protons (241-243), resiniferotoxin (244), retinoids (245), lysophosphatidic acid (LPA) (246) and also endogenous lipophilic vanilloid ligands such as Anandamide (195), 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (247), 15- HPETE, N- arachidonoyldopamine (NADA), N-oleoyldopamine (OLDA), Diacylglycerol (248), Allicin (168, 249) can activate TRPV1. Furthermore, TRPV1 can also be activated by strong depolarization, where application of depolarizing voltages at room temperature produces an outward current. TRPV1 voltage sensitivity can be functionally modulated by increasing the temperature or the concentration of vanilloid which produce leftward shift of voltage dependence (250).

Structural determinants of TRPV1

TRPV1 is a membrane protein of 838 aminoacids. TRPV1 was believed to be structurally similar to voltage gated K^{+} channels, since it displays six transmembrane domains, with a pore loop between TM5- TM6 and intracellular N and C termini. Later, structure of TRPV1 ion channel was studied by using electron cryo- microscopy. This substantiated that TRPV1 contains repeated ankyrin domains (ARD) at the cytoplasmic N terminal followed by six transmembrane segments with a pore loop between TM5 and TM6 and a TRP domain (23- 25 amino acid) which is found in many TRP family members followed by a cytoplasmic C terminal domain (251) **(figure- 9)**.

Ankyrin repeat domains (ARD). The N terminus contains six repeated ankyrin domains (TRPV1- ARD) which are responsible for many protein- protein interactions and also participate in the formation of tetrameric channel. The N terminal domain is also an important region which determines the desensitization of the channel, containing competitive

binding sites for ATP (which prevents desensitization) and Calmodulin (CaM) (induces desensitization) (252). Basal TRPV1 ARD is ATP bound, upon activation of TRPV1, Ca^{2+} and Mg^{2+} enters the cell, where Mg^{2+} chelates ATP, prompting ARD accessible for Ca^{2+}-CaM.

CaM binding sites. Calmodulin (CaM) directly binds to TRPV1 upon activation by Ca^{2+} (253). CaM interacts with both N- (122- 189 aa) and C- termini (767- 801 aa) of TRPV1. Primary function of Ca^{2+}/ CaM on TRPV1 activity is to decrease the open probability, thus operating as a TRPV1 channel inhibitor (253). The interaction of CaM at N terminus is Ca^{2+} dependent whereas at C terminus is Ca^{2+} independent. In addition, experiments using CaM antibody, abolished CaM mediated TRPV1 inhibition, which confirms its vital role in desensitization (252, 254).

PIP2 interaction. Phosphotidylinositol 4, 5- bisphosphate (PIP2) binds directly on C-terminus of TRPV1 and competes with CaM for binding. TRPV1 activation leads to increase in Ca^{2+} ions induces PLC activation which depletes PIP2 in the membrane making the site accessible for CaM, thereby lessening TRPV1 channel activity. Intracellular application of ATP generates depleted PIP2 and increases the maximal conductance of TRPV1 (252, 255).

Pore loop & C terminal. TRPV1 pore region is formed by the transmembrane domains TM5 and TM6 and the connecting loop. Mutations in the pore region affects both ion permeation (256) and direct channel gating (257). Activation of TRPV1 involves plasticity at the upper and lower gate of the pore. This implicates that dual gating mechanism of TRPV1 occurs upon binding of different agonists (258). The C terminus was found to be essential for TRPV1 functioning and plays a prominent role in tetramerization (259, 260), channel gating (261-264) and for the interaction with other proteins like tubulin (265). A complete mutational mapping of TRPV1 reveals the function of mutation at distinct sites affecting channel activity from activation to functional modulation of the channel (137).

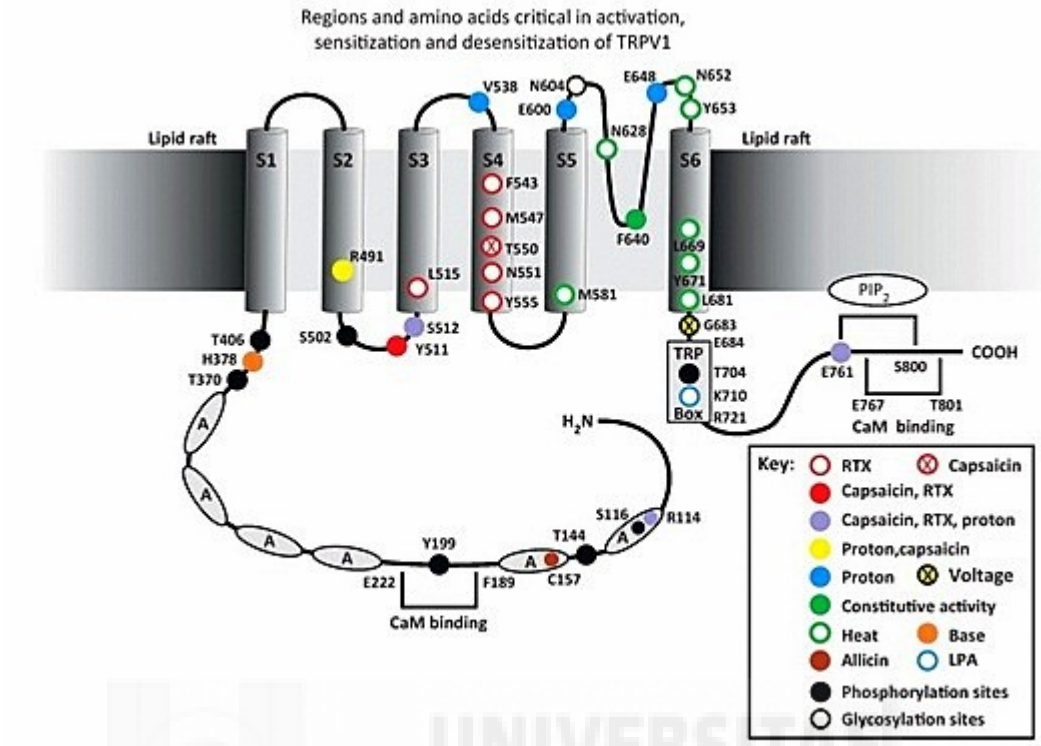

Figure- 9: **Transient receptor potential vanilloid type 1 (TRPV1) channel topology highlighting key residues and aminoacids involved in gating function evoked by different stimuli are indicated**. The TRP domain conserved in TRP channels is required for PIP_2 activation; Black circles, phosphorylation sites involved in sensitizing actions of protein kinase C (PKC) and protein kinase A (PKA) (266).

Heterotetramers of TRPV1

Although TRPV1 is predominantly expressed as homotetramer, TRPV1 can also be expressed as heterotetramers with TRPV2 (267, 268) and TRPV3 (208). Moreover, recent studies discovered the existence of a functional interaction between TRPV1 and TRPA1 heteromers (269).

MODULATION OF TRPV1 FUNCTION

TRPV1 channels suffer two major modulatory actions:

1) Agonist induced desensitization
2) Inflammatory sensitization

Agonist induced desensitization

Desensitization is a common process found in all cell surface proteins including ion channels after prolonged stimuli activation. This mechanism acts as a protective system that prevents potential excitotoxicity of the cell due to continuous activation and inhibits increased entry of Ca^{2+} ions through negative feedback mechanism.

TRPV1 response to agonists and the presence of external Ca^{2+} displays two distinct mechanism of desensitization (270, 271).

1. **Acute desensitization**: inactivation of the current during a prolonged application of capsaicin.

As mentioned above, acute desensitization is critically dependent on external Ca^{2+} and abolished by removal of Ca^{2+}. Ca^{2+} induces activation of Ca^{2+}/calmodulin- dependent serine/ threonine phosphatase 2B (Calcineurin), which dephosphorylates the channel and induces strong desensitization (271, 272). Conversely, Ca^{2+} dependent desensitization of TRPV1 channel can be reversed by activating PKA (272) and PKC (273). S116 is a potent phosphorylation site for the reversal of desensitization by PKA and this phosphorylation site is ligand independent. A similar mechanism is also observed when TRPV1 is activated by protons (pH 5) (274).

2. **Tachyphylaxis**: diminution of the maximal current amplitude during successive deliveries of the same agonist concentration.

Similar to acute desensitization, tachyphylaxis of TRPV1 current is also dependent on the presence of external Ca^{2+} (275). Tachyplylaxis is abolished when cells are exposed to ATP/ GTP containing internal solution (270). Capsaicin induced TRPV1 tachyphylaxis is also mediated by calcium activated calcineurin and it is sensitive to CsA- CyP (Cyclosporin A- Cyclophilin A), an inhibitor of calcineurin (protein phosphatase B). Moreover, pretreatment of cells with forskolin, an activator of adenylyl cyclase, reduces tachyphylaxis of TRPV1. Similar mechanism is also observed in proton induced tachyphylaxis of TRPV1 (272).

3. Desensitization through endocytosis:

Prolonged activation of TRPV1 by its agonists capsaicin and resiniferotoxin (RTX) induces desensitization through internalization of the receptor which is due to the activation of endocytosis and lysosomal degradation pathway. This process requires TRPV1 activation and calcium influx through the receptor and it is strongly modulated by PKA dependent phosphorylation, the same manner as in acute desensitization and tachyphylaxis process (276).

Inflammatory sensitization of TRPV1

Inflammatory pain is initiated by tissue damage/ inflammation. It is characterized by pain hypersensitivity at the site of damage and also at adjacent tissue. This is mainly accompanied by allodynia (stimuli that would not produce pain normally) and hyperalgesia (enhanced pain sensation to a normal painful stimulus). Sensitization of TRPV1 is one among the prominent mechanisms involved during inflammatory conditions. Most of the inflammatory mediators like ATP, BK, PG, serotonin, adenosine, acidic pH are released *in vivo* during tissue inflammation and can sensitize TRPV1 channel activity through phosphorylation mechanism and increased expression of new TRPV1 channels in the membrane through exocytosis.

1. TRPV1 phosphorylation mechanisms

TRPV1 can be phosphorylated by several kinases including PKC, PKA, Ca^{2+}/CaM-dependent kinase II (CaM kinase II), PI3 kinase and Src kinase **(figure- 10)**.

Protein kinase C (PKC). Phosphorylation of TRPV1 by PKC is triggered by the activation of metabotropic G protein coupled receptors (GPCRs) upon binding of the ligand (inflammatory mediators). This induces downstream intracellular signalling cascade by stimulating PLC which in turn converts PIP2 to IP_3 (acting on IP_3R on ER, releasing calcium from internal stores) and DAG (which activates PKC). Henceforth, putative phosphorylation sites of desensitized TRPV1 become phosphorylated and exhibit potentiated TRPV1 currents. Sensitization of TRPV1 by PKC also reduces the temperature threshold of TRPV1 activation.

Abundant experimental studies unravelled the mechanism of PKC induced potentiation of TRPV1. PKC directly modulates desensitized TRPV1 channel, by phosphorylating the putative sites S502 and S800 (273). Different inflammatory mediators induce phosphorylation of TRPV1 channel through PKC signalling like ATP (277), BK (76, 278, 279), PGI2 (80), PGE2 through EP1 receptor (80), Histamine (280), 5-Hydroxytryptamine (5 HT) through 5- HT_{2A} receptor (79), Endothelin (281), LPA (Lysophosphatidic Acid) (282), Activin (283), Macrophage inflammatory protein 1α (CCL-3) (284), Prokinectin (285) and SP (286, 287).

Protein kinase A (PKA). Phosphorylation of TRPV1 by PKA is triggered by the activation of specific GPCRs upon binding of the ligand (inflammatory mediators) which induces downstream intracellular signalling cascade by stimulating Gs adenylyl cyclase – PKA pathway.

PGE2 through EP4 receptor (80) and 5- Hydroxytryptamine (5 HT) through 5- HT_7 receptor (79) induce PKA mediated sensitization of TRPV1. PKA dependent phosphorylation potentially regulates the interaction of TRPV1 with PKA anchoring protein – AKAP 150 (rodent ortholog) and AKAP79 (human ortholog). Hence, PKA mediated thermal hypersensitivity is dependent on AKAP 79/ 150 (288, 289). Furthermore, PKA mediated phosphorylation plays a significant role in TRPV1 desensitization, where S116 putative site for desensitization becomes phosphorylated.

Sensitization by other kinases. Together with PKC and PKA, other phosphorylation mechanisms also potentiates TRPV1 currents. NGF induced activation of PI3 kinase, CAMKII, ERK and Src kinase pathways can phosphorylate TRPV1 (290-295). Chemokine ligand (CCL2) - also potentiates TRPV1 currents through PI3 kinase pathway (296).

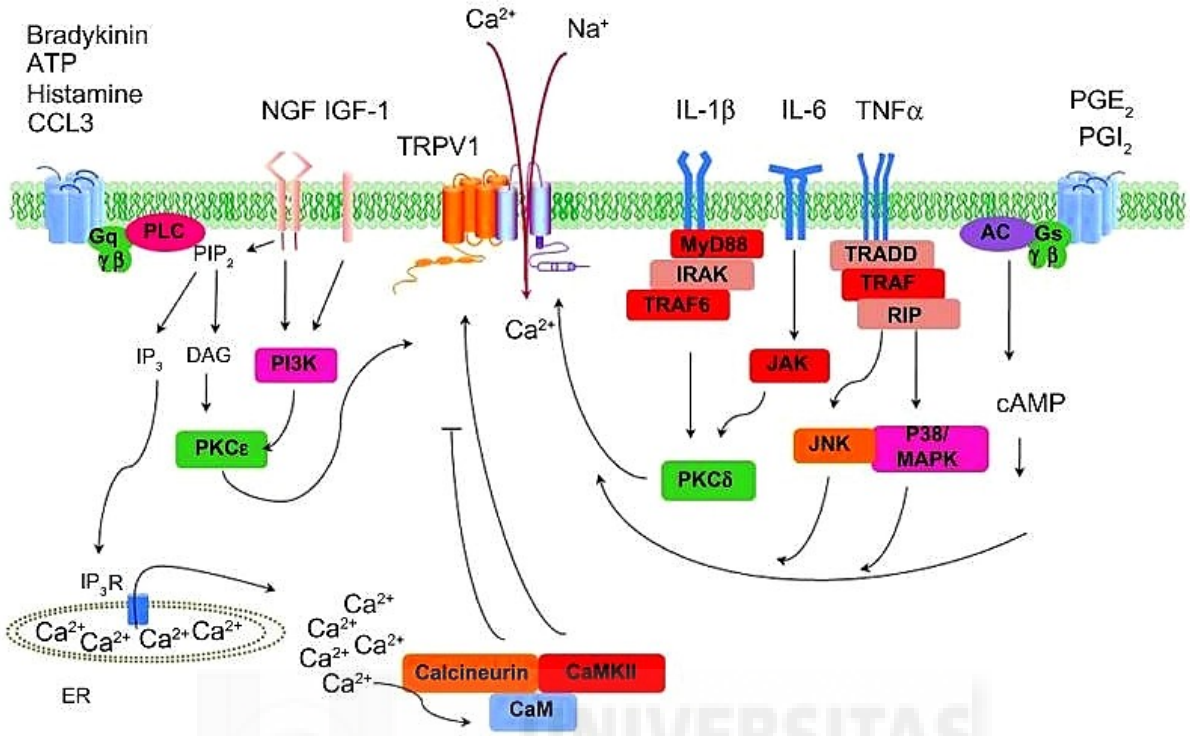

Figure- 10: Regulation of TRPV1 function and expression by proinflammatory mediators. Acute post-translation modification of transient receptor potential vanilloid 1 function. Activation of PLC C/PKC, PKA, CAMK, and other intracellular signaling cascades increase TRPV1 activity and cytosolic Ca^{2+} levels (297).

2. TRPV1 trafficking and exocytosis

Membrane proteins expression, including ion channel, is controlled by altering the number of proteins expressed at the cell surface to maintain the homeostasis. This process is mediated either by the release of newly synthesized proteins or by the activation of their degradation pathway. TRPV1 expression, similar to other proteins, is regulated by differential factors. Experimental evidences show that expression level of TRPV1 plays a vital role in pain hypersensitivity under chronic conditions (298). Increased TRPV1 levels have been observed in neuropathic pain conditions. Akin to other proteins, TRPV1 expression can be regulated by both constitutive and regulated secretory pathway. In constitutive secretory pathway (CSP), TRPV1 containing secretory vesicles are constantly transported and fused with the membrane without forming storage pool. Constitutive vesicles release is independent of Ca^{2+} and external signal. Nevertheless, constitutive vesicles are regulated by cascade of protein- protein interactions. In regulated secretory pathway (RSP), secretory materials are

accumulated in secretory vesicles as storage sites. These vesicles are arrested later and proceeds only when appropriate stimulus is applied mostly Ca^{2+} (299).

Constitutive TRPV1 expression. Structurally, TRPV1 interacts with numerous protein partners which enhances the surface expression and stability of the receptor. $GABA_A$ receptor associated protein (GABARAP) interacts with TRPV1, forming a signal complex which enhances channel trafficking and membrane expression. Furthermore, the presence of GABARAP increases the interaction of tubulin with the C terminus of TRPV1 (300). Cyclin dependent kinase 5 (CDK5), a main promoter of motor cargo association, positively regulates TRPV1 surface localization. CDK5 phosphorylates KIF13B (kinesin 3 family member 13B), a major protein involved in the intracellular transport of various cargos. KIF13B strongly interacts with TRPV1 carrying vesicles and promotes the transportation of TRPV1 to the membrane (301). In addition, Transforming growth factor- β (TGF-β) an inflammatory cytokine potentiates Cdk5 activity which in turn enhances TRPV1 activity. This corroborates the role of Cdk5 in nociceptive pain transduction (302). Studies also reported that functional interaction with TRPV1 also induces trafficking and increased membrane expression. One such example is P85β subunit of PI3 kinase which strongly interacts with TRPV1 and enhances NGF induced membrane insertion of TRPV1 channel (293-295).

As stated above, in addition to constitutive secretion, degradation pathways also regulate membrane expression of TRPV1. E3 ubiquitin ligase MYCBP2 (Myc- binding protein 2), known to be involved in receptor and ion channel internalization, specifically regulates thermal hyperalgesia through internalization of TRPV1 in primary sensory neurons. Lack of MYCBP2 activates p38 MAPK that leads to prolonged thermal hyperalgesia. Furthermore, loss of MYCBP2 also prevents capsaicin induced TRPV1 desensitization and its internalization (303). Recent studies on HeLa cells have been found that degradation of TRPV1 is also mediated by autophagy (298).

Regulated exocytosis of TRPV1. In addition to the constitutive secretion of TRPV1 protein, increased TRPV1 expression is also observed in the plasma membrane through vesicular exocytosis upon stimulation (regulated). This pathway supports rapid modulation of TRPV1 protein and its role in enhanced nociception **(figure- 11)**.

Studies of TRPV1 interaction with vesicular proteins using yeast two hybrid screening model revealed that the N- terminus of TRPV1 strongly interacts with two vesicular proteins namely Snapin and Synaptotagmin IX (Syt IX). These proteins associate with SNARE complex, a major regulator of neuronal exocytosis. Moreover, coexpression of TRPV1 with Syt IX in primary DRG cultures validates the stronger interaction of TRPV1 with vesicular proteins (304).

As mentioned before, PKC a major kinase, activated during inflammatory conditions potentiates TRPV1 currents. Application of BoNT A, a blocker of regulated exocytosis, potently abolishes the sensitization of TRPV1 current by PKC. This suggests an increased release of new TRPV1 channels from the vesicles to the membrane through SNARE mediated exocytosis. In trigeminal ganglion neurons, application of BoNT A abolishes the induced release of CGRP by both inflammatory mediators and depolarization (305). Numerous studies have reported the anti nociceptive action of BoNT A to treat different pain conditions (306-309). Despite, its high potential therapeutic usage, BoNT A produces higher neurotoxicity. Studies have been performed to identify and synthesize peptides to inhibit SNARE complex formation thereby controlling Ca^{2+} induced exocytotic release. Extensive studies on SNAP 25, a major protein involved in SNARE complex formation, revealed that a synthetic peptide patterned after the N terminus of SNAP 25 protein is a potent inhibitor of SNARE complex formation. This peptide inhibits Ca^{2+} induced neuronal exocytosis by disrupting the binary complex formed by SNAP 25 and syntaxin (310, 311).

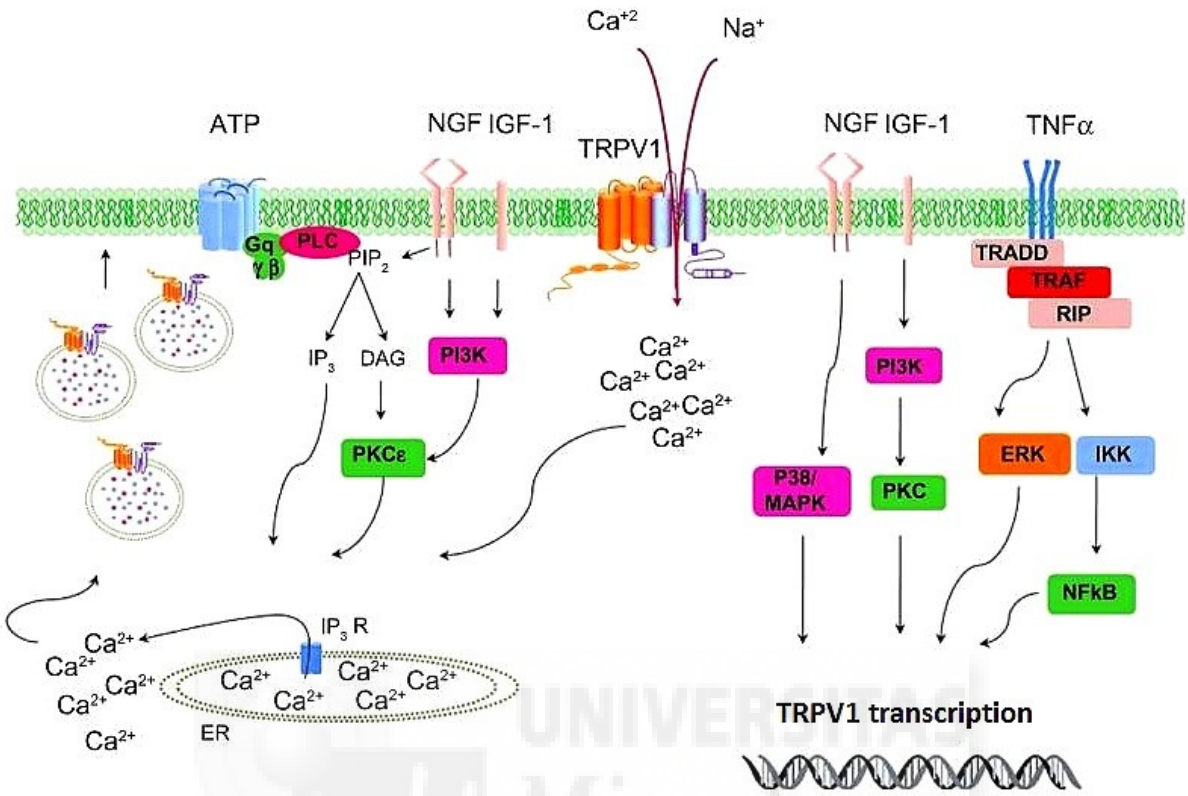

Figure- 11: Regulation of TRPV1 function and expression by proinflammatory mediators. 1) Rapid receptor translocation to the cell surface from the vesicular reservoir (left side). 2) Long-term upregulation of protein levels by transcription/translation process (right side) (297).

Furthermore, *in vitro* experiments revealed that inflammatory mediators can sensitize TRPV1 through exocytosis and phosphorylation mechanism. Inflammatory mediators like ATP, NGF and Insulin growth factor-1 (IGF-1) induce TRPV1 sensitization through the release of new channels from the vesicles to the plasma membrane. Sensitization of TRPV1 through exocytosis is inhibited by using the peptide DD04107, an inhibitor of neuronal exocytosis (312). This suggests a strong anti nociceptive potential of the peptide upon inflammatory sensitization. TRPV1 sensitization by BK, Interleukin- 1β (IL- 1β) and artemin is insensitive to the peptide DD04107. This suggests a possible modulatory role on TRPV1 channel gating through phosphorylation mechanism. In addition, *in vivo* studies confirm that the peptide DD04107 exhibits prolonged antinociceptive activity on different pain models (CFA, osteosarcoma, chemotherapy and diabetic neuropathy) (313).

Aforesaid studies provide an insight that inflammatory potentiation of TRPV1 is mediated by two distinct mechanisms; channel gating regulation (phosphorylation) and

release of new TRPV1 channels from the vesicles to the plasma membrane. It is still unclear if inflammatory sensitization of TRPV1 in nociceptor subpopulations follows a general mechanism or a distinct one exists.

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OBJECTIVES

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GENERAL OBJECTIVES

We hypothesize that the inflammatory recruitment of TRPV1 channels can occur in peptidergic nociceptors. An earlier study revealed that inflammatory sensitization of TRPV1 by ATP is exocytosis dependent whereas BK induced sensitization of TRPV1 is exocytosis independent (312). ATP and BK signal by activating $G\alpha_{q/11}$ pathways through GPCR and stimulating the β isoforms of PLC (PLC β), which in turn catalyzes the hydrolysis of PIP2, resulting in the generation of IP_3 and DAG. IP_3 regulates intracellular Ca^{2+} concentration through Ca^{2+} release from internal stores and DAG regulates PKC. Ca^{2+} ions and PKC participate in diverse signalling mechanism.

We wanted to elucidate the mechanism behind inflammatory sensitization of TRPV1 in nociceptors upon exposure to ATP and BK. Furthermore, we have also explored the possible involvement of two neuropeptides αCGRP and SP on sensitizing TRPV1 activity, by using a double knockout mice model of $\alpha CGRP^{-/-}xTac1^{-/-}$ together with patch clamp and micro electrode array techniques.

SPECIFIC OBJECTIVES

- To characterise the excitable properties of nociceptive neurons and its modulation by ATP and BK.
- To evaluate ATP mediated inflammatory potentiation of TRPV1.
- To examine BK mediated inflammatory potentiation of TRPV1.
- To assess the extent of inflammatory soup (ATP- BK pH 6.2) induced potentiation of TRPV1 evoked excitability.

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RESULTS

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CHARACTERISATION OF RAT NOCICEPTORS

For the characterisation of intrinsic membrane properties of nociceptors, DRG neurons from neonatal rats were used. Classification of subpopulation of nociceptors was done using IB4- Alexa 568 that binds to alive neurons and discriminates between peptidergic (does not bind to the plant lectin B4) and nonpeptidergic (binds to the plant lectin B4) subpopulations as shown in **figure- 12**. From our DRG cultures, 60 % of the neurons were positive for both TRPV1 and IB4. Experiments were made using patch clamp technique in whole cell configuration mode. Nociceptors ($IB4^-$ and $IB4^+$) were held at -60 mV.

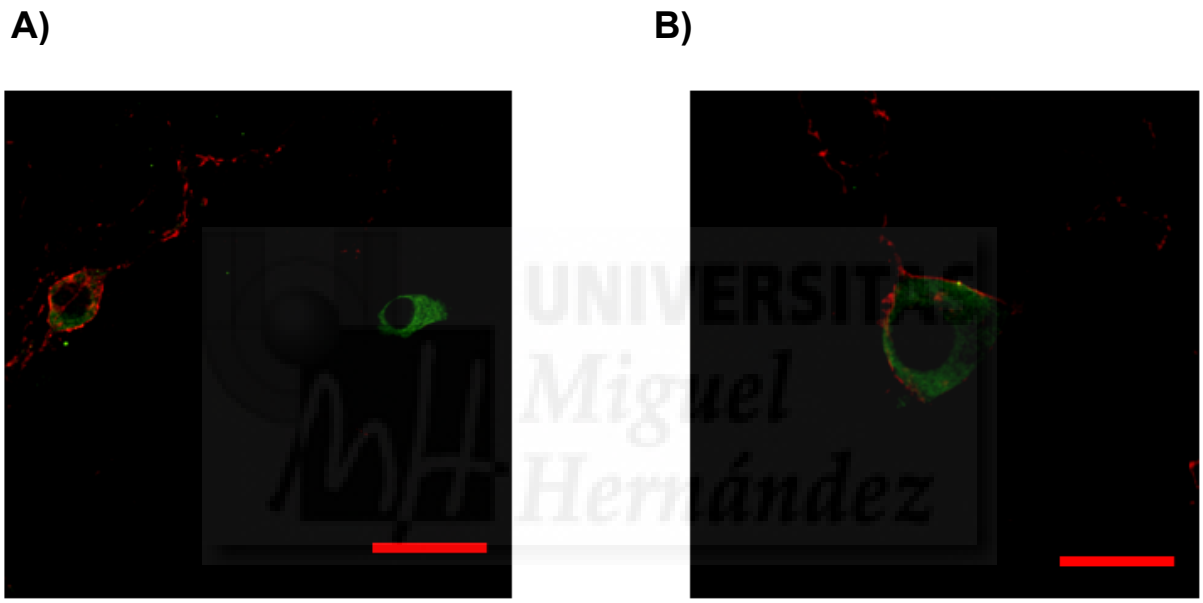

Figure- 12: Representative images of neonatal rat DRG neurons labelled with IB4-alexa 568 (red) and TRPV1 (green). A) Immunocytochemical images of $IB4^-$ ($TRPV1^+$) peptidergic and $IB4^+$ ($TRPV1^+$) nonpeptidergic neurons. B) Enlarged view of co staining of IB4 alexa 568 (red) with TRPV1 (green). Scale bar- 20µm.

Mean cell capacitance (pF) of $IB4^-$ and $IB4^+$ neurons was not significantly different. **Figure- 13** shows representative current traces from neurons ($IB4^-$, $IB4^+$) voltage clamped at -60 mV and stimulated with depolarizing voltage steps from -70 mV to +60 mV to evaluate voltage dependent ion channels, mainly Nav and Kv. As seen, both nociceptor types evoked Nav and Kv type currents. To ensure patched cells were neurons we used this protocol before and after experiments. Note also that IB4 binding does not affect functional properties of DRG neurons as previously reported (56).

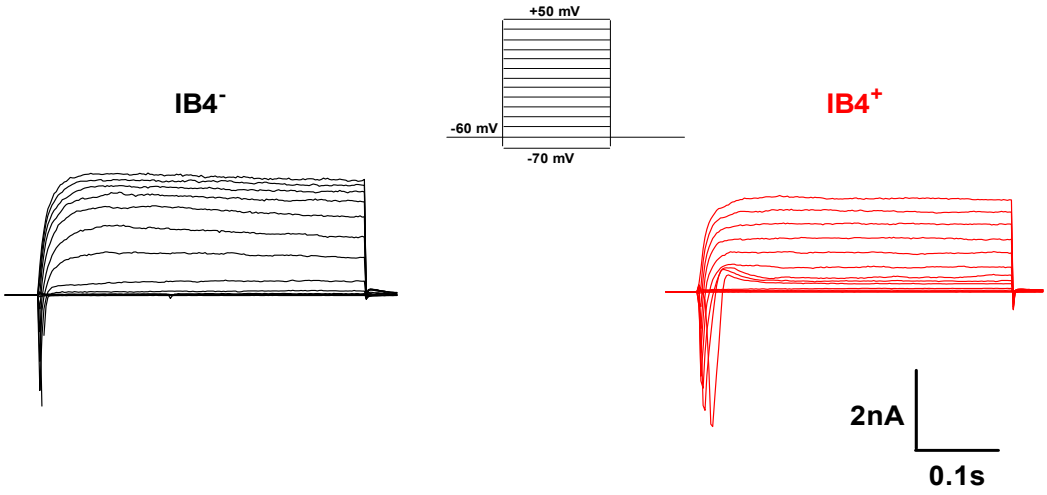

Figure- 13: Representative traces of ionic currents evoked in IB4$^-$ (black) and IB4$^+$ (red) neurons. Cells from IB4$^-$ and IB4$^+$ sub populations were injected with depolarizing voltage steps from -70 mV to +60 mV, which evoked typical neuronal Na^+- K^+ currents.

Differential changes in resting membrane potential and evoked discharges in IB4$^-$ and IB4$^+$ neurons

Electrical properties of peptidergic and nonpeptidergic neurons were analyzed for the following parameters: resting membrane potential, threshold potential, current threshold, amplitude of action potential, overshoot of action potential and duration of action potential as shown in **figure- 14**. To characterise the excitability of primary sensory neurons, we have examined primarily the spontaneous activities (within 300 ms) and evoked discharges (activated by 1 s, 300 pA depolarizing current pulse) in nociceptors having both peptidergic (IB4$^-$) and nonpeptidergic (IB4$^+$) subpopulations.

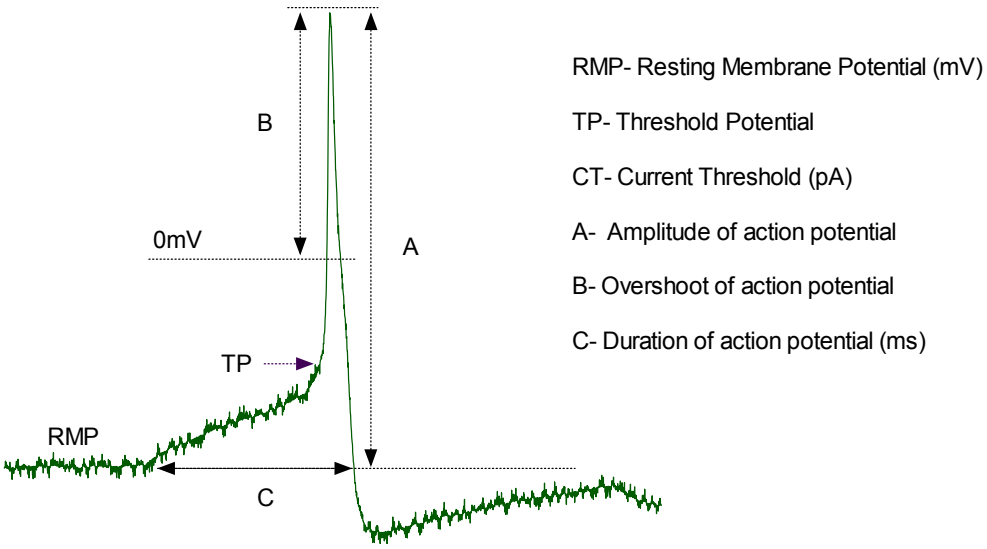

Figure- 14: Measured values for an action potential recorded from small-sized DRG neurons ($IB4^-$, $IB4^+$). A: amplitude of action potential, B: overshoot of action potential, C: duration of action potential, RMP: Resting membrane potential, TP: Threshold potential, CT: Current Threshold.

We have not observed spontaneous discharges in either subpopulation. Interestingly, $IB4^-$ neurons displayed more depolarized resting membrane potentials than $IB4^+$ neurons as shown in **figure- 15 (A)**. The hyperpolarized resting potential of $IB4^+$ could be due to the selective expression of TREK2, a leak K^+ channel that augments the K^+ permeability in these nociceptors. Evoked action potential upon current injection was readily observed in $IB4^-$ nociceptors as shown in **figure- 15 (B)**. As seen, 77% (33/43) of $IB4^-$ nociceptors exhibited evoked action potentials, compared with 21% (10/48) of $IB4^+$, consistent with the enhanced excitability of peptidergic sensory neurons.

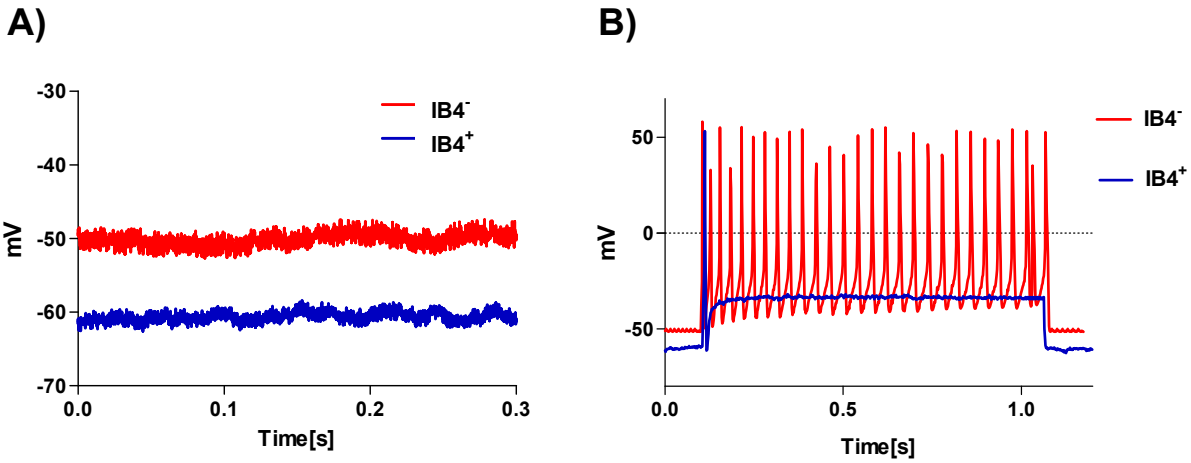

Figure- 15: Membrane resting potential and evoked discharges in small-sized DRG neurons from neonatal rats. (A) Representative resting membrane potential of $IB4^-$ (red) and $IB4^+$ (blue) neurons. Note that peptidergic neurons in neonatal rats had more depolarized resting membrane potential and nonpeptidergic neurons had more hyperpolarized. (B) Representative traces of evoked discharges. Injection of 300 pA current in $IB4^-$ peptidergic neurons (red) evoked trains of action potential, whereas in $IB4^+$ nonpeptidergic neurons (blue) very few or no action potential trains were evoked.

$IB4^-$ neurons require lower current threshold to evoke an action potential than $IB4^+$ neurons

To examine eventual changes in the amount of currents required (CT- current threshold) to evoke an action potential, in both $IB4^-$ and $IB4^+$ sub populations, we injected a 100 ms step of depolarizing current pulse from 0 pA to 200 pA. We observed that all $IB4^-$ neurons (43/43, 100%) required low current threshold (<50 pA) **(figure- 16 A)**, whereas $IB4^+$ neurons required distinct threshold currents to evoke action potential ranging from low current threshold (7/48, 14%) (<50 pA) to medium current threshold (2/48, 4%) (50-100 pA), and high current threshold (30/48, 62%) (>100 pA) **(figure- 16 B)**. Statistical analysis confirmed that $IB4^-$ neurons had significantly lower current threshold (21±2 pA) to evoke action potential compared to $IB4^+$ neurons (142±21 pA) (***- $p<0.001$) **(figure- 16 C)**. This result validates that $IB4^-$ neurons display enhanced excitability properties.

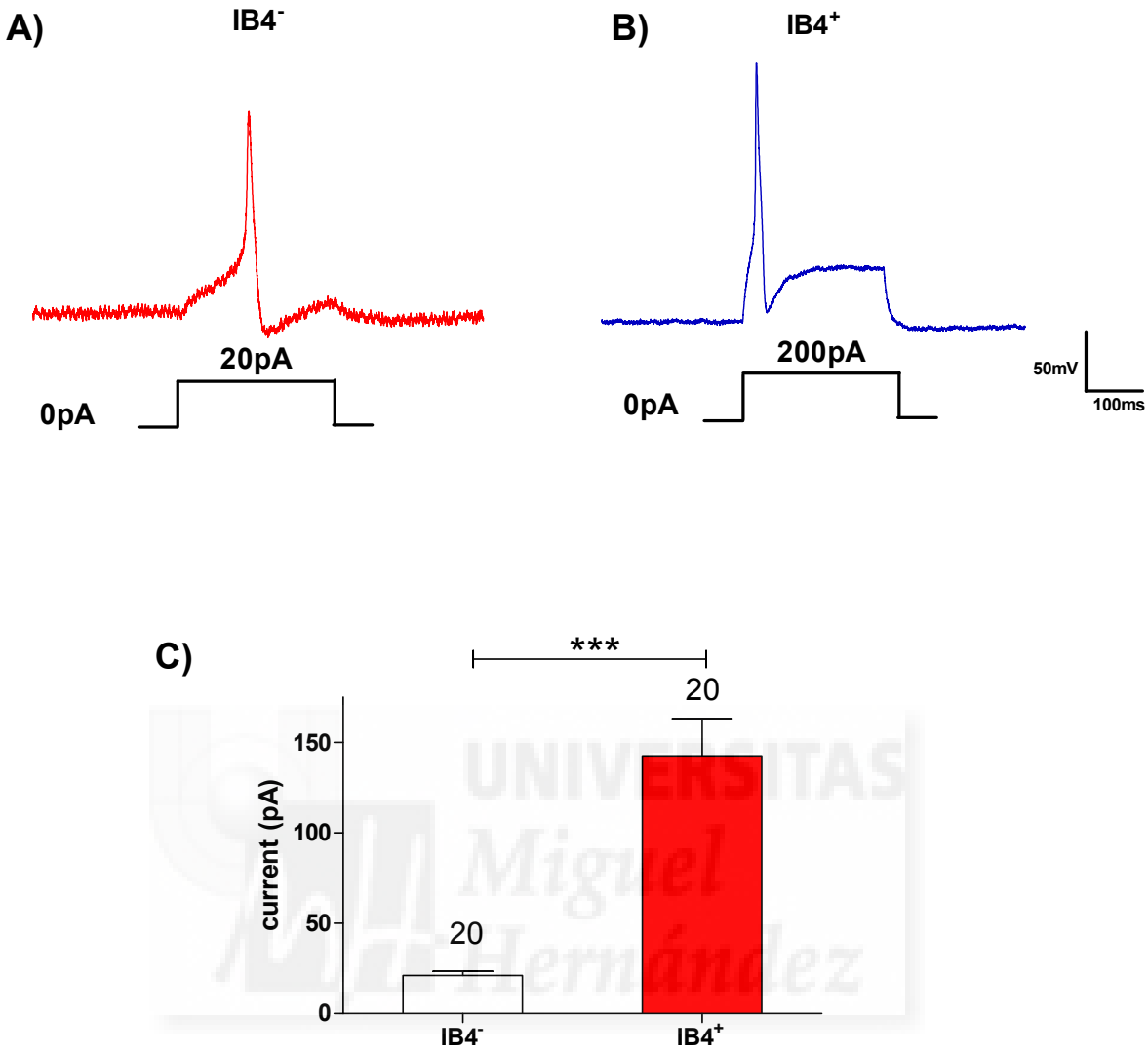

Figure- 16: Changes in current threshold (CT) in nociceptors from neonatal rat DRGs. A) 20 pA current injection was sufficient to evoke an action potential in IB4$^-$ neurons, B) 200 pA current injection evoked action potential in IB4$^+$ neurons. C) Mean threshold currents for IB4$^-$ and IB4$^+$ neurons. Number above the bars represent the number of neurons recorded, Number of cultures = 3. Statistical analysis was performed by unpaired Student's t- test (***- p<0.001).

Distinct electrogenic properties of IB4$^-$ peptidergic and IB4$^+$ nonpeptidergic rat DRG neurons

As shown in **figure- 17** IB4$^-$ neurons had a significantly depolarized resting membrane potential (-45±1 mV) compared to IB4$^+$ neurons (-54±2 mV) (***- p<0.001). Notably, a higher number of action potentials were evoked in IB4$^-$ neurons (16±2) than in IB4$^+$ neurons (6±1) (***- p<0.001). Similarly, IB4$^-$ neurons had lower amplitude of evoked action potential (92±4 mV) compared to IB4$^+$ neurons (106±4 mV) (*- p<0.05). In addition, the duration of action potential (ms) was slightly shorter (20±0.1 ms) in IB4$^-$ compared to

IB4$^+$ neurons (21±0.2 ms). Other parameters of action potentials like the threshold potential for IB4$^-$ neurons (-26±1 mV) compared to IB4$^+$ neurons (-26±1 mV) and the overshoot for IB4$^-$ neurons (49±2 mV) compared to IB4$^+$ neurons (52±2 mV) were not changed.

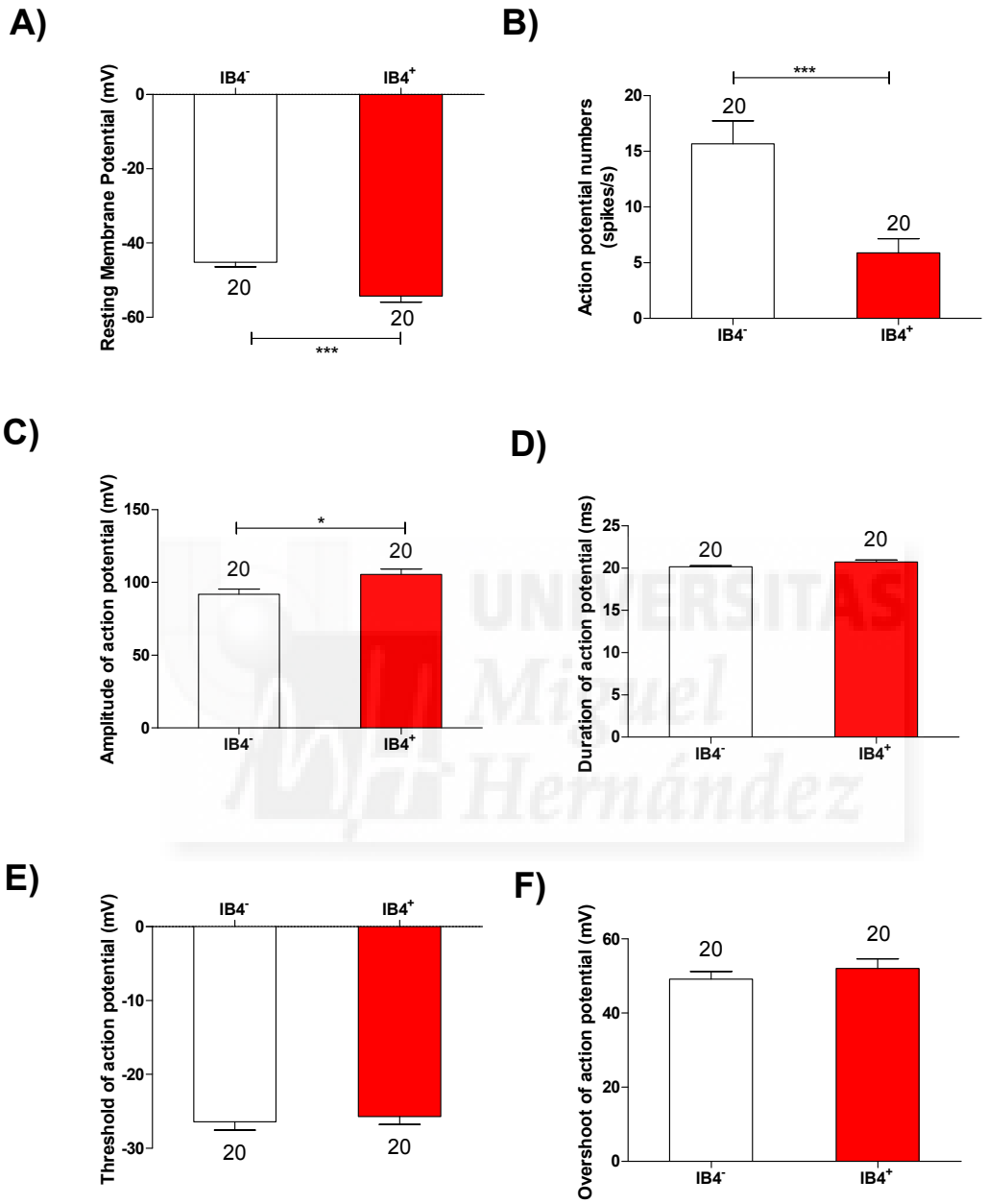

Figure- 17: Intrinsic electrogenic properties of IB4$^-$ (peptidergic) and IB4$^+$ (nonpeptidergic) rat DRG neurons. Summary of A- resting membrane potential, B- number of spikes, C- amplitude of action potential, D- duration of action potential, E- action potential threshold, F- overshoot of action potential evoked in IB4$^-$ and IB4$^+$ neurons. Numbers above the bars represent the number of neurons tested, Number of cultures = 3. Statistical analysis was performed by unpaired Student's t- test (*- p <0.05, ***- p<0.001).

NGF does not alter basal electrogenic properties of peptidergic and nonpeptidergic nociceptors

The differences we have observed in electrical properties of peptidergic and nonpeptidergic subpopulations reflect the possible involvement of differential ion channels. Nonetheless, they might be the result of different neurotrophic factors acting on both subpopulations and modulation of their electrical properties. In this regard $IB4^-$ neurons are NGF dependent while $IB4^+$ neurons shift towards GDNF dependency postnatally (between P4 to P10) (47).

To investigate the possibility that NGF alters the electrogenic properties of nociceptive neurons, subsequently we have measured evoked action potentials on peptidergic and nonpeptidergic neurons, which were cultured in the absence of NGF. There was no statistical difference observed between peptidergic and nonpeptidergic neurons with respect to cell capacitance (pF) in the absence of NGF. As shown in **figure- 18** peptidergic and nonpeptidergic subpopulations exhibited similar differences in the electrical properties as recorded in the presence of the neurotrophin. These properties includes the resting membrane potential, current threshold, threshold potential, action potential (amplitude, overshoot, and duration), and number of spikes akin to the cells exposed to NGF as shown in **table- 1**.

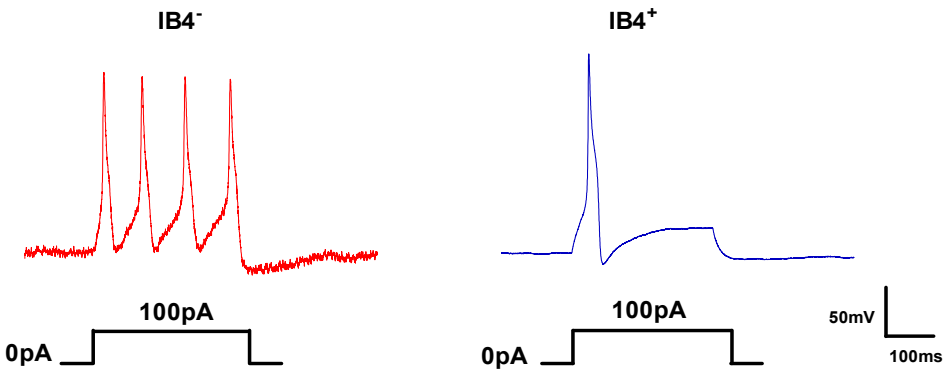

Figure- 18: Representative traces of evoked action potentials in $IB4^-$ (red) and $IB4^+$ (blue) neurons cultured in the absence of NGF. Nociceptors were injected with 100 pA current for 100 ms to evoke a train of action potentials.

Electrical parameters	(+) NGF		(-) NGF	
	IB4$^-$	IB4$^+$	IB4$^-$	IB4$^+$
RMP (mV)	-45 ± 1 (20)	-54 ± 2 (20)***	-46 ± 2 (13)	-59 ± 2 (8) ***
CT (pA)	21 ± 2 (20)	142 ± 20(20)***	30 ± 6 (13)	215 ± 33 (8) ***
TP (mV)	-26 ± 1 (20)	-26 ± 1 (20)	-21 ± 2 (13)	-24 ± 3 (8)
AP amplitude	92 ± 4 (20)	106 ± 4 (20)*	78 ± 4 (13)	106 ± 4 (8) ***
AP overshoot	49 ± 2 (20)	52 ± 2 (20)	44 ± 4 (13)	48 ± 4 (8)
AP duration (ms)	20 ± 0.1(20)	21 ± 0.2 (20)	20 ± 0.5 (13)	20 ± 0.2 (8)
No of spikes	16 ± 2 (20)	6 ±1.3(20)***	9 ± 3(13)	3 ± 2(8)

Table- 1: Electrical properties of IB4$^-$ and IB4$^+$ neurons cultured in the presence and absence of NGF. RMP- resting membrane potential, CT- current threshold, TP- threshold potential, AP- action potential. Data are expressed as mean ± SEM (n). Statistical analysis was performed by unpaired Student's t- test (*- p <0.05, ***- p<0.001).

Effect of ATP and BK on resting membrane potential and spontaneous activity in IB4$^-$ and IB4$^+$ neurons

Next we have characterised the functional changes induced by ATP and BK on excitability of nociceptors from both peptidergic and nonpeptidergic subpopulations. Before exposing the neurons (IB4$^-$ and IB4$^+$) to inflammatory mediators, their basal electrical properties were measured.

Later, spontaneous activity and changes in the membrane resting potential induced by both ATP and BK were individually assessed. Neurons were perfused with inflammatory mediators 10 μM ATP or 1 μM BK, applied continuously for 4 minutes (from 1st minute until 5th minute). DRG neurons in general are silent and exposure of neurons to ATP or BK leads to activation of purinergic and BK receptors, which induces a change in the membrane ionic currents leading to cell depolarization.

ATP induced modulation of nociceptor excitability

For ATP, 18 neurons in IB4$^-$ and 14 neurons in IB4$^+$ were tested. The response typically began within few seconds of ATP application. Mostly, depolarization of the membrane returned to basal level in seconds in both IB4$^-$ and IB4$^+$. Application of ATP induced robust depolarization of the membrane resting potential in peptidergic subpopulation

(-37±4 mV) compared to control (-42±2 mV). No such changes were observed in nonpeptidergic subpopulation where both control (-54±2 mV) and ATP (-57±3 mV) exhibited similar resting membrane potential. The magnitude of depolarization induced by ATP was significantly higher in $IB4^-$ neurons (20±5 mV) compared to $IB4^+$ neurons (11±1 mV) (*- $p<0.05$) as shown in **figure- 19**. Application of ATP activated most of the neurons tested (ATP responsive neurons: $IB4^-$- 13/18, $IB4^+$- 14/14).

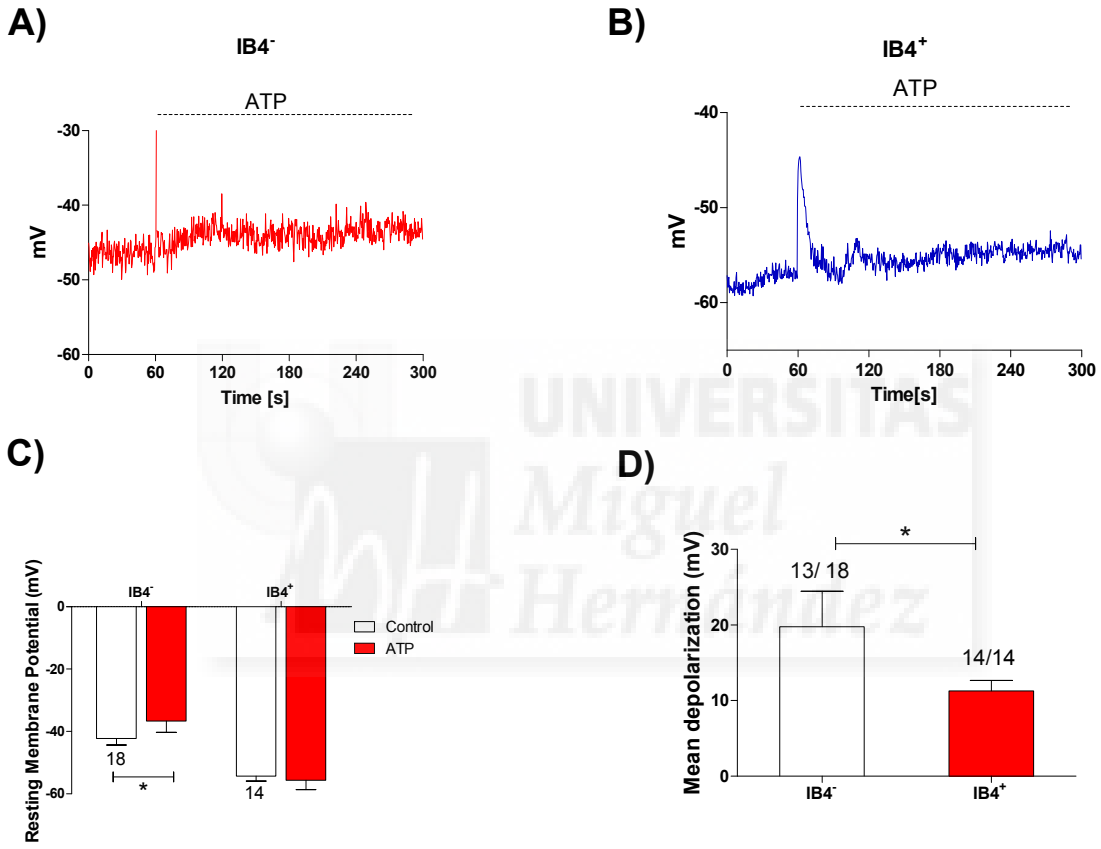

Figure- 19: ATP modulates excitability of $IB4^-$ and $IB4^+$ nociceptors from neonatal rat DRGs. A) Representative trace from peptidergic neuron exhibiting spontaneous activity with 10 µM ATP, B) representative trace from nonpeptidergic neuron exhibiting spontaneous activity with 10 µM ATP. ATP was applied from 60 seconds until 300 seconds, C) Resting membrane potential before and after 10 µM ATP D) Mean depolarization rate of peptidergic and nonpeptidergic neurons induced by 10 µM ATP. Numbers above the bars represent number of neurons tested, Number of cultures= 3. Statistical analysis was performed by paired and unpaired Student's t- test (*- $p<0.05$).

BK induced modulation of nociceptor excitability

For BK, 18 neurons in $IB4^-$ and 14 neurons in $IB4^+$ were tested. The response typically began within few seconds to one minute of BK application. Application of BK in $IB4^-$ neurons shown enhanced action potential discharge until washout, whereas $IB4^+$ neurons

exhibited little depolarization of the membrane. BK induced robust depolarization of the membrane resting potential in peptidergic subpopulation (-36±4 mV) compared to control (-42±2 mV). No such changes were observed in nonpeptidergic subpopulation where both control (-54±2 mV) and BK (-59±2 mV) exhibited similar resting membrane potential. The magnitude of depolarization was significantly higher in $IB4^-$ neurons (29±7 mV) exposed to BK compared to $IB4^+$ neurons (8±2 mV) (**- p<0.01) as shown in **figure- 20**. Application of BK activated most of the neurons tested (BK responsive neurons: $IB4^-$- 13/18, $IB4^+$- 11/14).

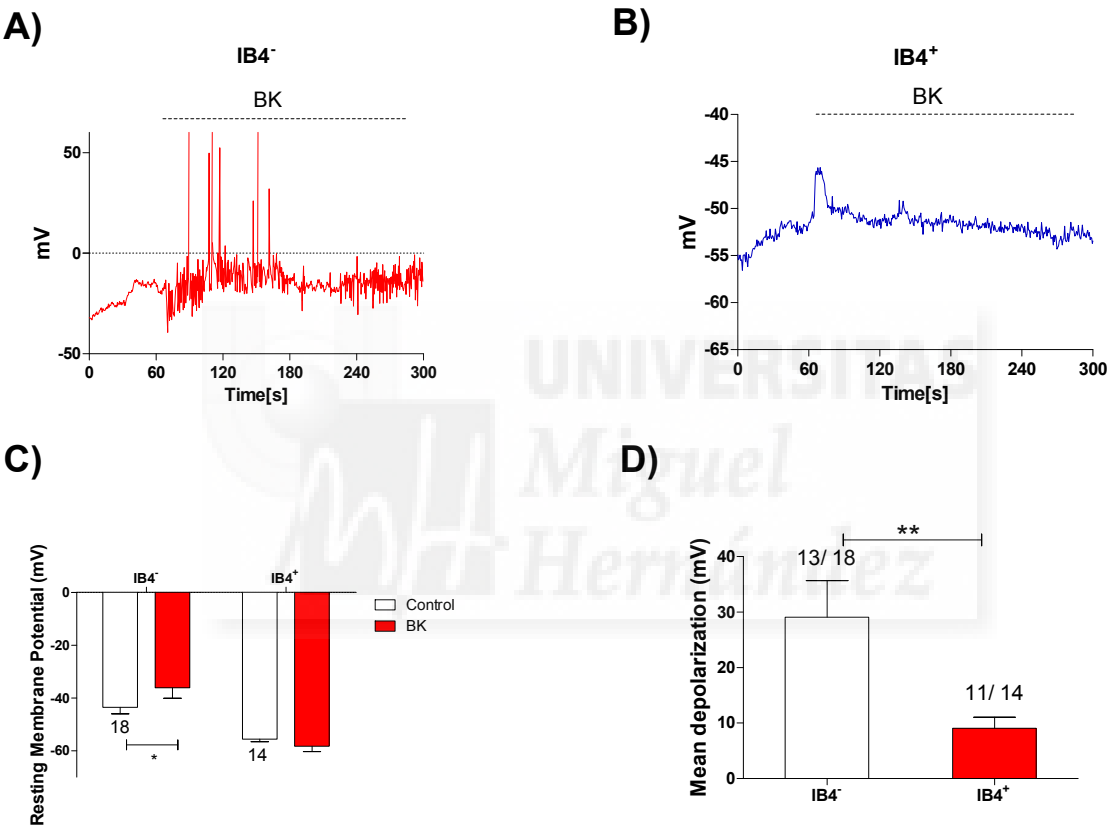

Figure- 20: BK modulates excitability of $IB4^-$ and $IB4^+$ nociceptors from neonatal rat DRGs. A) Representative trace from peptidergic neuron exhibiting spontaneous activity with 1 µM BK, B) representative trace from nonpeptidergic neuron exhibiting spontaneous activity with 1 µM BK. BK was applied from 60 seconds until 300 seconds. C) Resting membrane potential before and after 1 µM BK , D) Mean depolarization rate of peptidergic and nonpeptidergic neurons induced by 1 µM BK. Numbers above the bars represent the number of neurons tested, Number of cultures = 3. Statistical analysis was performed by paired and unpaired Student's t- test (*- p <0.05, **- p <0.01).

Inflammatory mediators induced changes in evoked excitability of rat nociceptors

Furthermore, we wanted to examine the modulatory role of inflammatory mediators (ATP and BK) on evoked excitability of nociceptors. We injected a depolarizing current of 100 pA for 100 ms. Neurons ($IB4^-$ and $IB4^+$) were initially examined for evoked excitability in basal conditions (vehicle). Inflammatory mediators (ATP and BK) were then applied individually for 5 minutes and again tested for changes in evoked excitability. Increase in excitability or sensitization by inflammatory mediators is associated with lower current threshold requirement to evoke an action potential and more evoked action potentials in response to previously induced stimuli. From our experiments, we did not observe any huge changes in the excitability of $IB4^-$ neurons after the exposure to inflammatory mediators: only a little number of neurons with an increase in evoked spikes was observed for both ATP (1/18 neurons) and BK (2/18 neurons). The rest of the $IB4^-$ neurons had the same number of evoked spikes. Most of the neurons in $IB4^+$ subpopulation exhibited either one or two evoked action potentials under basal conditions (vehicle). After exposure to inflammatory mediators, an increase in the number of neurons with evoked spikes was observed for both ATP (6/14 neurons) and BK (4/14 neurons) as shown in **figure- 21 and figure- 22**.

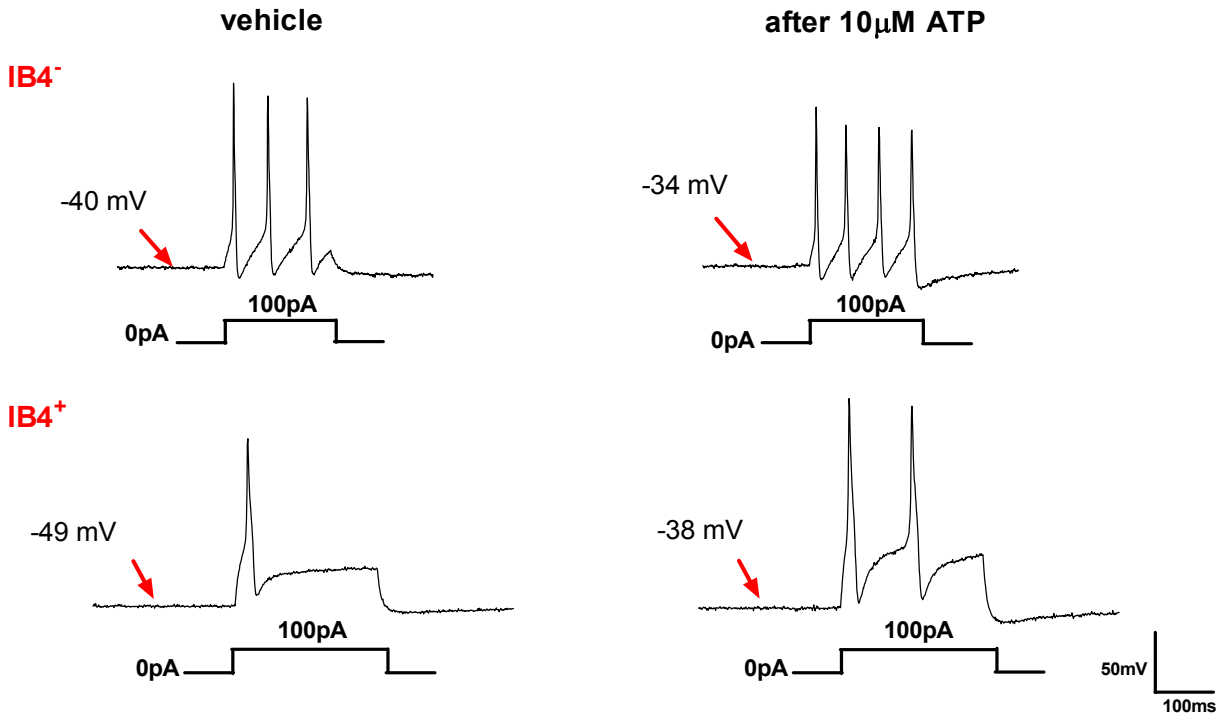

Figure- 21: ATP modulates evoked excitability of IB4$^-$ and IB4$^+$ somata to depolarizing current. Representative traces of 100 ms depolarizing current injection at a strength of 100 pA delivered to the soma through the recording electrode before and after application of 10 μM ATP (applied for 4 minutes) exhibited depolarization of membrane resting potential and increase in the number of spikes evoked, Top: IB4$^-$ Bottom: IB4$^+$.

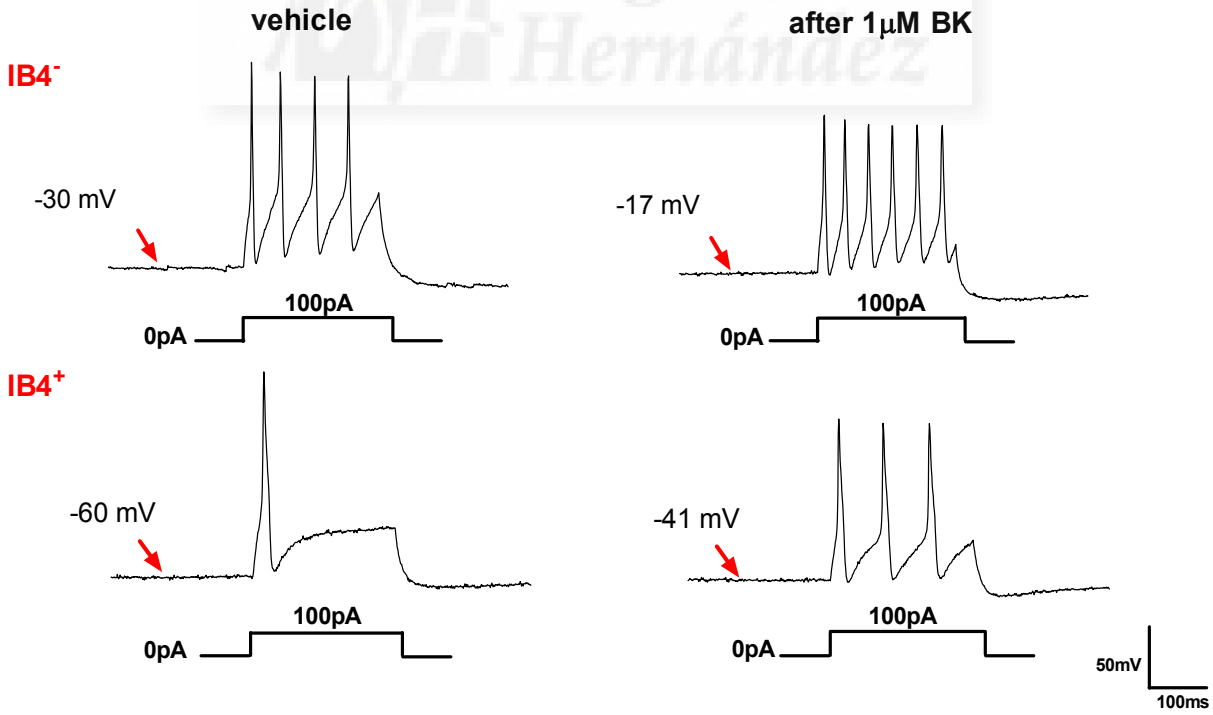

Figure- 22: BK modulates evoked responses of IB4$^-$ and IB4$^+$ somata to depolarizing current. Representative traces of 100 ms depolarizing current injection at a strength of 100 pA delivered to the soma through the recording electrode before and after application of 1 μM BK (applied for 4 minutes) exhibited depolarization of membrane resting potential and increase in the number of spikes evoked, Top: IB4$^-$ Bottom: IB4$^+$.

Facilitatory effect of combined application of ATP and BK on nociceptive neuron excitability

Preceding experiments revealed that individual applications of ATP and BK induced changes in the membrane resting potential of peptidergic subpopulations. No such effect was observed in nonpeptidergic subpopulations after ATP and BK exposure, although the number of evoked action potentials was increased. Furthermore, both ATP and BK exhibited robust spontaneous activity on peptidergic neurons and significantly increased their mean depolarization as well. Notwithstanding, nonpeptidergic neurons also exhibited spontaneous activity after ATP and BK exposure, although it was lesser compared to peptidergic neurons.

Under inflammatory conditions, release of different inflammatory mediators markedly increases the excitability of nociceptors. As mentioned before, both ATP and BK strongly depolarized the membrane resting potential in peptidergic subpopulations but had no effect on nonpeptidergic neurons. Since in all our experiments we applied inflammatory mediators separately and they exert their effect on excitability through discrete receptor signalling mechanisms. We questioned whether co application of both inflammatory mediators ATP and BK may facilitate enhanced spontaneous action potential firing and depolarize the resting membrane potential in both peptidergic and nonpeptidergic subpopulations. Akin to earlier experiments, we used the same protocol and measured the electrical properties of nociceptive neurons before and after application of 10 μM ATP- 1 μM BK. As shown in **figure- 23**, exposure of neurons to ATP- BK exhibited potent spontaneous activity in peptidergic subpopulations. In addition, nonpeptidergic neurons also exhibited higher depolarization rate though no spontaneous activity was observed ($IB4^-$: 41±7 vs $IB4^+$: 14±3 mV). Noticeably, ATP- BK induced strong depolarization of membrane resting potential in peptidergic (Control: -53±2 vs ATP- BK: -44±8 mV) and in nonpeptidergic neurons (Control: -60±3 vs ATP- BK: -55±3 mV) as well. In resume, combined application of ATP and BK enhanced the excitability of both peptidergic and nonpeptidergic subpopulations. Both inflammatory mediators induce depolarization of the nociceptor through inhibition of Kv7 channels and increasing the activity of TRPV1 and CACC, consistent with the effects observed (314, 315).

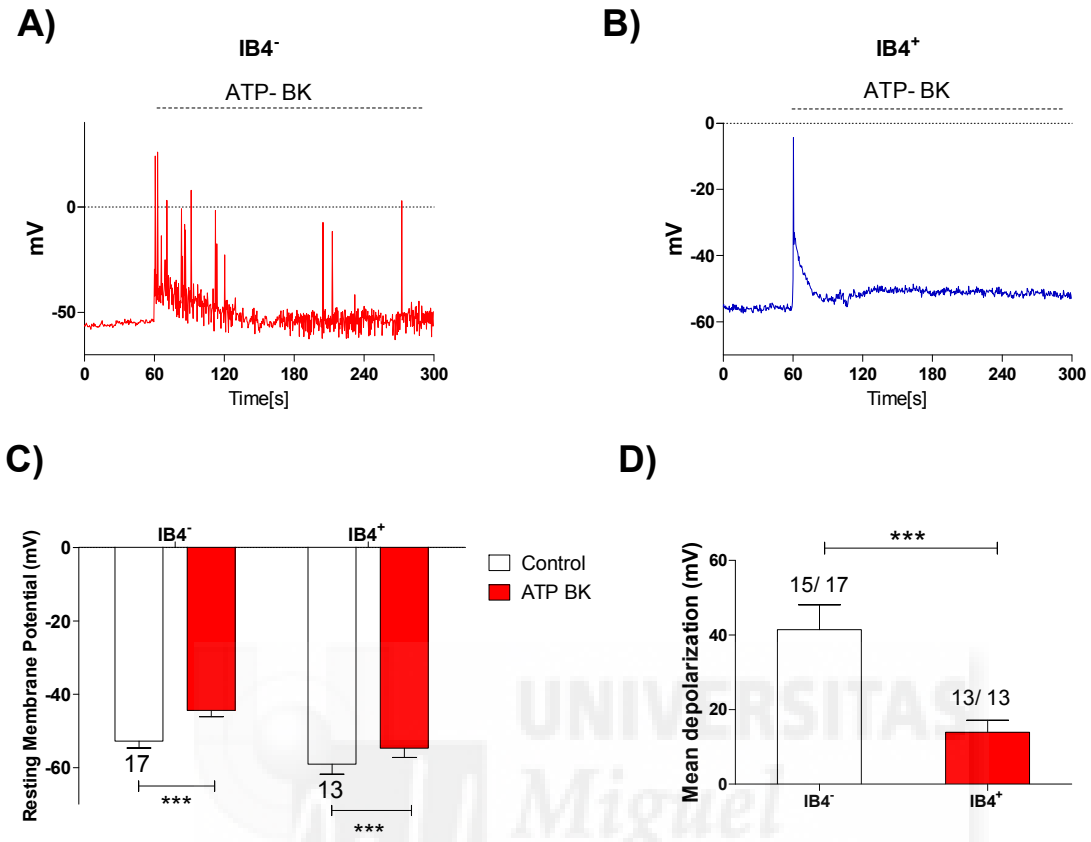

Figure- 23: Co application of ATP- BK modulates excitability of IB4$^-$ and IB4$^+$ nociceptors from neonatal rat DRGs. A) Representative trace from peptidergic neuron exhibiting spontaneous activity with 10 µM ATP- 1 µM BK, B) representative trace from nonpeptidergic neuron exhibiting spontaneous activity with 10 µM ATP- 1 µM BK. ATP- BK was applied from 60 seconds until 300 seconds, C) Resting membrane potential before and after 10 µM ATP- 1 µM BK, D) Mean depolarization rate of peptidergic and nonpeptidergic neurons induced by 10 µM ATP- 1 µM BK. Numbers above the bars represent the number of neurons tested, Number of cultures = 3. Statistical analysis was performed by paired and unpaired Student's t- test (***- $p<0.001$).

ATP INDUCED INFLAMMATORY SENSITIZATION OF TRPV1

Capsaicin evoked neuronal spikes on whole neuronal population using micro electrode array

Prior experiments on peptidergic and nonpeptidergic nociceptors from neonatal rat DRGs corroborated that enhanced excitability occurs primarily in peptidergic subpopulations, while nonpeptidergic subpopulations have a milder increase in excitability upon exposure to the inflammatory mediators ATP/BK. Furthermore, we characterised the excitability of neurons using Micro Electrode Array (MEA) technique where DRG neurons formed a cluster of cultured network resembling *in vivo* pattern. This technique does not allow differentiating subpopulations. MEA technique has been used in our experiments to study inflammation induced sensitization of TRPV1 evoked excitability. All our experiments evaluated capsaicin induced activation of TRPV1 and its sensitization by inflammatory mediators like ATP, BK and co- application of ATP- BK. We also evaluated tissue acidosis condition that occurs during inflammatory microenvironment, by applying ATP- BK at pH 6.2. Prior to our sensitization experiments, we characterised capsaicin induced TRPV1 spikes, followed by desensitization of TRPV1 evoked spikes induced by repetitive capsaicin applications on DRG neurons.

Capsaicin evoked neuronal firing in silent nociceptors

The spike signal obtained in MEA was either from single neuron or a group of neurons surrounding the electrode. **Figure- 24** shows planar microelectrode array (A and B), where neurons are in contact with the electrodes (C and D) and a capsaicin evoked spike (E). In MEA chips, smaller the distance between the cell and the electrode, higher the extracellular signals.

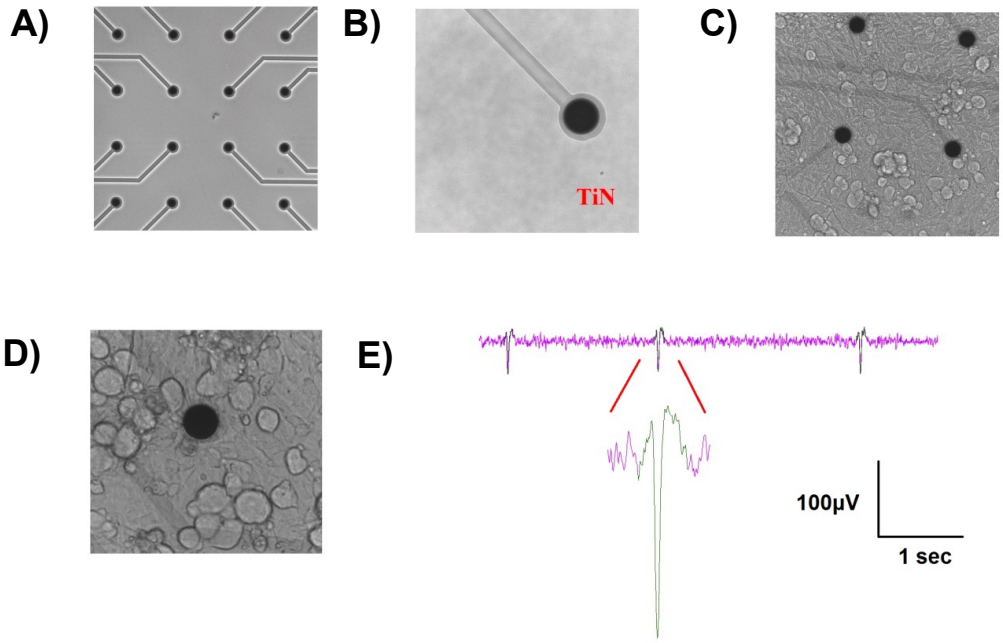

Figure- 24: Capsaicin evoked neuronal spikes measured using microelectrode array. (A, B) Planar micro electrode array, (C) cultured neonatal rat DRG neurons over a substrate array of planar microelectrodes (Multichannel Systems, Reutlingen, Germany) 2 days after seeding. The electrodes are 30 µm wide; electrode spacing is 200 µm. (D) Cluster of cells coupled to a microelectrode of the array and (E) a trace of 500 nM Capsaicin evoked spike.

Capsaicin evoked neuronal spikes were measured from neonatal rat DRG neurons which are silent without any spontaneous activity. As shown in **figure- 25** before capsaicin (Cps) application no spikes were evoked. During capsaicin application downward neuronal spikes were evoked through activation of TRPV1, which were completely removed after washing out the vanilloid.

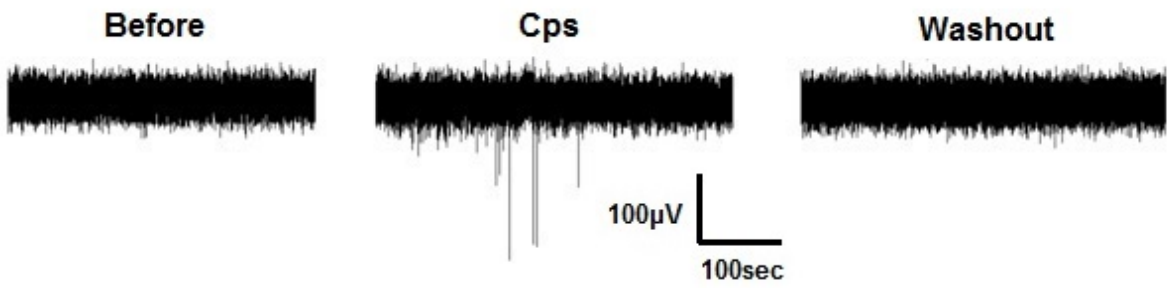

Figure- 25: Representative traces of 500 nM Capsaicin evoked neuronal firing. Exposure to 500 nM Capsaicin on silent neonatal rat DRG neurons evoked electrical activity mediated through the opening of TRPV1 channels. Capsaicin was applied for 15 seconds. Neurons did not evoke any electrical activity after washing capsaicin with standard external solution.

Capsaicin induced tachyphylaxis of TRPV1 evoked neuronal spikes

It is reported that the repeated activation of TRPV1, leads to desensitization of the channel activity, a phenomenon known as tachyphylaxis (270). We wanted to study the process of TRPV1 mediated tachyphylaxis of neuronal spikes in our MEA system. Three repetitive applications of 500 nM Cps for 15 seconds resulted in desensitization of TRPV1 mediated neuronal spikes as shown in **figure- 26**. A continuous protocol was used for 16 minutes where three repetitive pulses of 500 nM capsaicin was applied (P1 –1st pulse at 1st minute, P2- 2nd pulse at 5th minute, P3- 3rd pulse at 14:30 minutes). Upon consecutive application of capsaicin, TRPV1 evoked neuronal spikes exhibited heterogeneous responses with either a partial or complete desensitization of neuronal spikes. This protocol was used for all the MEA experiments. Later, data was processed to extract and compare Mean Spike Frequency for all the three pulses (P1, P2, and P3) using offline MC RACK program.

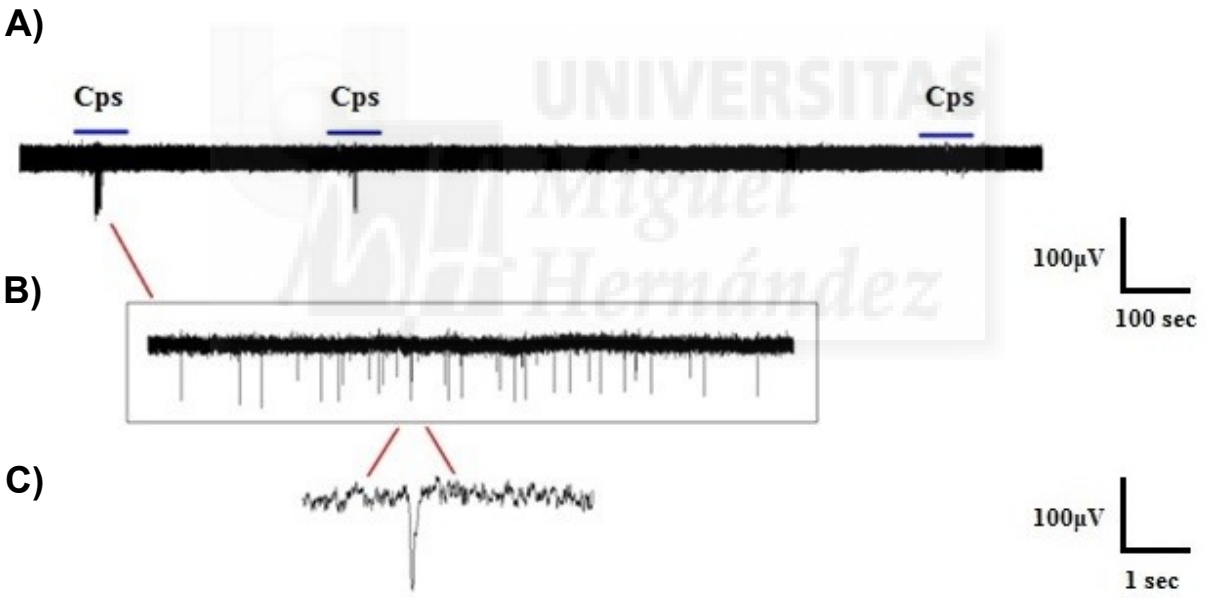

Figure- 26: Capsaicin induced tachyphylaxis of TRPV1 evoked neuronal spikes. A) An example of tachyphylaxis to three consecutive applications of 500 nM capsaicin (horizontal bars) given at 5 min intervals. B, C) represents enlarged view of capsaicin evoked neuronal firing from first pulse. TRPV1 mediated neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in each group and represented as Mean spike frequency (Hz).

The brief application of capsaicin pulses for 15 seconds was insufficient to extract the mean spike frequency. There was a delay in some electrodes between the initial exposure to capsaicin and the activation of neuronal spikes, which ranges from 4 to 5 seconds. This could be due to the time required for capsaicin to reach all the electrodes. Furthermore, the

heterogeneous response to capsaicin suggests a possible involvement of other membrane associated ion channels. For this reason, we have applied a time frame during analysis of MEA data to analyse extra 30 seconds after capsaicin application for all the three pulses (P1, P2, P3).

Peptide DD04107 does not interfere with basal TRPV1 activity

To study the effect of inhibiting neuronal exocytosis on TRPV1 sensitization, we used a botulinomimetic peptide DD04107 (EEMQRR), which disrupts the SNARE complex formation. First we wanted to characterise the peptide under control conditions to know if it interferes with the basal TRPV1 activity, by using both MEA and patch clamp techniques. For MEA experiments DRG neurons were preincubated with 20 µM palmitoylated DD04107 for one hour. Application of 500 nM capsaicin on DRG neurons seeded in MEA evoked neuronal spikes mediated through TRPV1 (P1). Re-application of capsaicin after 5 minutes interval twice (P2, P3) resulted either in a response of greatly reduced neuronal spikes or in no response, which indicated that desensitization of TRPV1 had occurred. The mean spike frequency of a second pulse of capsaicin never exceeded the frequency of neuronal spikes of the first pulse and it was further reduced with the third pulse (P3). Thus the desensitization of neuronal spikes evoked by TRPV1 was not readily reversible. Preincubation of neurons with DD04107 did not affect capsaicin evoked neuronal spikes and its desensitization as shown in **figure- 27**.

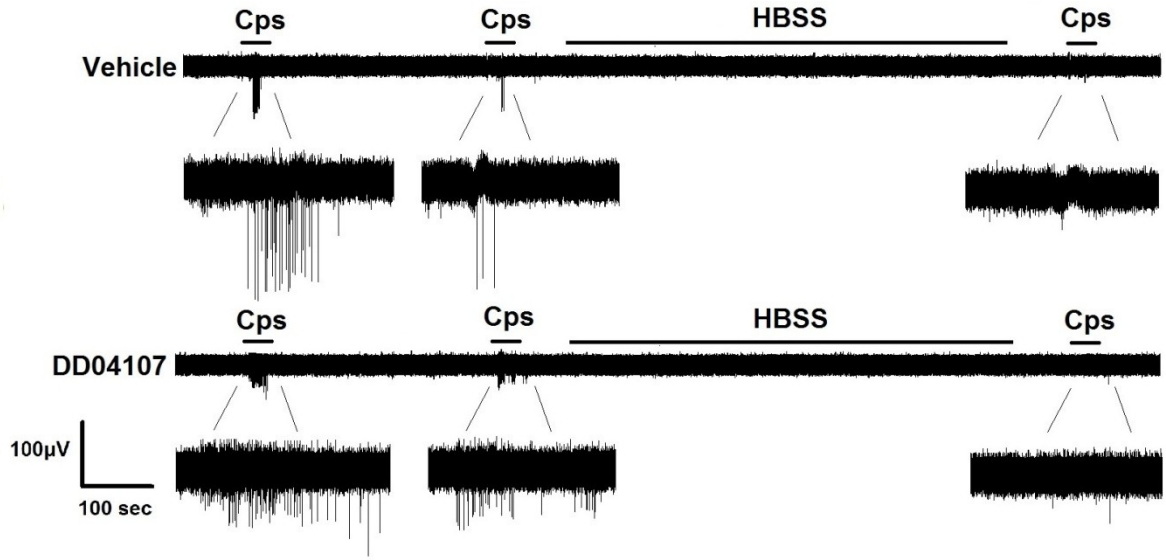

Figure- 27: DD04107 does not affect TRPV1 evoked neuronal spikes and its tachyphylaxis. Application of capsaicin (500 nM, 15 s) on neonatal rat DRG neurons induced TRPV1 evoked neuronal excitability in both vehicle and DD04107 treated groups (1st pulse- P1). Repeated exposure of DRG neurons to capsaicin (2nd pulse (P2) and 3rd pulse (P3)) evoked fewer action potentials compared to the 1st pulse due to the desensitization of TRPV1 channel activity.

We also tested the effect of nonpalmitoylated DD04107 on basal TRPV1 activity in $IB4^-$ and $IB4^+$ nociceptors using patch clamp technique under whole cell configuration mode. In both subpopulations 100 µM nonpalmitoylated DD04107 (represented as Peptide) was applied through patch pipette, and the recordings were started 10 minutes after forming the seal. Application of 1 µM capsaicin induced TRPV1 currents on both subpopulations, which also exhibited desensitization upon repetitive capsaicin application. Neurons treated with nonpalmitoylated DD04107 also exhibited activation of TRPV1 currents and its desensitization in both subpopulations as shown in **figure- 28**.

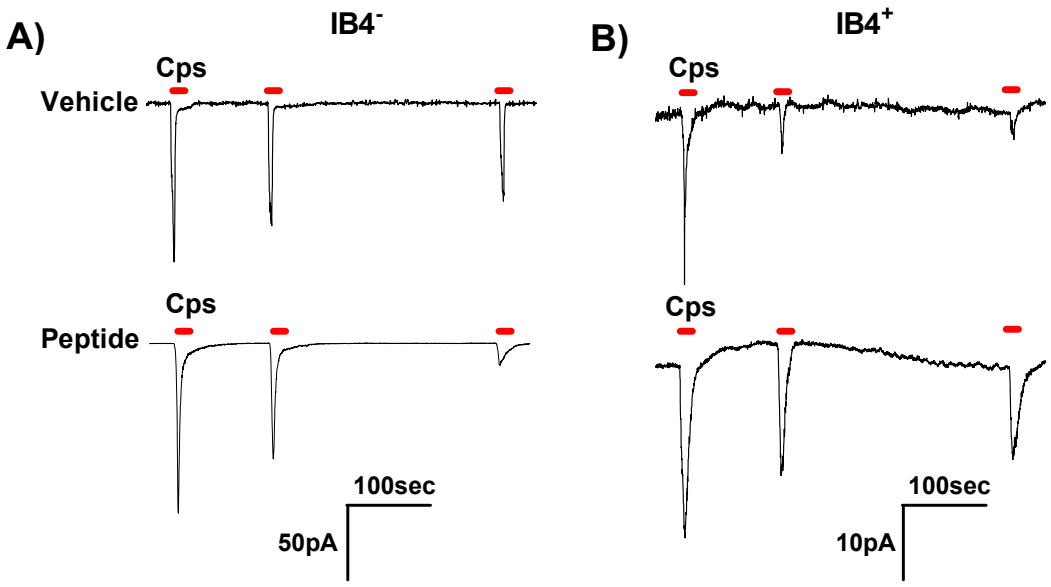

Figure- 28: **Representative voltage clamp recordings of currents elicited by capsaicin (1 µM, 10 s) in IB4$^-$ and IB4$^+$ nociceptors treated with nonpalmitoylated DD04107** (A, B) 100 µM nonpalmitoylated DD04107 represented as Peptide was given through the patch pipette and incubated for 10 minutes after forming the seal. Three repetitive pulses of capsaicin at 1 µM concentration were given for the continuous protocol.

In addition, we wanted to check if nonpalmitoylated DD04107 (Peptide) interferes with the basal TRPV1 current density in nociceptors. We calculated the ratio of initial TRPV1 current responses (pA) to their corresponding cell capacitance (pF) and compared between Vehicle and Peptide treated groups in both subpopulations (IB4$^-$, IB4$^+$). As shown in **figure- 29**, DD04107 did not affect basal TRPV1 current density in both IB4$^-$ and IB4$^+$ nociceptors.

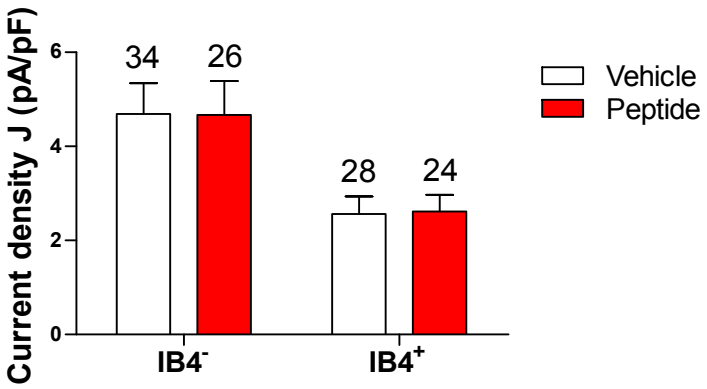

Figure- 29: Nonpalmitoylated DD04107 (Peptide) does not affect basal TRPV1 currents in IB4$^-$ and IB4$^+$ neurons. Current density was calculated by taking the ratio of initial TRPV1 current responses (pA) to their corresponding cell capacitance (pF). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of neurons registered. Number of cultures = 3. Statistical analysis was performed by two- way ANOVA with Bonferroni's post hoc test.

Pharmacological blockage of neuronal exocytosis reduces ATP induced sensitization of TRPV1 excitability

ATP induced sensitization of TRPV1 excitability in rat nociceptors was evaluated using MEA technique. As seen before, consecutive application of capsaicin led to desensitization in the number of spikes evoked and desensitized responses could be significantly recovered with ATP sensitization (10 μM ATP was applied between P2 and P3) as shown in **figure- 30 (Vehicle)**. ATP induced sensitization of TRPV1 excitability was blocked by 20 μM DD04107, an inhibitor of neuronal exocytosis, as shown in **figure- 30 (DD04107)**. This indicates that ATP induced TRPV1 sensitization was predominantly due to the release of new TRPV1 channels from the vesicles and their insertion into the plasma membrane of DRG neurons. Although we observed a less significant potentiation of TRPV1 activity by ATP in DD04107 treated group (*-$p<0.05$) compared to Vehicle group (***-$p<0.001$), we still have potentiation of TRPV1 evoked excitability **(figure- 31)**. This confirms that there is a subpopulation of nociceptors involved in channel recruitment. Therefore, it was noteworthy to evaluate the fold increase in the potentiation of TRPV1 evoked spikes induced by ATP between Vehicle and DD04107 treated groups.

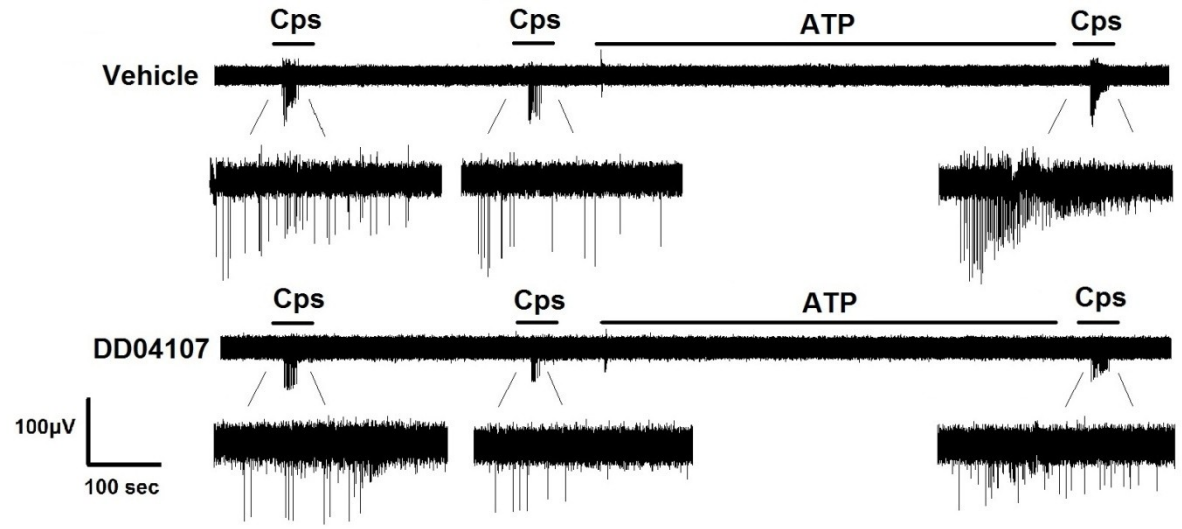

Figure- 30: DD04107 abrogates, ATP sensitized TRPV1- mediated nociceptor excitability. Representative traces of potentiation of capsaicin (500 nM, 15 s) responses elicited by applying 10 µM of ATP between the second (P2) and third (P3) vanilloid pulse (top), in neonatal rat DRG neurons. Preincubation of the neurons with DD04107 (bottom) also induced potentiation of TRPV1 but it was less significant compared to Vehicle (top).

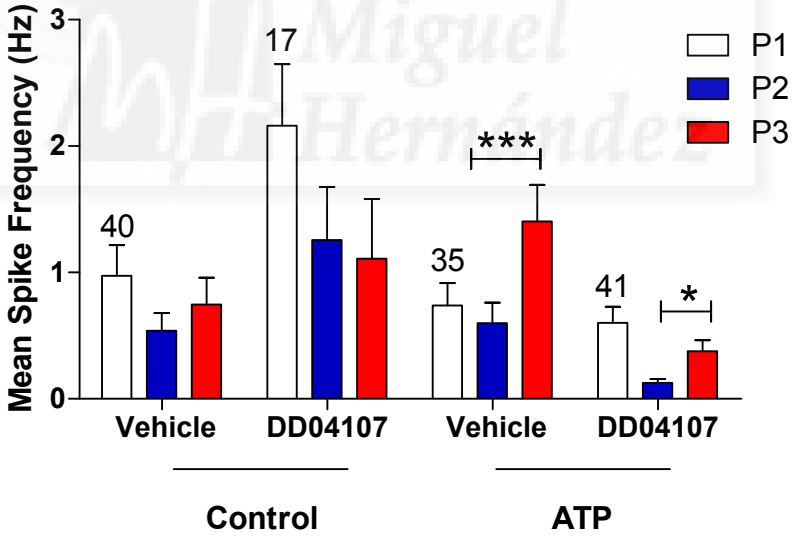

Figure- 31: DD04107 abrogates ATP induced inflammatory sensitization of TRPV1 mediated neuronal firing in rat DRG neurons. Effect of 20 µM DD04107 on 10 µM ATP mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in each groups and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (***- $p<0.001$, *- $p<0.05$).

We have calculated the ratio of third pulse to second pulse (P3/P2) in both Vehicle and DD04107 treated groups exposed to ATP. Then, the impact of DD04107 on blocking the

potentiation of TRPV1 channel induced by ATP treatment was compared between Vehicle and DD04107 groups as shown in **figure- 32**. A significant blockage of TRPV1 potentiation on ATP treated neurons pre incubated with DD04107 (2.6±0.5) compared to the Vehicle (5.5±1.5) (**- $p<0.01$) was observed. Although ATP induced TRPV1 exocytosis is mainly due to the release of intracellular Ca^{2+}, no such potentiation was observed under control conditions, which confirms that external Ca^{2+} ions entering through TRPV1 were not involved.

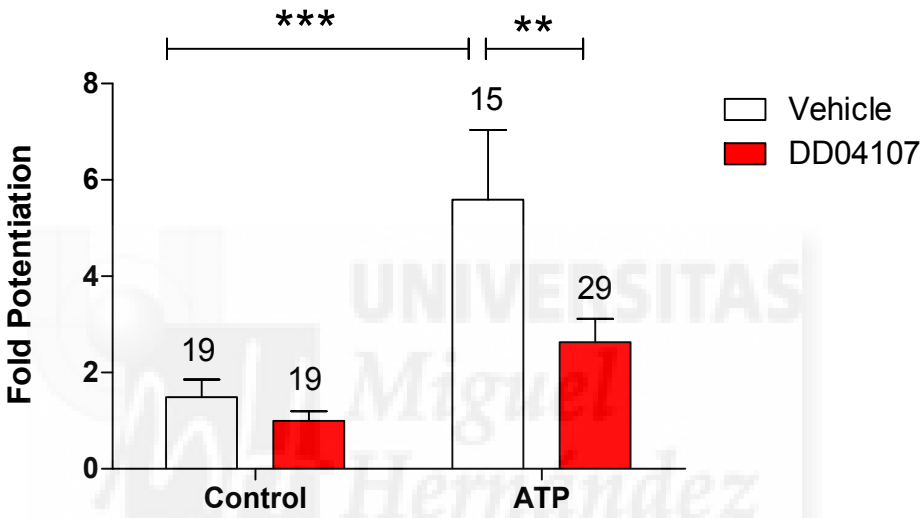

Figure- 32: ATP mediated TRPV1 sensitization requires channel recruitment in a subset of nociceptors. Effect of 20 µM DD04107 on 10 µM ATP mediated fold potentiation of TRPV1 activity was determined by comparison of capsaicin-mediated neuronal spikes before and after ATP incubation (ratio P3/P2) of the groups (vehicle, DD04107). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by two- way ANOVA with Bonferroni's post hoc test (**- $p<0.01$, ***- $p<0.001$).

Neuronal exocytosis is responsible for ATP induced TRPV1 sensitization in $IB4^-$ neurons but not for $IB4^+$ neurons

Using MEA technique, we found that ATP induced sensitization of TRPV1 excitability was due to the release of new TRPV1 channels and their insertion into the membrane in a subset of nociceptors. Next we wanted to know which subtype of nociceptor was involved in mobilization of TRPV1 channels upon ATP exposure. For that purpose ATP induced TRPV1 sensitization was analysed in both peptidergic ($IB4^-$) and nonpeptidergic ($IB4^+$) subpopulations. Capsaicin induced desensitization of TRPV1 currents in $IB4^-$ nociceptors was potentiated by 10 µM ATP as shown in **figure- 33 A (Vehicle)**. Notably,

ATP induced TRPV1 sensitization in IB4$^-$ nociceptors was blocked by 100 µM nonpalmitoylated DD04107 (represented as Peptide) delivered to the neuronal cytosol through the pipette as shown in **figure- 33 A (Peptide)**. Similarly in IB4$^+$ neurons, ATP induced TRPV1 sensitization was observed, but it was insensitive to the blocker of neuronal exocytosis peptide as shown in **figure- 33 (B)**.

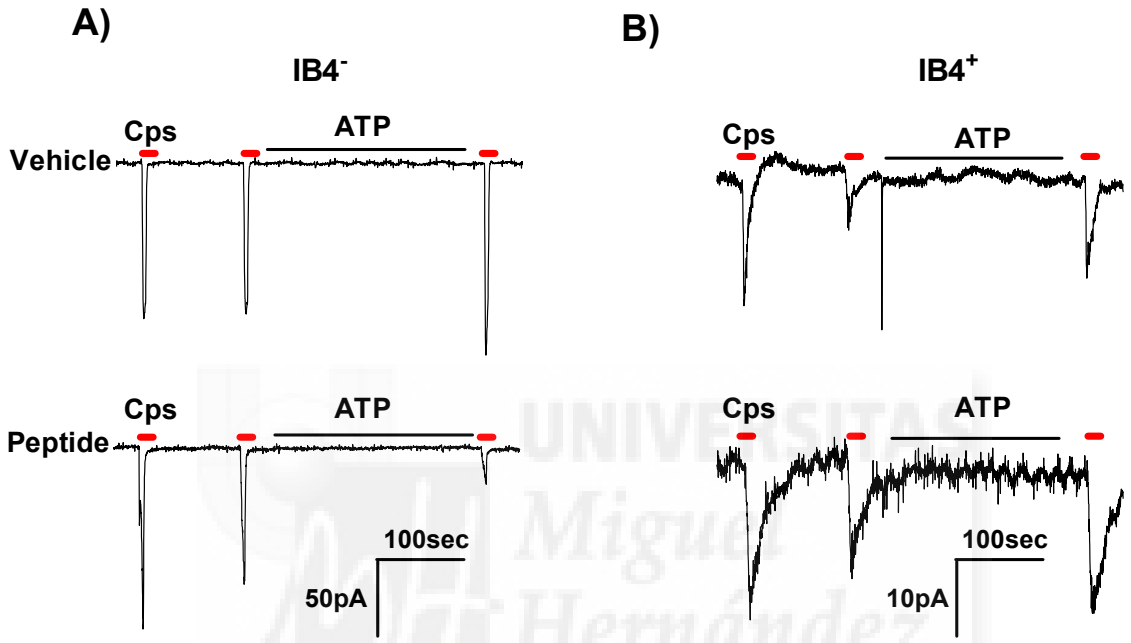

Figure- 33: Nonpalmitoylated DD04107 (Peptide) mediated abrogation of ATP induced TRPV1 sensitization occurs primarily in peptidergic nociceptors. Representative voltage clamp recordings of ATP induced sensitization of TRPV1 currents elicited by capsaicin (1 µM, 10 s) and the abrogating effect of the peptide in IB4$^-$ and IB4$^+$ nociceptors (A, B). Cells were held at -60 mV. 100 µM nonpalmitoylated DD04107 was given through the patch pipette and incubated for 10 minutes after forming the seal. Three repetitive pulses of capsaicin at 1 µM concentration were given for the continuous protocol. 10 µM ATP was applied between second and third pulse of capsaicin.

Fold potentiation of TRPV1 currents was calculated by taking the ratio of P3 to P2. As shown in **figure- 34** both IB4$^-$ and IB4$^+$ subpopulations had no significant changes in fold potentiation between Vehicle and nonpalmitoylated DD04107 groups under control conditions. In IB4$^-$ nociceptors, fold increase in current intensity was significantly higher after ATP incubation compared to control Vehicle group (*- $p<0.05$). ATP induced increase in TRPV1 potentiation was significantly blocked by 100 µM nonpalmitoylated DD04107 (**- $p<0.01$). Similarly, in IB4$^+$ nociceptors fold increase in current intensity was significantly higher after ATP incubation compared to control Vehicle group (**- $p<0.01$),

whereas ATP induced increase in TRPV1 potentiation was not blocked by 100 µM nonpalmitoylated DD04107 although a tendency was observed. This could be due to the possible involvement of differential TRPV1 sensitization mechanism involved in IB4$^-$ (exocytosis) and IB4$^+$ (phosphorylation) nociceptors.

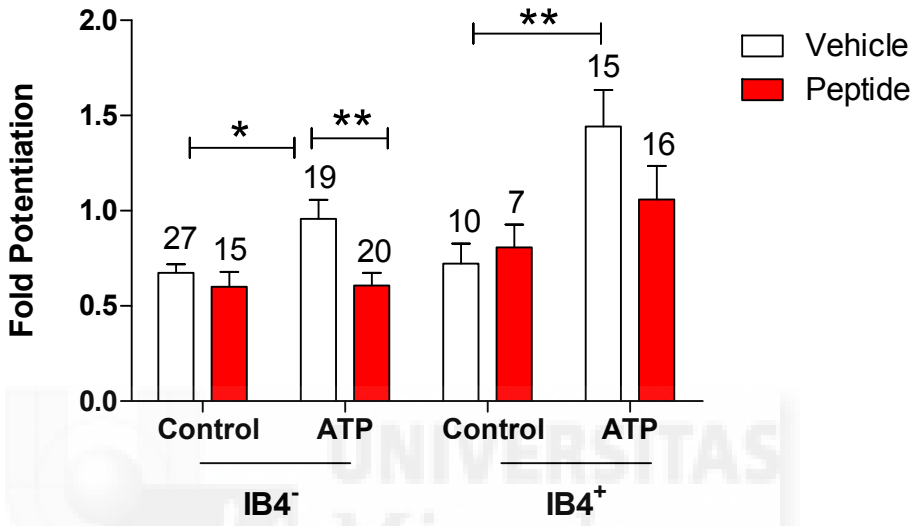

Figure- 34: Nonpalmitoylated DD04107 lessens ATP induced inflammatory sensitization of TRPV1 channel in peptidergic but not in nonpeptidergic nociceptors. Effect of 100 µM nonpalmitoylated DD04107 on 10 µM ATP mediated fold potentiation of TRPV1 activity was determined by comparison of capsaicin-mediated current intensities before and after incubation of ATP (ratio P3/P2) of the groups (Control Vehicle, Control Peptide, ATP Vehicle, ATP Peptide) for both IB4$^-$ and IB4$^+$ subpopulations. Data are expressed as mean ± SEM. The numbers above the bars represent the total number of neurons registered. Number of cultures = 4. Statistical analysis was performed by two-way ANOVA with Bonferroni's post hoc test (*- $p<0.05$, **- $p<0.01$).

Further we focussed on the fold potentiation (ratio P3/P2) to check the percentage of neurons that exhibited sensitization of TRPV1 induced by ATP in IB4$^-$ and IB4$^+$ subpopulations, as shown in **figure- 35**. There was a significant difference in the percentage of neurons that exhibited potentiated response to capsaicin after incubation with ATP, compared between Vehicle and nonpalmitoylated DD04107 treated groups. In fact in IB4$^-$ group, exposure to ATP induced 30 % of neurons in the Vehicle group with potentiated response, whereas in DD04107 treated group we observed a significant blockage of ATP induced TRPV1 potentiation with only 5 % of neurons being potentiated. In IB4$^+$ group, a higher percentage of neurons exhibited potentiation of TRPV1 currents induced by ATP in both Vehicle (60 % of neurons) and DD04107 treated groups (40 % of neurons). This substantiates that there is a possible involvement of two distinct potentiation mechanisms in IB4$^-$ (through exocytosis) and IB4$^+$ (through channel gating mechanism) subpopulations.

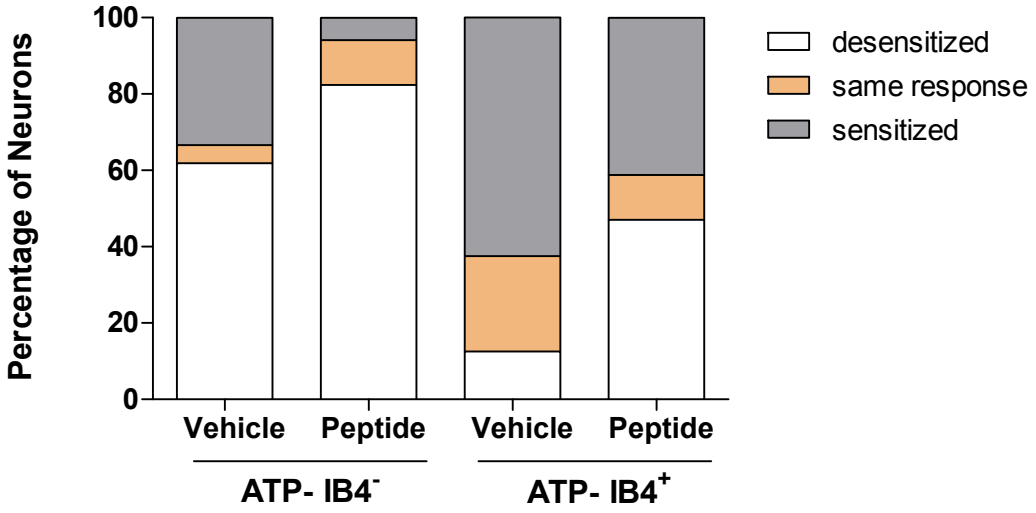

Figure- 35: Percentage of Neurons with ATP induced potentiation of TRPV1 activity in peptidergic and nonpeptidergic nociceptors treated with Vehicle and nonpalmitoylated DD04107 (Peptide). Percentage of neurons with potentiated TRPV1 activity when treated with 10 μM ATP were determined in both peptidergic and nonpeptidergic rat DRG neurons. The values of the ratio P3/P2 in both vehicle and peptide treated groups were calculated. The criteria is when the ratio is lesser than 0.9 the neurons are desensitized; ratio of 0.9-1.1 there is no significant difference between the second and third pulses; ratio greater than 1.1 the channel is getting sensitized with ATP.

ATP mediated TRPV1 potentiation in IB4$^+$ but not in IB4$^-$ subpopulation is PKC dependent

Since we found that the potentiation of TRPV1 by ATP was due to the differential mechanism involved in IB4$^-$ (primarily due to exocytosis) and IB4$^+$ (primarily due to channel gating) neurons, we could not exclude the possibility of phosphorylation mechanism involved in both subpopulations. To confirm that we used a general PKC inhibitor and examined whether PKC is involved in ATP induced TRPV1 potentiation in both IB4$^-$ and IB4$^+$ neurons. Bisindolylmaleimide (BIM) was used at 1 μM concentration and given through the patch pipette (10 minutes incubation after forming the seal). Application of three repetitive pulses of 1 μM capsaicin induced desensitization of TRPV1 channel that was significantly recovered by the application of 10 μM ATP between the second and third pulses. As shown in **figure-36** ATP induced sensitization of TRPV1 currents was not affected in IB4$^-$ neurons treated with BIM, whereas in IB4$^+$ neurons, potentiation of TRPV1 currents by ATP was strongly inhibited by BIM.

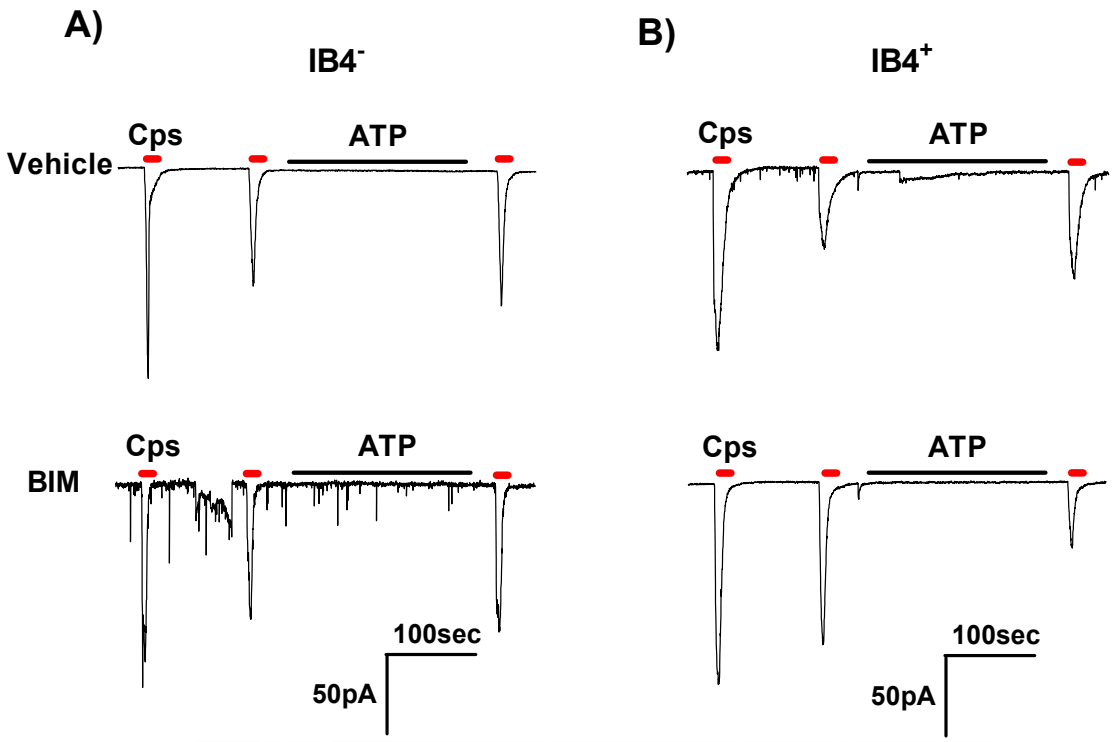

Figure- 36: PKC inhibitor (BIM) prevents the potentiation of TRPV1 induced by ATP in IB4$^+$ but not in IB4$^-$ neurons. Representative voltage clamp recordings of currents elicited by capsaicin (1 µM, 10 s) in IB4$^-$ and IB4$^+$ nociceptors (A, B). Cells were held at -60 mV. 1 µM BIM was given through the patch pipette and incubated for 10 minutes after forming the seal. Three repetitive pulses of capsaicin at 1 µM concentration were given for the continuous protocol. 10 µM ATP was applied between second and third pulse of capsaicin.

Fold potentiation of TRPV1 currents was calculated by taking the ratio of P3 to P2. **Figure- 37** shows that in IB4$^-$ and IB4$^+$ subpopulations under control conditions no significant changes in fold potentiation were observed in both Vehicle and BIM treated groups. In IB4$^-$ nociceptors, fold increase in current intensity was significantly higher after ATP incubation (*- $p<0.05$) compared to control Vehicle group. ATP induced TRPV1 potentiation was not affected by 1 µM BIM. Similarly, in IB4$^+$ nociceptors fold increase in current intensity was significantly higher after ATP incubation (**- $p<0.01$) compared to control Vehicle group. ATP induced TRPV1 potentiation was significantly blocked by 1 µM BIM (*- $p<0.05$).

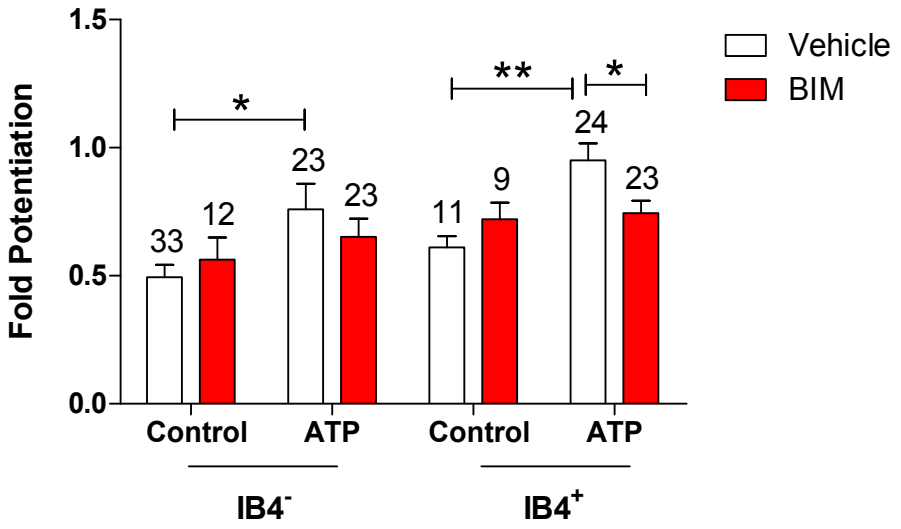

Figure- 37: BIM (PKC inhibitor) abolishes ATP induced inflammatory sensitization of TRPV1 channel in nonpeptidergic nociceptors. Effect of 1 μM BIM on 10 μM ATP mediated fold potentiation of TRPV1 activity was determined by comparison of capsaicin-mediated current intensities before and after incubation of ATP (ratio P3/P2) of the groups (Control Vehicle, Control BIM, ATP Vehicle, ATP BIM) for both $IB4^-$ and $IB4^+$ subpopulations. Data are expressed as mean ± SEM. The numbers above the bars represent the total number of neurons registered. Number of cultures = 4. Statistical analysis was performed by two-way ANOVA with Bonferroni's post hoc test (*- $p<0.05$, **- $p<0.01$).

In addition, we focused on the fold potentiation (ratio P3/P2) to check the percentage of $IB4^-$ and $IB4^+$ neurons that exhibited potentiation of TRPV1 induced by ATP as shown in **figure- 38**. There was a significant difference in the percentage of neurons that exhibited potentiated response to capsaicin after incubation with ATP, compared with control groups. In fact, in $IB4^-$ group exposure to ATP induced 18 % of neurons with a potentiated response in the Vehicle group. We have not observed blockage of ATP induced TRPV1 potentiation in BIM treated neurons which showed 13 % of neurons with a potentiated response, although a tendency was observed. Similarly, in $IB4^+$ group exposure to ATP induced a higher percentage of neurons with potentiated responses in Vehicle group (16 % of neurons), whereas neurons treated with BIM exhibited a complete blockage of TRPV1 potentiation induced by ATP. Hence, it confirms that ATP induced sensitization of TRPV1 in $IB4^+$ nonpeptidergic neurons is PKC dependent, but not in $IB4^-$ peptidergic neurons where TRPV1 exocytosis is primarily involved, though a possible role of PKC cannot be excluded.

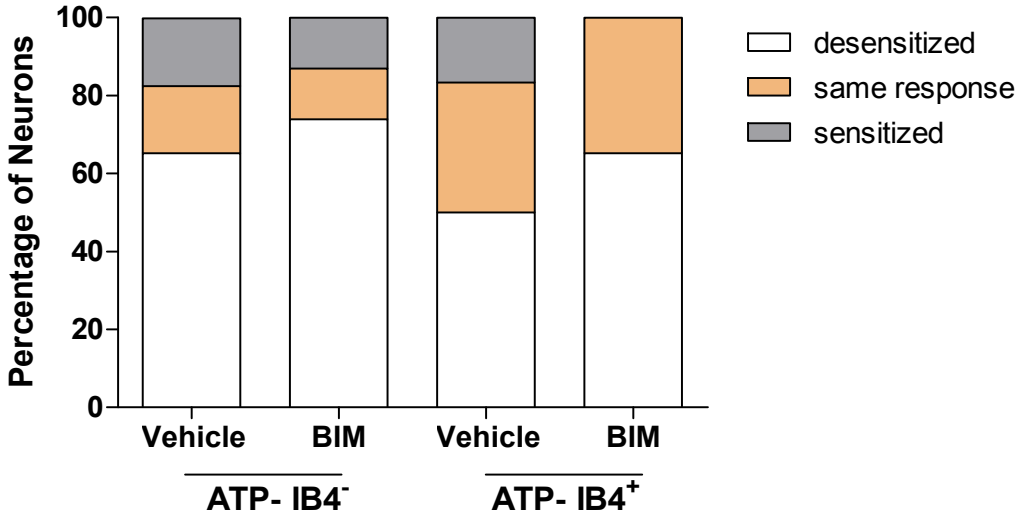

Figure- 38: Percentage of Neurons with ATP induced potentiation of TRPV1 activity in peptidergic and nonpeptidergic nociceptors treated with Vehicle and BIM. Percentage of neurons with potentiated TRPV1 activity when treated with 10 µM ATP was determined in both peptidergic and nonpeptidergic rat DRG neurons. The value of the ratio P3/P2 in both Vehicle and BIM treated groups was calculated. The criteria is when the ratio is lesser than 0.9 the neurons are desensitized; ratio of 0.9-1.1 there is no significant difference between the second and third pulses; ratio greater than 1.1 the channel is getting sensitized with ATP.

Role of αCGRP and Tac1 on ATP induced inflammatory sensitization of TRPV1

Experiments from rat DRG neurons have confirmed that peptidergic nociceptors are primarily involved in recruiting new TRPV1 channels from the vesicles to the plasma membrane upon ATP exposure. We further extended our studies to know the functional mechanism behind exocytotic release of TRPV1 during inflammation. We focused on the role of two primary neuropeptides, αCGRP and Tac1 (encoding SP) in sensitizing TRPV1. We hypothesize that there is a possible involvement of these two neuropeptides in sorting TRPV1 channels in large dense core vesicles (LDCVs) and driving their regulated exocytosis upon inflammatory (ATP) insult. We used $\alpha CGRP^{-/-}$ and $Tac1^{-/-}$ gene knockout mice and generated a double knockout mice model of $\alpha CGRP^{-/-}xTac1^{-/-}$ (referred to as DKO). We studied ATP induced potentiation of TRPV1 in $\alpha CGRP^{-/-}xTac1^{-/-}$compared with wild type mice.

Recently, it has been reported that TRPV1 is primarily restricted to peptidergic subpopulation during development in mice and it does not co localise with IB4 (a marker for nonpeptidergic nociceptors) (199). All our experiments were done in adult mice for both wild

type and double knockout models with 12- 15 weeks of age to be sure that the observed TRPV1 responses we studied were from peptidergic nociceptors. We studied ATP induced potentiation of TRPV1 evoked neuronal spikes in wild type and DKO mice models using MEA technique.

Removal of neuropeptides did not affect basal TRPV1 responses and the intrinsic membrane properties of nociceptors

Before performing sensitization experiments we wanted to know if the removal of neuropeptides potentially affects the basal TRPV1 responses and the electrical properties of nociceptors. Capsaicin responses and intrinsic electrogenic properties in both wild type and DKO mice were studied using patch clamp technique.

A capsaicin dose response curve on TRPV1^{+} DRG neurons from wild type and DKO mice exhibited no significant changes with no alteration in the EC_{50} (0.7±0.1 µM for WT vs 0.5±0.1 µM for DKO) as shown in **figure- 39**.

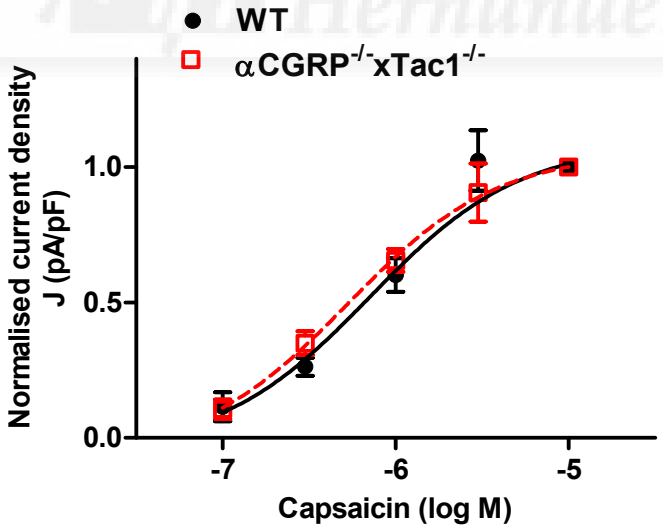

Figure- 39: Capsaicin dose-response curves from wild type and αCGRP$^{-/-}$xTac1$^{-/-}$ DRG at -60 mV. Ionic currents evoked by different concentrations of capsaicin were measured in wild type and αCGRP$^{-/-}$xTac1$^{-/-}$ nociceptors, by patch clamp in whole-cell configuration and standard external solution was used.

Also we studied electrogenic properties of the nociceptors from wild type and DKO mice positive for TRPV1. As shown in **table- 2** we did not observe any significant changes in

the electrical properties measured for resting membrane potential, current threshold, threshold potential, action potential amplitude, action potential overshoot, action potential duration and after hyperpolarization amplitude in both wild type and DKO mice.

	WT	αCGRP$^{-/-}$xTac1$^{-/-}$
RMP (mV)	-45.6 ± 2.3 (13)	-44.9 ± 2.2 (21)
CT (pA)	12.5 ± 2.5 (13)	14.7 ± 3.0 (21)
TP (mV)	-32.8 ± 1.6 (13)	-33.5 ± 1.8 (21)
AP amplitude (mV)	67.0 ± 4.1 (13)	68.6 ± 4.3 (21)
AP overshoot (mV)	23.0 ± 2.6 (13)	19.5 ± 2.5 (21)
AP duration (ms)	21.5 ± 0.4 (13)	21.2 ± 0.5 (21)
AHP amplitude (mV)	9.2 ± 1.3 (13)	8.9 ± 1.6 (21)

Table- 2: Intrinsic electrogenic properties of TRPV1 sensitive DRG neurons in wild type and αCGRP$^{-/-}$ xTac1$^{-/-}$ mice. RMP: resting membrane potential; CT: the depolarized current threshold for evoking the 1st action potential; TP: threshold potential; AHP: afterhyperpolarization; AP: action potential. No changes were observed in αCGRP$^{-/-}$ x Tac1$^{-/-}$ compared to wild type DRG neurons as assessed by the intrinsic electrogenic parameters. Data are expressed as mean ± SEM (n). Statistical analysis was performed by Unpaired Student's t-test.

ATP induced sensitization of neuronal firing mediated through TRPV1 in wild type and αCGRP$^{-/-}$xTac1$^{-/-}$mice nociceptors

ATP induced sensitization of TRPV1 excitability in nociceptors from wild type and DKO mice DRG neurons was evaluated using MEA technique. Application of capsaicin led to activation of TRPV1 channel in DRG neurons, inducing changes in ionic flow, which was detected as a train of evoked neuronal spikes, as shown in Vehicle for both wild type and DKO mice neurons **(figure- 40 A and B)** and represented as Mean Spike Frequency (Hz). In wild type nociceptors, repetitive capsaicin pulses induced desensitization of the vanilloid-evoked electrical responses. Desensitized capsaicin responses could be recovered with ATP sensitization and blocked by DD04107 **(figure- 40 A and C)**. In DKO nociceptors, capsaicin also induced neuronal activity. Vanilloid-induced TRPV1 tachyphylaxia was partially overturned by ATP **(figure- 40 B)** and virtually insensitive to DD04107 **(figure- 40 D)**.

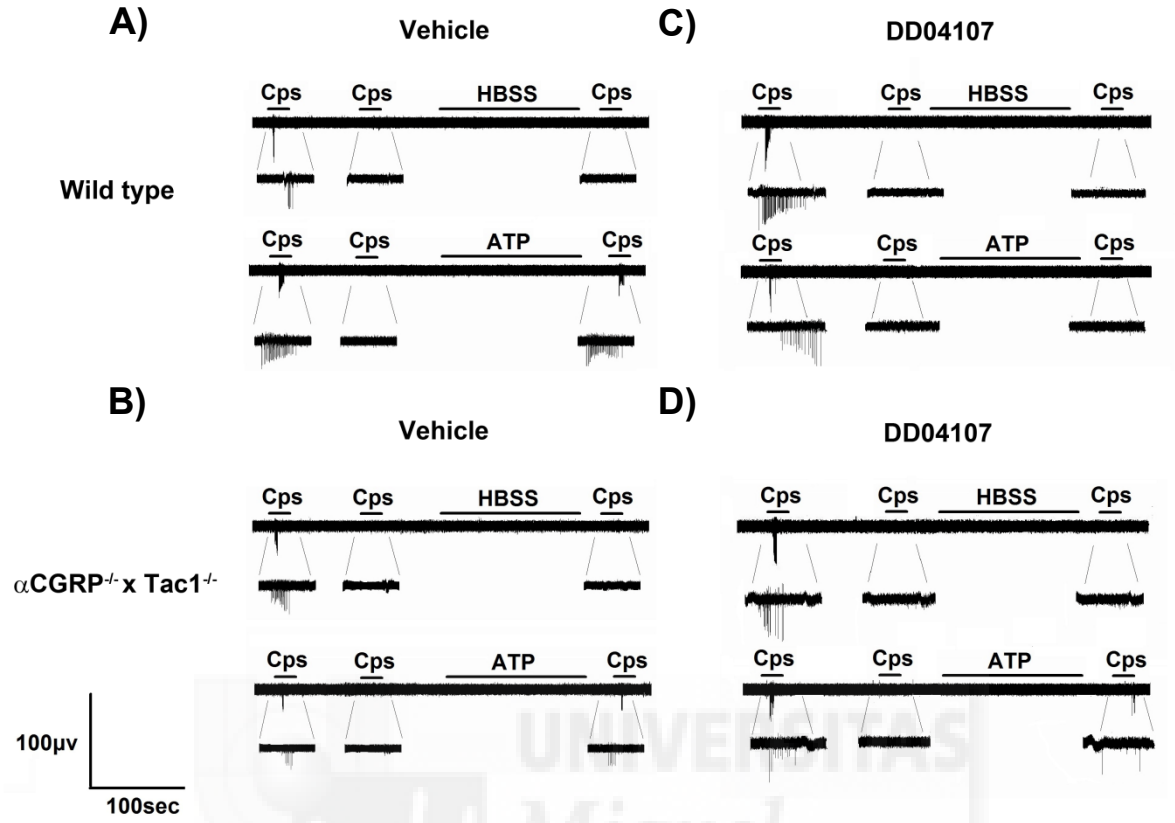

Figure- 40: **ATP induced sensitization of neuronal firing mediated through TRPV1 in wild type and αCGRP$^{-/-}$xTac1$^{-/-}$mice nociceptors**. Repetitive application of capsaicin 500 nM induced tachyphylaxis of TRPV1 mediated neuronal firing and it was reversed by sensitizing the neurons with 10 μM ATP in wild type and αCGRP$^{-/-}$xTac1$^{-/-}$ mice nociceptors.

DD04107 abrogates ATP induced sensitization of TRPV1 evoked neuronal spikes in wild type nociceptors

Consecutive application of capsaicin led to desensitization in the number of spikes evoked and desensitized responses could be significantly recovered (***- $p<0.001$) with ATP sensitization (10 μM ATP was applied between P2 and P3) in wild type nociceptors. Some neurons exposed to ATP evoked fast neuronal spikes, due to the activation of ionotropic P2X3 receptor, which is widely expressed in nociceptors. ATP induced sensitization of TRPV1 evoked excitability from wild type DRG neurons was blocked by DD04107, indicating that ATP induced TRPV1 sensitization was mainly due to the insertion of new TRPV1 channels into the plasma membrane, as shown in **figure- 41**.

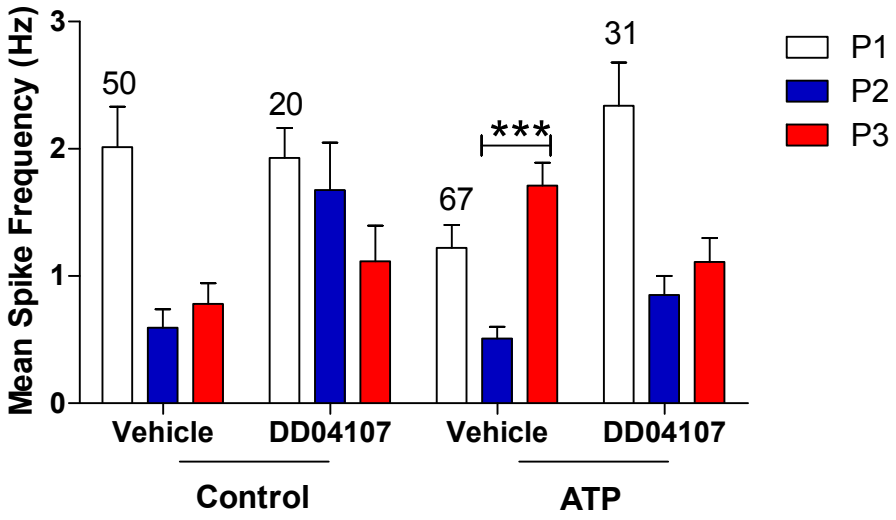

Figure- 41: DD04107 abolishes ATP induced inflammatory sensitization of TRPV1 mediated neuronal firing in wild type mice DRG neurons. Effect of 20 µM DD04107 on 10 µM ATP mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in each group and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 4. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (***- $p<0.001$).

DD04107 did not affect ATP induced sensitization of TRPV1 evoked neurons spikes in αCGRP$^{-/-}$xTac1$^{-/-}$ nociceptors

In DKO mice neurons also repetitive application of capsaicin induced desensitization in the number of spikes evoked and it was significantly recovered (*- $p<0.05$) with ATP sensitization, though it was less significant compared to wild type DRG neurons. However DRG neurons from DKO mice model did not show any blockage of TRPV1 potentiation after treatment with DD04107 as we see a significant potentiation of TRPV1 activity (*- $p<0.05$) by ATP in DD04107 treated group as shown in **figure- 42**. Hence these results show the significant role of neuropeptides on promoting TRPV1 exocytosis. The remnant potentiation we observed in DD04107 treated group could be due to the modification of channel gating through phosphorylation process and not through TRPV1 exocytosis.

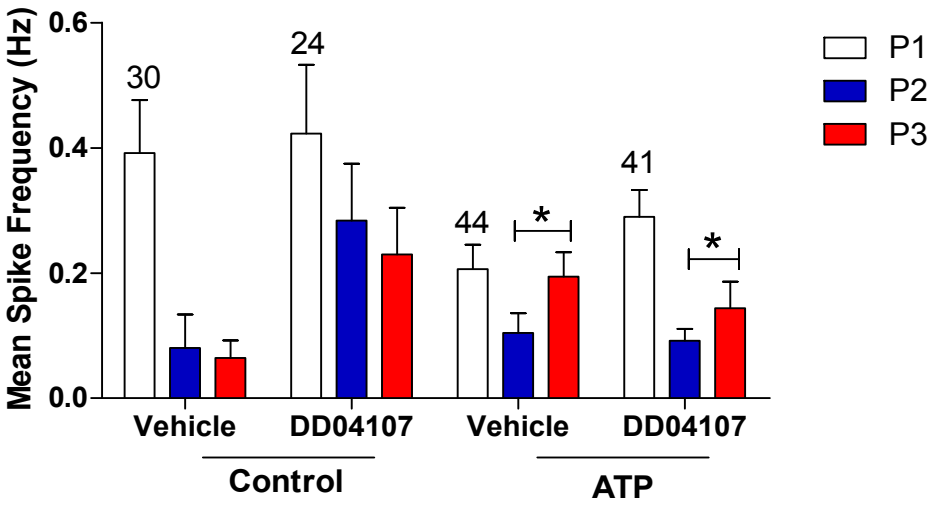

Figure- 42: DD04107 does not abolish ATP induced inflammatory sensitization of TRPV1 mediated neuronal firing in αCGRP$^{-/-}$xTac1$^{-/-}$ mice DRG neurons. Effect of 20 μM DD04107 on 10 μM ATP mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in each group and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures =4. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (*- $p<0.05$).

Neuropeptides did not sensitize TRPV1 through autocrine signalling

The reduction of TRPV1 potentiation by ATP in DKO mice models could be due to the strong impact of the neuropeptides αCGRP and Tac1. It is possible that the release of neuropeptides along with TRPV1 from the vesicles could activate their own receptors CGRP1 and NK1 through autocrine signalling, thereby potentiating TRPV1 responses. Hence we evaluated the effect of blocking these receptors on ATP mediated TRPV1 potentiation from wild type DRG neurons. As shown in **figure- 43** blockade of CGRP1 and NK1 receptors with their specific antagonists did not suppress ATP induced potentiation of TRPV1 evoked neuronal excitability. These results indicate that ATP induced potentiation of TRPV1 in peptidergic nociceptors is not due to the activation of CGRP1 and NK1 receptors.

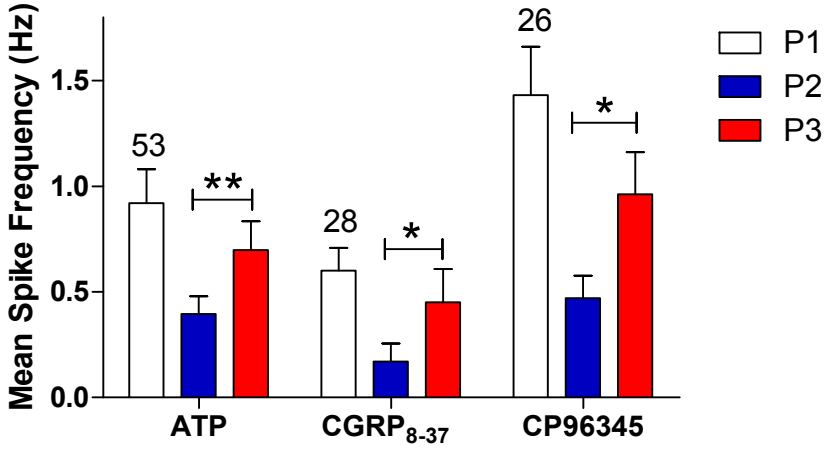

Figure- 43: Neuropeptides CGRP and SP does not sensitize TRPV1 through autocrine signalling in wild type nociceptors. Effect of αCGRP (250 nM CGRP $_{8-37}$) and Tac1 (10 μM CP96345) receptor antagonists was studied by co application with 10 μM ATP on potentiation of TRPV1 evoked neuronal firing activity, determined by comparison of all the three pulses (P1, P2, P3) in each group and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes responded. Number of cultures = 3. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (*- $p<0.05$, **- $p<0.01$).

αCGRP is essential for ATP induced sensitization of TRPV1

In order to know which neuropeptide was involved in regulating TRPV1 exocytosis, we next studied the role of each neuropeptide in the nociceptors from αCGRP$^{-/-}$ and Tac1$^{-/-}$ single knockout mice. We evaluated ATP induced potentiation of TRPV1 evoked spikes in single knockouts compared with wild type mice. Our result shows that capsaicin induced desensitization of TRPV1 evoked neuronal spikes was completely reversed by ATP in nociceptors from Tac1$^{-/-}$ mice. Nevertheless, abrogation of αCGRP expression eliminated ATP induced increase in mean spike frequency of TRPV1 evoked neuronal firing as shown in **figure- 44**. Together, these results indicate that αCGRP expression is necessary for ATP induced TRPV1 potentiation in peptidergic nociceptors, whereas SP plays a marginal role.

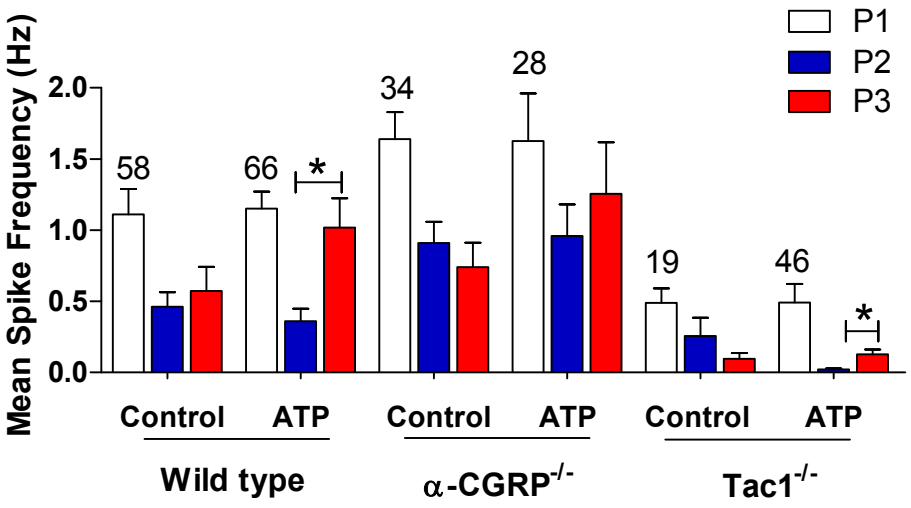

Figure- 44: αCGRP but not Tac1 is involved in ATP induced TRPV1 sensitization. Effect of 10 μM ATP mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in each group (wild type, $\alpha CGRP^{-/-}$ and $Tac1^{-/-}$) and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 4. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (*- $p<0.05$).

Experimental depolarization does not sensitize TRPV1

We found that ATP induced TRPV1 exocytosis is mainly due to the elevation of intracellular Ca^{2+} ions, which activates SNARE complex machinery. Many studies on DRG neurons revealed that experimental depolarization stimulated by higher KCl concentration could induce exocytosis by increasing intracellular Ca^{2+} concentration. Such depolarizing stimuli could induce the release of neuropeptides CGRP and SP. We found that the removal of these two neuropeptides significantly affects ATP induced TRPV1 potentiation. Thus, we questioned whether electrical stimulation of the neurons through depolarization could induce exocytosis of TRPV1 carrying vesicles and potentiate TRPV1 activity similar to ATP. Hence we performed experiments on nociceptors from wild type mice and compared depolarization induced TRPV1 potentiation with ATP treated groups. As shown in **figure- 45** depolarizing the neurons with 40 mM KCl for 8 minutes did not potentiate TRPV1 evoked neuronal firing compared to ATP treated neurons which showed a significant potentiation (***- $p<0.001$) of TRPV1 spikes. It evidently demonstrates that the inflammatory sensitization of TRPV1 due

to the recruitment of new channels from LDCVs to the neuronal surface is specific for inflammatory mediator ATP, but not for depolarizing pulses.

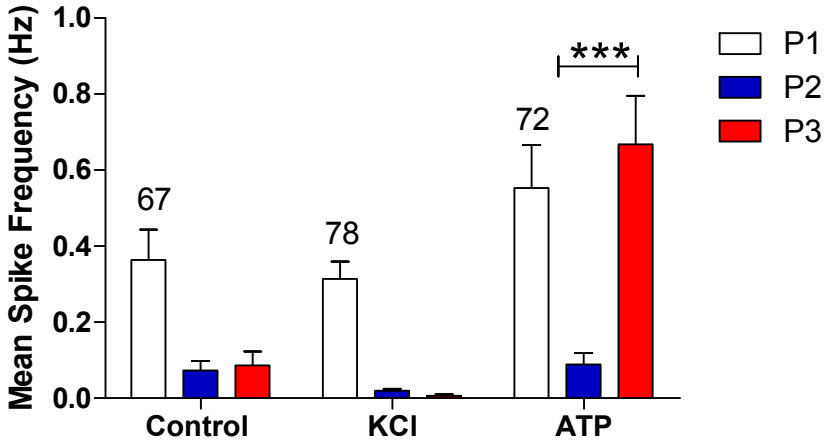

Figure- 45: Depolarization of nociceptors does not sensitize TRPV1 in wild type mice. Effect of 10 μM ATP and 40 mM KCl mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in each group and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (***- $p<0.001$).

BK INDUCED INFLAMMATORY SENSITIZATION OF TRPV1

Blockage of neuronal exocytosis also lessens BK induced TRPV1 excitability

Earlier experiments with ATP revealed that DD04107 significantly reduced the sensitization of TRPV1, which primarily occurs in peptidergic nociceptors. Furthermore, knocking down of the genes αCGRP and Tac1 corresponding to the neuropeptides αCGRP and SP had a high impact on exocytotic inflammatory sensitization of TRPV1, with αCGRP playing a major role in TRPV1 vesicular release. As we cited earlier bradykinin (BK), a potent inflammatory mediator, is also released during tissue injury. BK is reportedly involved in increasing the membrane expression and trafficking of opioid receptors in sensory neurons (316). Previous studies from our group reported that DD04107 abolished only ATP but not BK mediated TRPV1 potentiation suggesting that not all pro inflammatory mediators promote the exocytotic release of TRPV1 (312).

In addition, recent studies revealed that BK induced CGRP release is dose dependent: acute incubation (10 minutes) of DRG neurons with 1 µM BK enhanced the release of CGRP (317). Akin to ATP, BK also exerts its action through $G\alpha_{q/11}$ signalling pathway activated by BK2 receptor. Henceforth, we proposed that BK could also induce sensitization of TRPV1 through exocytotic vesicular release of new TRPV1 channels.

In order to unravel the mechanism behind BK induced TRPV1 sensitization in rat DRG neurons, we performed both MEA and patch clamp technique on $IB4^-$ and $IB4^+$ nociceptors, similar to ATP experiments. In MEA experiments consecutive application of capsaicin led to desensitization in the number of spikes evoked and desensitized responses could be significantly recovered after BK sensitization (1 µM BK was applied between P2 and P3) **(figure- 46 Vehicle)**. BK induced sensitization of TRPV1 evoked excitability was abrogated by DD04107 **(figure- 46 DD04107)**. This indicates that BK induced TRPV1 sensitization was mainly due to the release of new TRPV1 channels from the vesicles and their insertion into the plasma membrane in a subset of nociceptors.

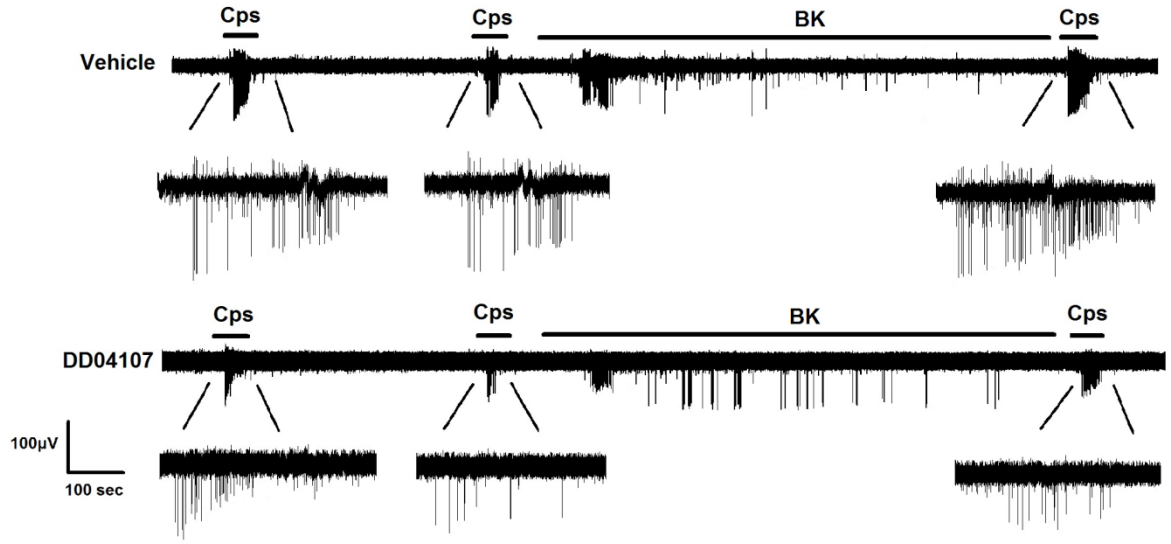

Figure- 46: DD04107 abrogates, BK sensitized TRPV1 evoked nociceptor excitability. Representative traces of potentiation of capsaicin (500 nM, 15 s) responses elicited by applying 1 μM of BK between the second (P2) and third (P3) vanilloid pulse (top), in neonatal rat DRG neurons. Preincubation of the neurons with 20 μM DD04107 (bottom) also potentiated TRPV1 but it was less significant compared to Vehicle (top).

Though we saw a less significant potentiation of TRPV1 activity by BK in DD04107 treated group (*- $p<0.05$) compared to Vehicle group (***- $p<0.001$), we still observed potentiation of TRPV1 evoked excitability as shown in **figure- 47**. Hence it was important to compare the fold increase in BK induced potentiation of TRPV1 between Vehicle and DD04107 treated groups.

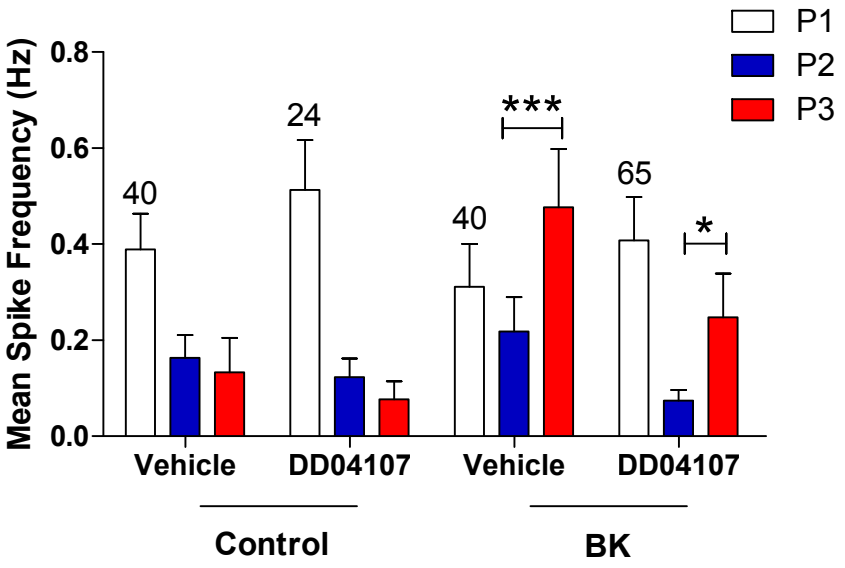

Figure- 47: DD04107 lessens BK induced inflammatory sensitization of TRPV1 in neonatal Rat DRG neurons. Effect of 20 µM DD04107 on 1 µM BK mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in each group and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (*- $p<0.05$, ***- $p<0.001$).

We analysed the ratio of third pulse to second pulse (P3/P2) in both vehicle and DD04107 treated groups exposed to BK. Then, the impact of DD04107 on blocking the potentiation of TRPV1 channel induced by BK treatment was compared between Vehicle and DD04107 groups. We observed a significant blockage of BK induced TRPV1 potentiation on neurons pre incubated with DD04107 (3±0.4) compared to the Vehicle (7±2.5) (*- $p<0.05$) as shown in **figure- 48**. BK induced TRPV1 exocytosis was mainly due to the release of intracellular Ca^{2+}. No such potentiation was observed under control conditions, which confirms that external Ca^{2+} ions entered through TRPV1 were not involved.

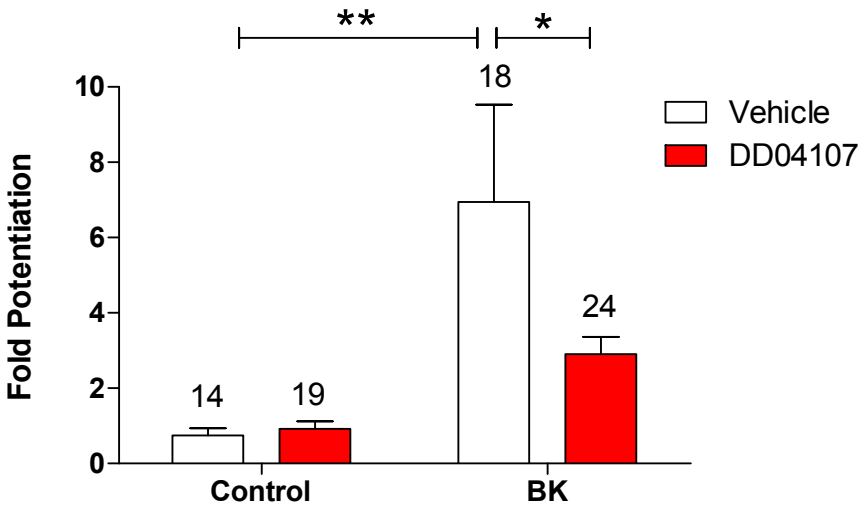

Figure- 48: BK mediated TRPV1 sensitization requires channel recruitment in a subset of nociceptors. Effect of 20 µM DD04107 on 1 µM BK mediated fold potentiation of TRPV1 activity was determined by comparison of capsaicin-mediated neuronal spikes before and after incubation of BK (ratio P3/P2) of the groups (Vehicle, DD04107). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by two- way ANOVA with Bonferroni's post hoc test (*- $p<0.05$, **- $p<0.01$).

Neuronal exocytosis is responsible for BK induced TRPV1 sensitization in IB4$^-$ neurons but not in IB4$^+$ neurons

Using MEA technique, we found that BK induced sensitization of TRPV1 excitability was due to the release of new TRPV1 channels and their insertion into the membrane in a subset of nociceptors. We wanted to identify which subtype of nociceptors (peptidergic or nonpeptidergic) was involved in TRPV1 mobilization upon BK exposure using patch clamp technique. Capsaicin induced desensitized ionic currents in IB4$^-$ nociceptors were potentiated by 1 µM BK (BK was applied between P2 and P3) as shown in **figure- 49 A Vehicle**. Notably, BK induced TRPV1 sensitization in IB4$^-$ sensory neurons was blocked by 100 µM nonpalmitoylated DD04107 (represented as Peptide) delivered to the neuronal cytosol through the pipette as shown in **figure- 49 A Peptide**. In contrast to IB4$^-$ neurons, BK induced TRPV1 sensitization in IB4$^+$ sensory neurons showed differential responses, since in IB4$^+$ neurons we did not observe significant TRPV1 potentiation as shown in **figure- 49 B**.

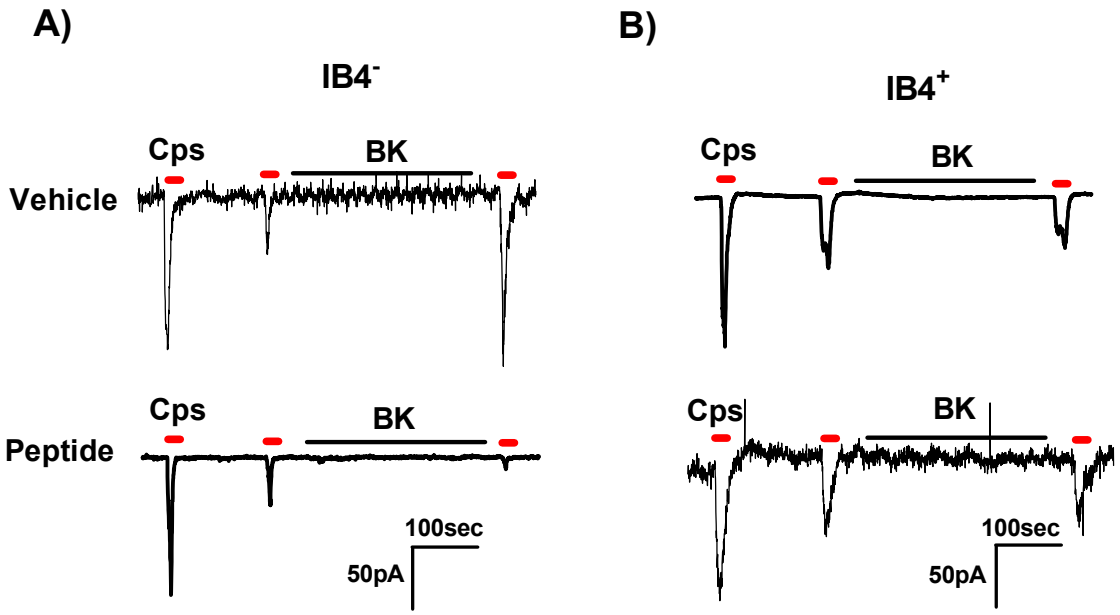

Figure- 49: Non-palmitoylated DD04107 (Peptide) mediated abrogation of BK induced TRPV1 sensitization occurs primarily in peptidergic nociceptors. Representative voltage clamp recordings of currents elicited by capsaicin (1 µM, 10 s) in $IB4^-$ and $IB4^+$ nociceptors (A, B). Cells were held at -60 mV. 100 µM non-palmitoylated DD04107 was given through the patch pipette and incubated for 10 minutes after forming the seal. Three repetitive pulses of capsaicin at 1 µM concentration were given for the continuous protocol. 1 µM BK was applied between second and third pulse of capsaicin.

Fold potentiation of TRPV1 currents was calculated by taking the ratio of P3 to P2 (P3/P2- since BK was applied between P2 and P3). **Figure- 50** shows that in $IB4^-$ and $IB4^+$ subpopulations no significant changes in fold potentiation were observed under control conditions in both Vehicle and non-palmitoylated DD04107 groups. In $IB4^-$ nociceptors, fold increase in TRPV1 current intensity was significantly higher after BK incubation compared to control Vehicle group (*- $p<0.05$). BK induced increase in TRPV1 potentiation was significantly blocked by 100 µM nonpalmitoylated DD04107 (**- $p<0.01$). In $IB4^+$ nociceptors no significant potentiation of BK induced fold increase in current intensity was observed.

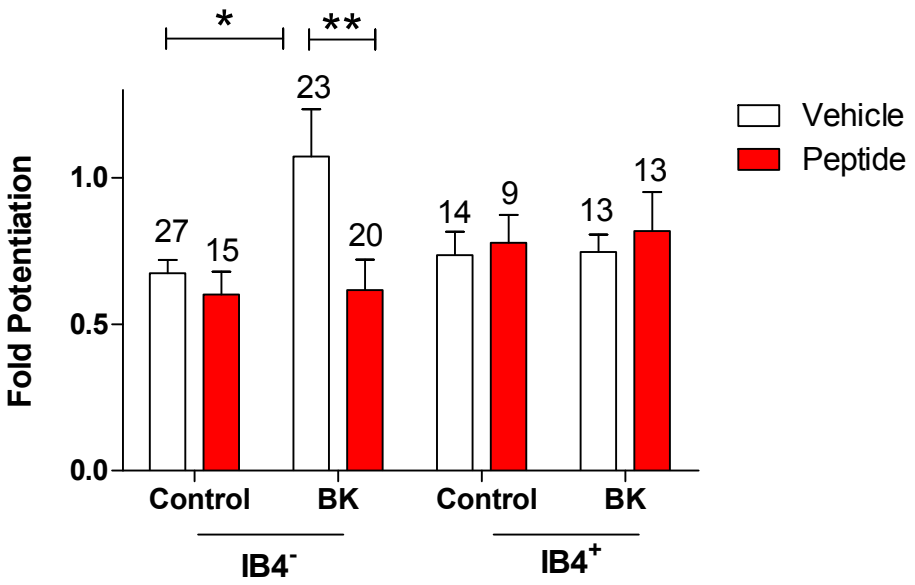

Figure- 50: Nonpalmitoylated DD04107 (Peptide) lessens BK induced inflammatory sensitization of TRPV1 channel in IB4$^-$. Effect of 100 μM non-palmitoylated DD04107 on 1 μM BK mediated fold potentiation of TRPV1 activity was determined by comparison of capsaicin-mediated current intensities before and after incubation of BK (ratio P3/P2) of the groups (Control Vehicle, Control Peptide, BK Vehicle, BK Peptide) for both IB4$^-$ and IB4$^+$ subpopulations. Data are expressed as mean ± SEM. The numbers above the bars represent total neurons analyzed. Number of cultures = 4. Statistical analysis was performed by two-way ANOVA with Bonferroni's post test (*- $p<0.05$, **- $p<0.01$).

In addition, we focused on the fold potentiation (ratio P3/P2) to check the percentage of IB4$^-$ and IB4$^+$ neurons that exhibited potentiation of TRPV1 induced by BK as shown in **figure- 51**. There was a significant difference in the percentage of neurons that showed potentiation of capsaicin induced currents after BK incubation compared with control Vehicle group. In fact, in IB4$^-$ group 44 % of neurons showed a potentiated response after the third pulse of capsaicin, whereas we observed a higher blockage of capsaicin induced currents in nonpalmitoylated DD04107 treated group exposed to BK, with only 15 % of neurons exhibiting potentiation. In IB4$^+$ group, we did not observe any neurons potentiated by BK in Vehicle group, whereas in nonpalmitoylated DD04107 group 23 % of neurons were potentiated by BK. The differential potentiated responses that we observed in IB4$^+$ neurons could be due to the lesser expression of BK1 receptor, or co expression of B2/B1 receptor in a subpopulation of IB4$^+$ neurons. Hence, we found that the mechanism behind BK induced TRPV1 potentiation in IB4$^-$ neurons is primarily due to exocytosis; although PKC mediated phosphorylation could play a vital role in sensitization process.

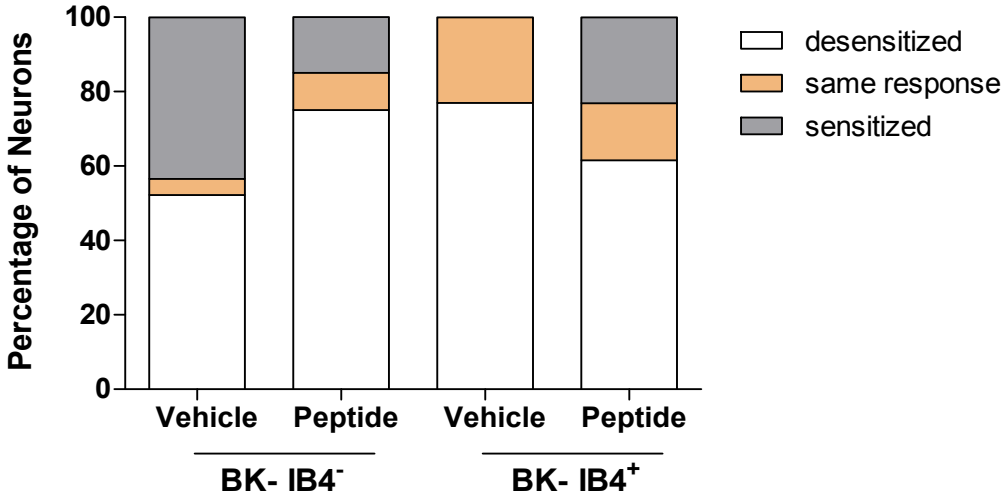

Figure- 51: Percentage of Neurons with BK induced potentiation of TRPV1 activity in peptidergic and nonpeptidergic nociceptors treated with vehicle and non-palmitoylated DD04107 (Peptide). Percentage of neurons with potentiated TRPV1 activity when treated with 1 μM BK was determined in both the sub populations of peptidergic and nonpeptidergic rat DRG neurons. The values of the ratio P3/P2 in both vehicle and peptide treated groups was calculated. The criteria is when the ratio is lesser than 0.9 the neurons are desensitized; ratio of 0.9-1.1 there is no significant difference between the second and third pulses; ratio greater than 1.1 the channel is getting sensitized with BK.

BK induced TRPV1 potentiation in IB4$^-$ subpopulation is PKC dependent

Since we found that BK significantly potentiates TRPV1 currents through exocytosis in IB4$^-$ subpopulations, we wanted to further elucidate if exocytosis is the prime mechanism involved in this sensitization process. Earlier studies proved that extracellular application of BK activates bradykinin receptor which is involved in the intracellular modification of TRPV1 sensitization. Numerous studies reported that BK induced sensitization of TRPV1 current is mainly due to the direct phosphorylation of TRPV1 channel at S800 by PKCε (318, 319). Thus similar to ATP experiments, we wanted to examine the impact of inhibiting PKC in BK induced TRPV1 potentiation.

We used Bisindolylmaleimide (BIM), a general PKC inhibitor, as a blocker in peptidergic nociceptors alone, since in nonpeptidergic neurons we did not observe any significant TRPV1 potentiation by BK. BIM was used at 1 μM concentration and given through patch pipette (10 minutes incubation after forming the seal). Then we applied three repetitive pulses of capsaicin which induced desensitization of TRPV1 channel that was significantly recovered by the application of BK between the second and third pulses. As

shown by the traces of IB4$^-$ neurons registered in **figure- 52**, application of BK induced sensitization of capsaicin activated currents and it was strongly inhibited in the neurons treated with BIM.

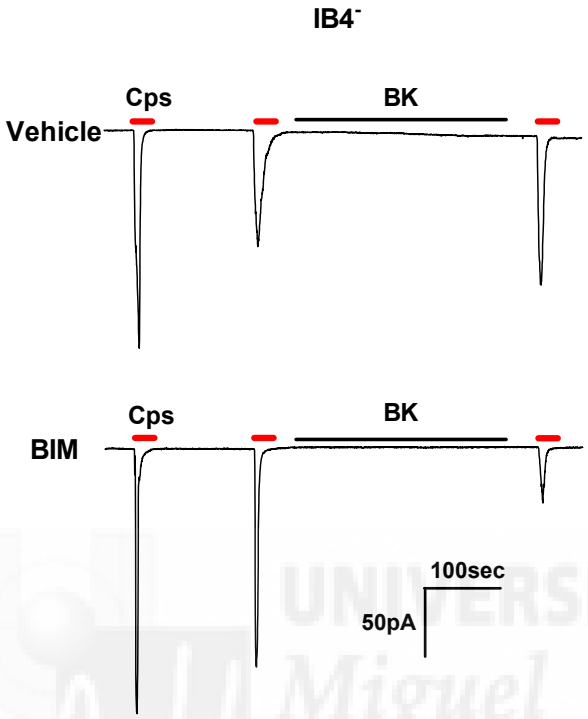

Figure- 52: BIM (PKC inhibitor) abolishes BK induced inflammatory sensitization of TRPV1 channel in rat peptidergic DRG neurons. Representative voltage clamp recordings of currents elicited by capsaicin (1 µM, 10 s) in IB4$^-$ nociceptors. Cells were held at -60 mV. 1 µM BIM was given through the patch pipette and incubated for 10 minutes after forming the seal. Three repetitive pulses of capsaicin at 1 µM concentration were given for the continuous protocol. 1 µM BK was applied between second and third pulse of capsaicin.

Fold potentiation of TRPV1 currents was calculated by taking the ratio of P3 to P2 (P3/P2- since BK was applied between P2 and P3). **Figure- 53** shows no significant changes in fold potentiation were observed under control conditions in both Vehicle and BIM treated groups. In IB4$^-$ nociceptors, fold increase in current intensity was significantly higher after BK incubation compared to control Vehicle group (**- $p<0.01$). BK induced increase in TRPV1 potentiation was significantly blocked by 1 µM BIM (*- $p<0.05$).

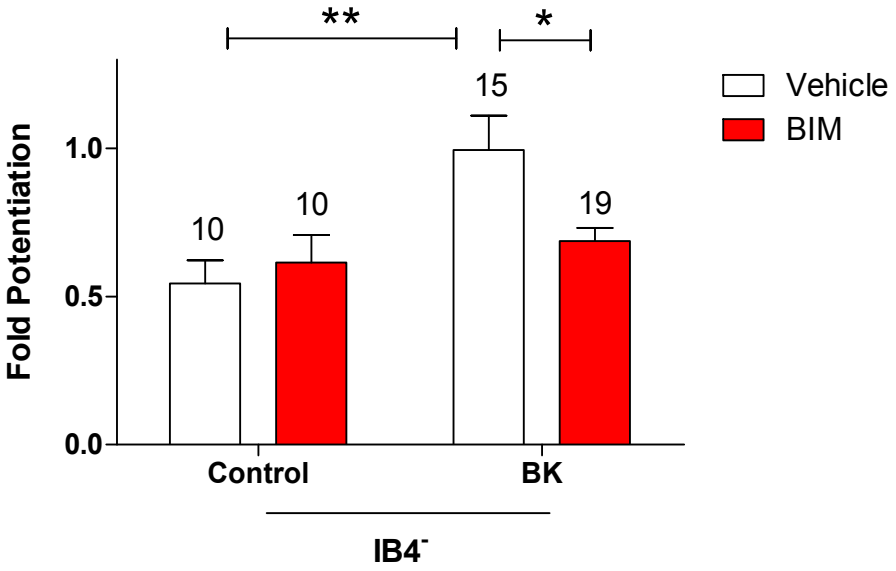

Figure- 53: BIM (PKC inhibitor) abolishes BK induced inflammatory sensitization of TRPV1 channel on rat peptidergic DRG neurons. Effect of 1 μM BIM on 1 μM BK mediated fold potentiation of TRPV1 activity was determined by comparison of capsaicin-mediated current intensities before and after incubation of BK (ratio P3/P2) of the groups (Control Vehicle, Control BIM, BK Vehicle, and BK BIM) for $IB4^-$ subpopulations. Data are expressed as mean ± SEM. The numbers above the bars represent the total number of neurons registered. Number of cultures = 4. Statistical analysis was performed by two-way ANOVA with Bonferroni's post hoc test (*- $p<0.05$, **- $p<0.01$).

Further we focused on the fold potentiation (ratio P3/P2) to check the percentage of $IB4^-$ neurons that exhibited BK induced potentiation of TRPV1 as shown in **figure- 54**. There was a significant difference in the percentage of neurons that exhibited potentiated response to capsaicin after BK incubation compared with control Vehicle group. In fact, in $IB4^-$ group exposure to BK showed 25 % of neurons with a potentiated response after the third pulse of capsaicin (ratio P3/P2) in the Vehicle group. We observed a complete blockage of BK induced TRPV1 potentiation by BIM. Hence two different mechanisms of potentiation of TRPV1 by BK in $IB4^-$ nociceptors exist, where BK can induce both exocytosis and phosphorylation of TRPV1 channel.

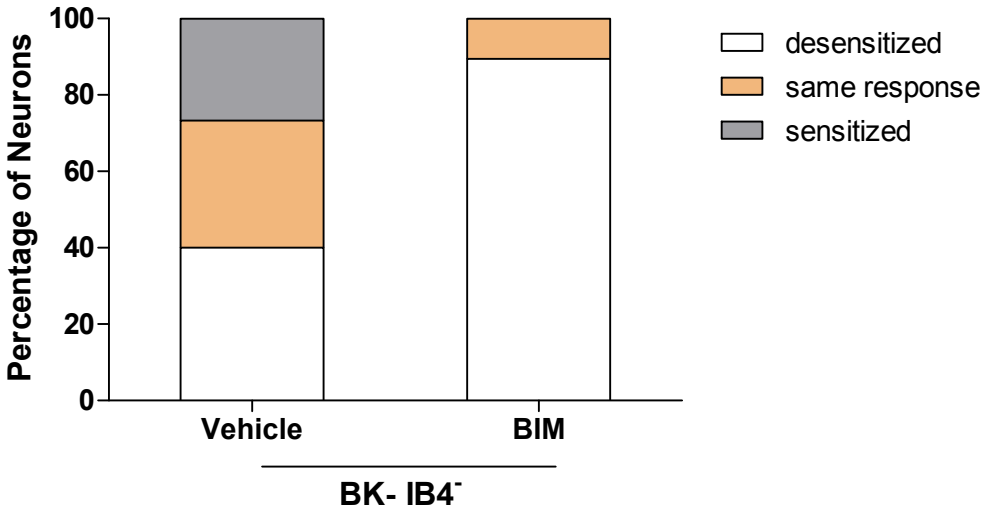

Figure- 54: Percentage of Neurons with BK induced potentiation of TRPV1 activity in peptidergic nociceptors treated with Vehicle and BIM. Percentage of neurons with potentiated TRPV1 activity when treated with 1 µM BK was determined in peptidergic sub population of rat DRG neurons. The values of the ratio P3/P2 in both vehicle and BIM treated groups was calculated by taking the ratio of P3 (third pulse of capsaicin) to P2 (second pulse of Capsaicin). The criteria is when the ratio is lesser than 0.9 the neurons are desensitized; ratio of 0.9-1.1 there is no significant difference between the second and third pulses; ratio greater than 1.1 the channel is getting sensitized with BK.

BK2 receptor contributes largely for BK mediated TRPV1 sensitization

Prior experiments with BK revealed that peptide DD04107 abrogated sensitization of TRPV1 mediated neuronal firing in nociceptors. Furthermore, patch clamp experiments in $IB4^-$ and $IB4^+$ neurons, showed that BK potently sensitized TRPV1 currents in peptidergic subpopulation which was sensitive to both inhibition of neuronal exocytosis and PKC. In contrast, no traceable potentiation was observed in $IB4^+$ subpopulations; consistent with the literature that BK induced potentiation is mainly mediated through BK2 receptors. BK2R is expressed constitutively in peptidergic nociceptors, whereas BK1 receptor expression is inducible during inflammation in nonpeptidergic nociceptors, thus contributing for chronic pain conditions. Nevertheless, many studies reported a basal constitutive expression of BK1 receptor, although it was less compared to BK2 receptor expression (320, 321).

In order to unravel which BK receptor subtype is involved in sensitization of TRPV1 in nociceptors we performed MEA experiments. A continuous protocol was used for 16 minutes where three repetitive pulses of 500 nM capsaicin were applied. Consecutive

application of capsaicin led to desensitization in the number of spikes evoked and desensitized responses could be significantly recovered with BK sensitization (1 μM BK was applied between P2 and P3). DRG neurons exposed to BK evoked slow and consistent neuronal spikes until washout, through the activation of metabotropic B2 or B1 receptors **(figure- 55)**. Akin to BK, BK2 receptor agonist ([Phe^{8}ψ(CH-NH)-Arg^9]-Bradykinin) was applied between P2 and P3 and significantly potentiated TRPV1 mediated neuronal spikes. Furthermore, to explore the role of basal BK1 receptor on BK induced TRPV1 sensitization, BK1 receptor agonist (Sar-[D- Phe^8]-des-Arg^9-Bradykinin) was applied between P2 and P3 which significantly potentiated TRPV1 mediated neuronal spikes. Application of both the agonists (BK1 and BK2) evoked neuronal spikes through activation of their corresponding receptors, although activation of BK1 receptor evoked less neuronal spikes compared to BK2 receptor.

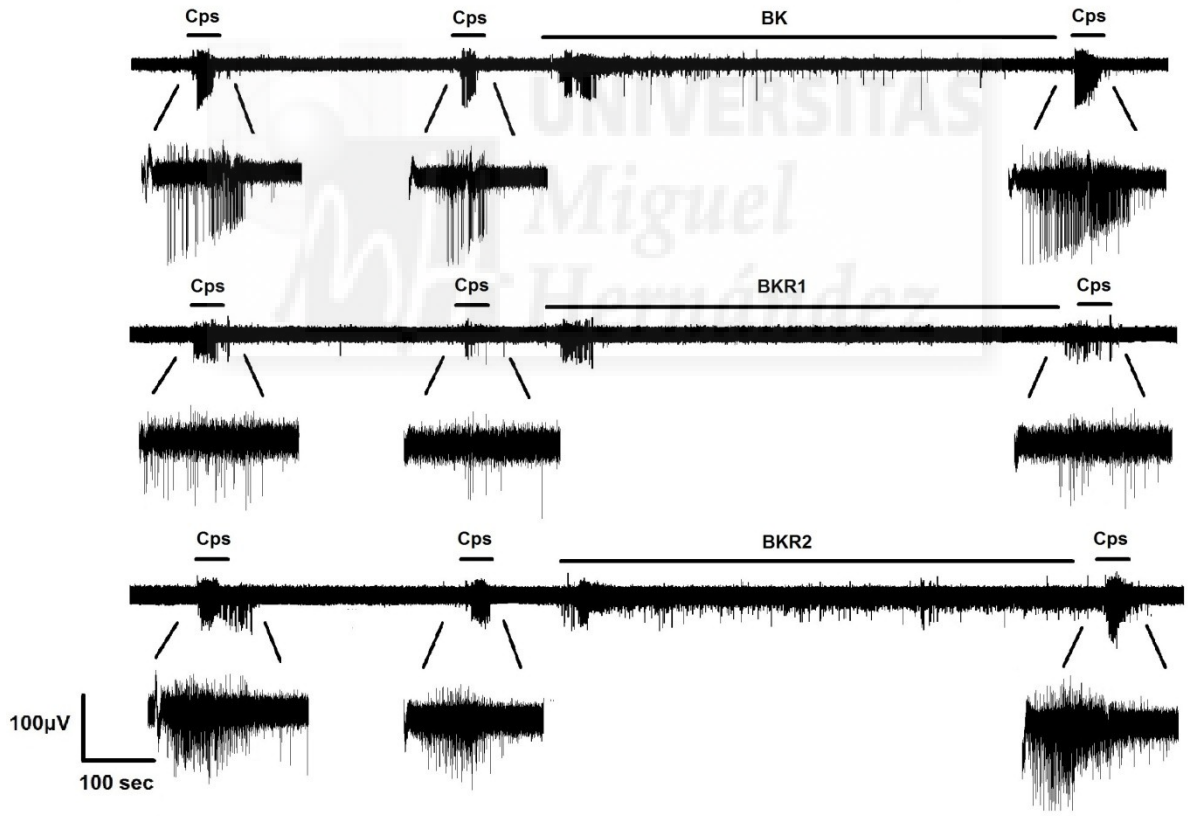

Figure- 55: BK1 and BK2 receptor agonists on sensitization of TRPV1 evoked neuronal spikes in neonatal rat nociceptors. Representative traces of potentiation of capsaicin (500 nM, 15 s) responses elicited by applying 1 μM of BK and its agonists 1 μM BK1 (Sar-[D- Phe^8]-des-Arg^9-Bradykinin) and 1 μM BK2 ([Phe^{8}ψ(CH-NH)-Arg^9]-Bradykinin) between the second (P2) and third (P3) vanilloid pulse in neonatal rat DRG neurons.

There was a significant potentiation of TRPV1 evoked neuronal spikes observed after BK application (***- p<0.001) **(figure- 56)**. Specific activation of BK receptors- BK1 (*- p<0.05) and BK2 (***- p<0.001) also exhibited significant potentiation of TRPV1 evoked neuronal spikes.

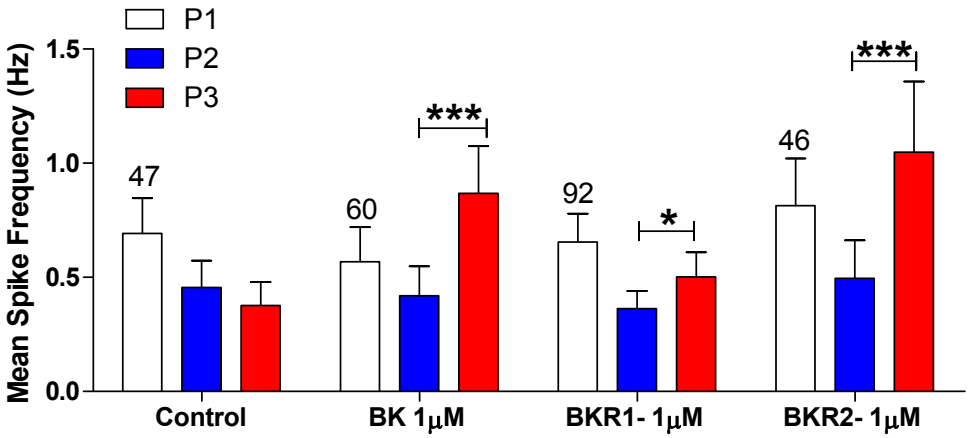

Figure- 56: BK1 and BK2 receptor agonists on sensitization of TRPV1 evoked neuronal spikes in neonatal rat nociceptors. Effect of 1 μM BK1 (Sar-[D- Phe^{8}]-des-Arg^{9}-Bradykinin) and 1 μM BK2 ([Phe^{8}ψ(CH-NH)-Arg^{9}]-Bradykinin) receptor agonists was determined by comparison of all the three pulses (P1, P2, P3) in each group and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (***-p<0.001, *-p<0.05).

In order to know which receptor induced response is similar to that evoked by BK we analysed the ratio of third pulse to second pulse (P3/P2) (since BK and its agonists was applied between P2 and P3). We observed a significant increase in fold potentiation of TRPV1 evoked neuronal spikes in BK treated groups 2.6±0.4 compared to control 1±0.2 (**p<0.01). Similar increase in fold potentiation of TRPV1 evoked neuronal spikes was observed upon activation of BK2 receptor 3±0.3 compared to control (***-p<0.001). No significant increase in fold potentiation of TRPV1 evoked neuronal spikes was observed in neurons treated with BK1 receptor agonist **(figure- 57)**.

Though the degree of sensitization was less compared to both BK and BK2 receptor agonist, a possible involvement of BK1 receptor on TRPV1 sensitization cannot be excluded. This could be due to the experimental conditions, where *in vitro* application of BK specifically activates BK2 receptor, but not BK1 receptor.

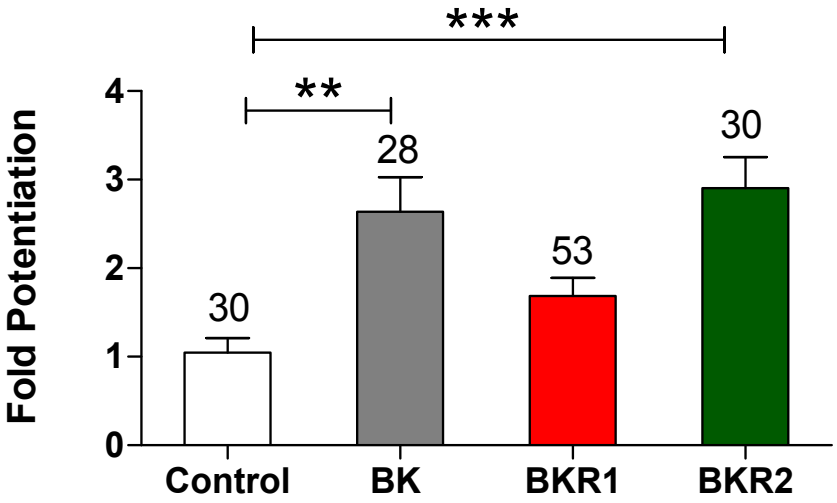

Figure- 57: BK2 receptor is mainly involved in BK induced sensitization of TRPV1 evoked neuronal spikes in neonatal rat nociceptors. Fold potentiation of BK and its agonists induced potentiation of TRPV1 mediated neuronal firing activity was calculated by taking the ratio of P3 to P2 (ratio P3/P2). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by one- way ANOVA followed by Bonferroni's post hoc test (**- $p<0.01$, ***- $p<0.001$).

Role of αCGRP and Tac1 on BK induced sensitization of TRPV1 evoked neuronal spikes

In addition, we wanted to examine the modulatory role of αCGRP on BK induced sensitization of TRPV1, since from our earlier experiments with ATP we validated that αCGRP plays a major role on TRPV1 sensitization through exocytosis. Also experiments from neonatal rat DRG neurons confirmed that BK induced TRPV1 potentiation involves exocytotic release of new TRPV1 channel carrying vesicles. We proposed that akin to ATP, BK could also require αCGRP for TRPV1 vesicular release.

BK induced sensitization of TRPV1 excitability in nociceptors from wild type, $Tac1^{-/-}$ and $\alpha CGRP^{-/-}$ knockout mice were evaluated using MEA technique.

DD04107 abrogates BK induced sensitization of TRPV1 evoked neuronal spikes

In wild type nociceptors, repetitive capsaicin pulses induced desensitization of the vanilloid-evoked electrical responses. Consecutive application of capsaicin led to

desensitization in the number of spikes evoked and desensitized responses were significantly recovered (*- p<0.05) with BK sensitization (1 µM BK was applied between P2 and P3) in wild type. Some neurons exposed to BK evoked fast neuronal spikes, due to the activation of metabotropic BK2 receptor, which is widely expressed in nociceptors. BK induced TRPV1 sensitization of excitability was blocked by DD04107, indicating that BK induced TRPV1 sensitization was mainly due to the insertion of new TRPV1 channels into the plasma membrane of nociceptors as shown in **figure- 58**.

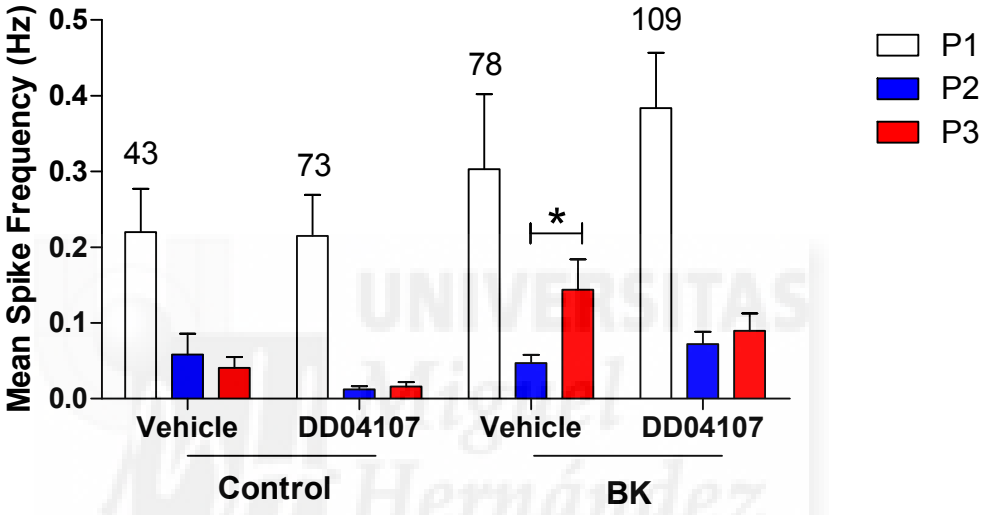

Figure- 58: DD04107 abrogates BK induced inflammatory sensitization of TRPV1 mediated neuronal firing in wild type mice nociceptors. Effect of 20 µM DD04107 on 1 µM BK mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in each group and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 4. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (*- p<0.05).

αCGRP is essential for BK induced TRPV1 sensitization

In order to know which neuropeptide is involved in regulating TRPV1 exocytosis, we next studied the role of each neuropeptide in the nociceptors from αCGRP$^{-/-}$ and Tac1$^{-/-}$ single knockout mice. We evaluated BK induced potentiation of TRPV1 evoked spikes in single knockout mice. Our result shows that capsaicin induced desensitization of TRPV1 evoked neuronal spikes was completely reversed by BK in nociceptors from Tac1$^{-/-}$ mice (***- p<0.001) as shown in **figure- 59**.

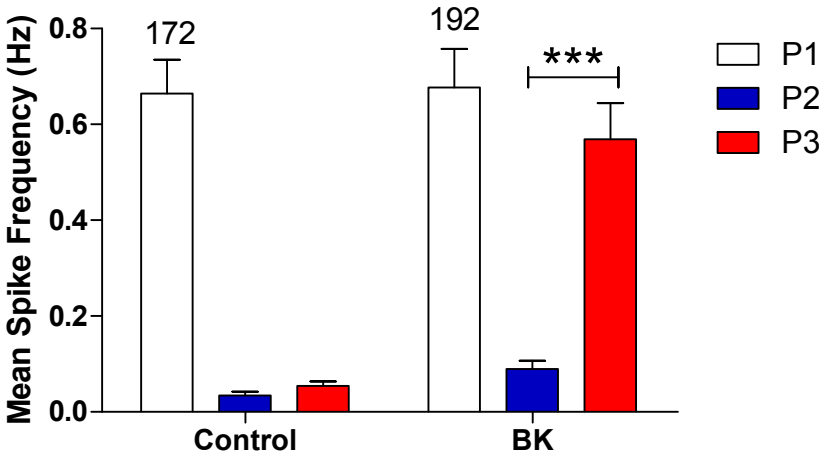

Figure- 59: Silencing of Tac1 in nociceptors does not affect BK induced inflammatory sensitization of TRPV1 mediated neuronal firing. 1 µM BK mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in control and BK groups and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (***- $p < 0.001$).

Nevertheless, abrogation of αCGRP expression eliminated BK induced increase in mean spike frequency of TRPV1 evoked neuronal firing as shown in **figure- 60**. Together, these results indicate that αCGRP expression is necessary for BK induced TRPV1 potentiation in peptidergic nociceptors.

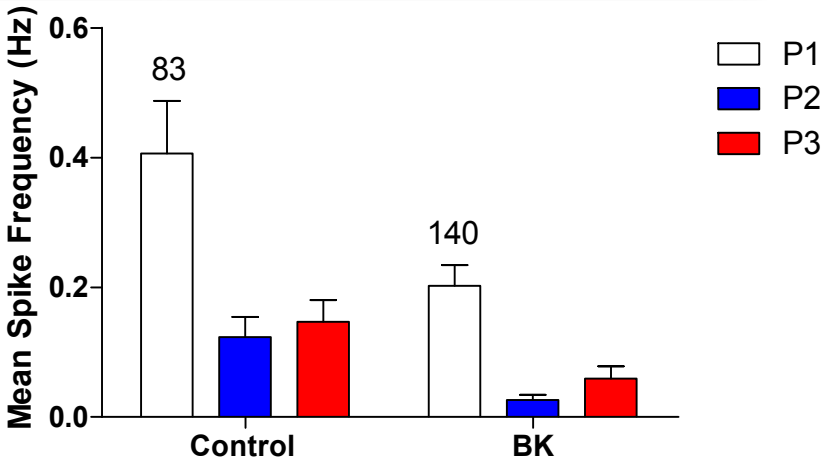

Figure- 60: Silencing of αCGRP$^{-/-}$ abrogates BK induced inflammatory sensitization of TRPV1 mediated neuronal firing. 1 µM BK mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in control and BK groups and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 4. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test.

FACILITATORY EFFECTS OF ATP AND BK CO- APPLICATION ON INFLAMMATORY SENSITIZATION OF TRPV1

Blockage of neuronal exocytosis reduces ATP- BK induced TRPV1 excitability

Until now we have evaluated the effect of pro- inflammatory mediators ATP and BK individually on potentiation of TRPV1. We found that the mechanism of TRPV1 potentiation by ATP and BK is different in both peptidergic and nonpeptidergic nociceptors. DD04107 significantly abrogated TRPV1 potentiation induced by ATP and BK in peptidergic subpopulations. The ultimate aim of our experiments was to study the impact of blocking neuronal exocytosis in a complicated environment like tissue injury, where the immune cells adjacent to the primary sensory neurons discharge a mixture of inflammatory compounds with a drop in pH leading to tissue acidosis. Hence we wanted to test the effect of these two inflammatory mediators ATP and BK, applied concurrently in the form of a simple cocktail, on potentiation of TRPV1 evoked spikes and its abrogation by DD04107.

ATP- BK induced sensitization of TRPV1 excitability in rat nociceptors was evaluated using MEA technique. Consecutive application of capsaicin led to desensitization in the number of spikes evoked and desensitized responses could be significantly recovered with ATP- BK sensitization (10 μM ATP- 1 μM BK was applied between P2 and P3) as shown in **figure- 61 (Vehicle)**. ATP-BK induced sensitization of TRPV1 evoked spikes was blocked by DD04107 (ATP- BK Peptide). This corroborated that ATP- BK induced TRPV1 sensitization is mainly due to the insertion of new TRPV1 channels into the plasma membrane in a subset of nociceptors as shown in **figure- 61 (DD04107)**.

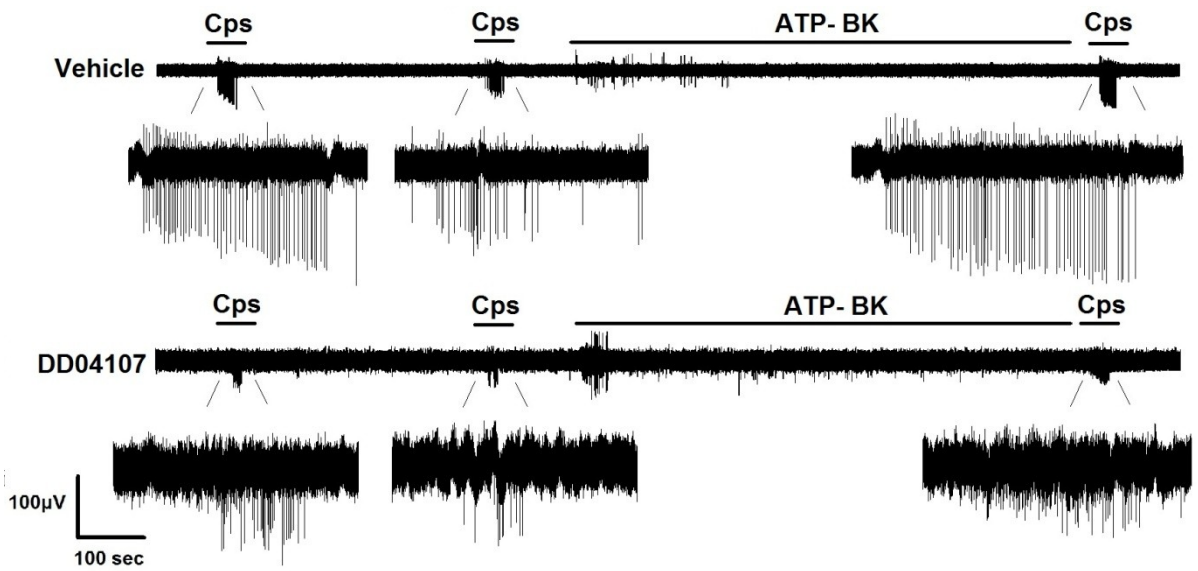

Figure- 61: DD04107 abrogates ATP-BK sensitized TRPV1- mediated nociceptor excitability. Representative traces of potentiation of capsaicin (500 nM, 15 s) responses elicited by applying the cocktail of 10 µM ATP- 1 µM BK between the second (P2) and third (P3) vanilloid pulse, in neonatal rat DRG neurons (top). Preincubation of the neurons with DD04107 (bottom) also potentiated TRPV1 but it was less significant compared to Vehicle (top).

Though we saw a less significant potentiation of TRPV1 activity by ATP- BK in DD04107 (*- $p<0.05$) compared to Vehicle group (***- $p<0.001$), we still observed potentiation of TRPV1 evoked spikes as shown in **figure- 62**. Hence it was important to compare ATP- BK induced fold increase in the potentiation of TRPV1 between Vehicle and DD04107 treated groups.

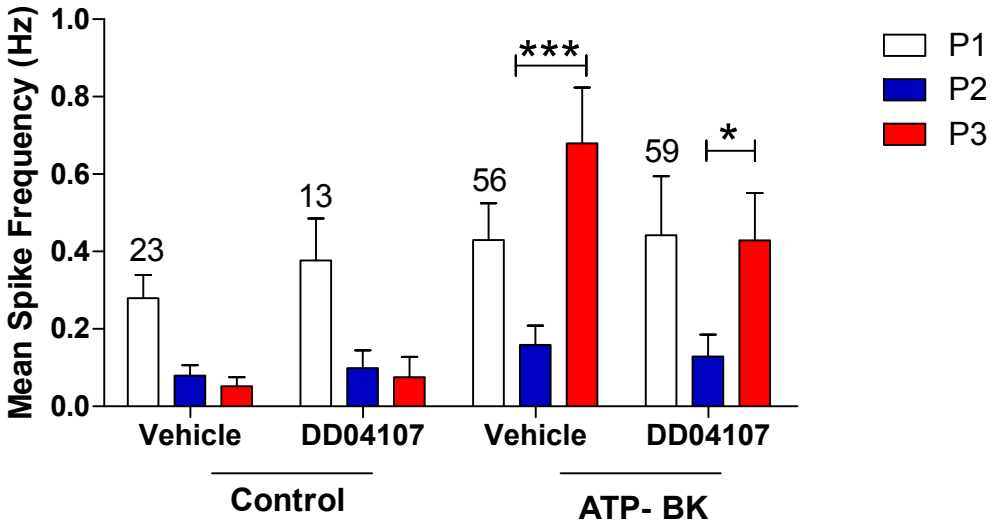

Figure- 62: DD04107 abrogates ATP- BK induced inflammatory sensitization of TRPV1 mediated neuronal firing in rat DRG neurons. Effect of 20 µM DD04107 on 10 µM ATP, 1 µM BK mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in each group and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (*- $p<0.05$, ***- $p<0.001$).

To quantify the extent of TRPV1 potentiation, we calculated the ratio of third pulse to second pulse (P3/P2) in both Vehicle and DD04107 treated groups exposed to ATP- BK. Then, the impact of DD04107 on blocking the potentiation of TRPV1 channel induced by ATP- BK treatment was compared between Vehicle and DD04107 groups as shown in **figure- 63**. We observed a significant blockage of TRPV1 potentiation on ATP- BK treated neurons pre incubated with DD04107 (5±0.6) compared to Vehicle (12±3) (*- $p<0.05$).

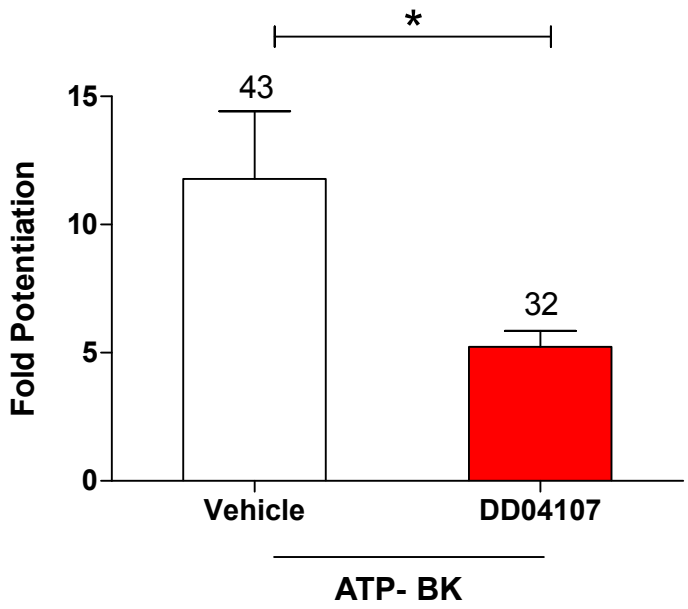

Figure- 63: ATP- BK mediated TRPV1 sensitization requires channel recruitment in a subset of nociceptors. Effect of 20 µM DD04107 on 10 µM ATP - 1 µM BK mediated potentiation of TRPV1 activity was determined by comparison of capsaicin mediated neuronal spikes before and after incubation of ATP- BK (ratio P3/P2) of the groups Vehicle and DD04107. Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by Unpaired Student's t- test (*- $p<0.05$).

DD04107 does not abrogate ATP- BK induced TRPV1 sensitization in rat nociceptors under acidic conditions

Combined application of ATP- BK significantly potentiated TRPV1 evoked neuronal excitability which was abolished by blocking exocytotic release of TRPV1 channels with DD04107. In addition, we wanted to apply our inflammatory cocktail in conditions similar to tissue acidosis microenvironment. It has been reported that pH 6.1 was sufficient to evoke nociceptive pain and to facilitate the action of inflammatory mediators (322). Nevertheless, experimenting at low pH is a complex mechanism, since DRG neurons express proton sensitive ASIC channels that can be activated at pH 6.4. Furthermore, TRPV1 itself can be activated by protons at pH 5.6. Therefore we didn't neglect that the resultant inflammatory cocktail actions could also be due to the involvement of activation of ASIC channels, but we directed our study towards the functional modulation of TRPV1.

Hence we prepared our cocktail ATP- BK at pH 6.2 and studied their effect on potentiation of TRPV1 evoked neuronal excitability and its abrogation by DD04107.

Application of ATP- BK pH 6.2 undergoes a complex mechanism where purinergic, bradykinin and proton receptors are activated and exert their effect in both depolarization of the membrane potential and potentiation of TRPV1.

ATP- BK pH 6.2 induced sensitization of TRPV1 excitability in rat nociceptors was evaluated using MEA technique. Consecutive application of capsaicin led to desensitization in the number of spikes evoked and desensitized responses could be significantly recovered with ATP- BK pH 6.2 treatment (10 µM ATP- 1 µM BK- pH 6.2 was applied between P2 and P3) as shown in **figure- 64 (Vehicle)**. ATP-BK pH 6.2 induced TRPV1 sensitization of excitability was not blocked by DD04107 as shown in **figure- 64 (DD04107)**.

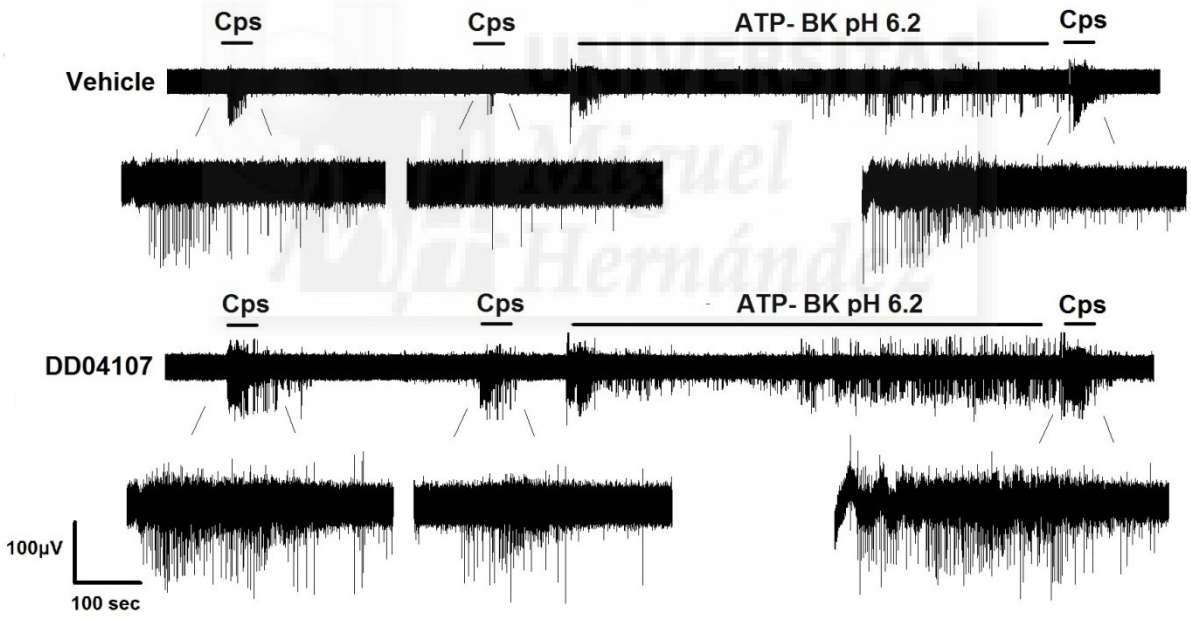

Figure- 64: DD04107 does not affect ATP-BK sensitized TRPV1- mediated nociceptor excitability at pH 6.2. Representative traces of potentiation of capsaicin (500 nM, 15 s) responses elicited by applying the cocktail of 10 µM ATP- 1 µM BK at pH 6.2 between the second (P2) and third (P3) vanilloid pulse (top), in neonatal rat DRG neurons. Pre incubation of the neurons with the peptide DD04107 (bottom) had significantly higher potentiation of TRPV1 compared to Vehicle (top).

We observed a significantly higher potentiation of TRPV1 evoked neuronal spikes in DD04107 compared to the Vehicle groups as shown in **figure- 65**. To compare ATP- BK pH 6.2 induced fold increase in the potentiation of TRPV1 between Vehicle and DD04107

treated groups, we calculated the ratio of third pulse to second pulse (P3/P2) in both Vehicle and DD04107 treated groups exposed to ATP- BK pH 6.2.

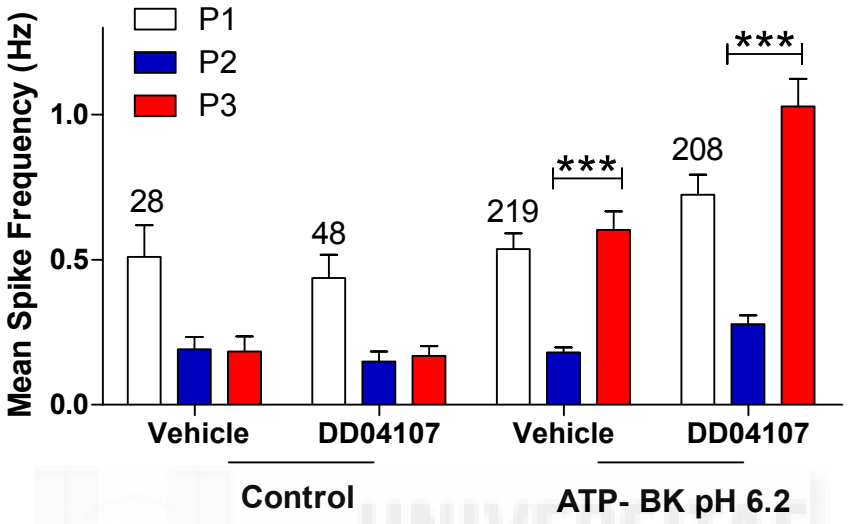

Figure- 65: DD04107 peptide does not affect ATP, BK –pH 6.2 induced inflammatory sensitization of TRPV1 mediated neuronal firing in rat DRG neurons. Effect of 20 µM DD04107 on 10 µM ATP, 1 µM BK at pH 6.2 mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in each group and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (***- $p<0.001$).

Then, we compared the ratio P3/P2 of Vehicle and DD04107 treated groups exposed to ATP- BK pH 6.2. We observed a significantly higher TRPV1 potentiation on ATP- BK pH 6.2 treated neurons pre incubated with DD04107 (8.5±1.3) compared to the Vehicle (5.6±0.6) (*- $p<0.05$) as shown in **figure- 66**.

This could be due to the presence of distinct subpopulations of nociceptors in the culture, where pH 6.2 could function as a modulator of TRPV1. Hence the blockage effect of DD04107 that we observed earlier at physiological pH 7.4 could be suppressed by the sensitization of TRPV1 induced by pH 6.2 in both nonpeptidergic C-fibers and medium sized Aδ fibers.

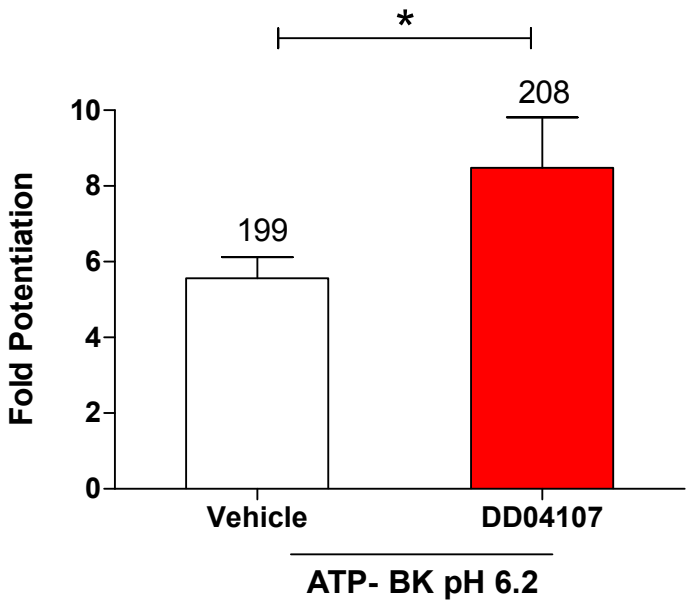

Figure- 66: Inhibition of neuronal exocytosis does not affect ATP- BK –pH 6.2 induced inflammatory sensitization of TRPV1 mediated neuronal firing in rat DRG neurons. Effect of 20 µM DD04107 on 10 µM ATP– 1 µM BK with pH 6.2 mediated potentiation of TRPV1 activity was determined by comparison of capsaicin-mediated neuronal spikes before and after incubation of ATP- BK pH 6.2 (ratio P3/P2) of the groups Vehicle and DD04107. Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by Unpaired, Students t- test (*- $p<0.05$).

pH 6.2 potentiates TRPV1 excitability

Since we did not see an impact of DD04107 on ATP- BK pH 6.2 cocktail, we focused our experiments on pH 6.2 alone, to know if a reduction in pH could modulate TRPV1 activity. It has been reported that pH 6.2 enhances TRPV1 activity by decreasing the EC_{50} of the agonist from µM to nM (323). Hence we used pH 6.2 as a sensitizing stimulus and studied its effect on potentiation of TRPV1 evoked neuronal excitability.

As we mentioned before, we did not exclude the possibility that the application of pH 6.2 could also activate proton receptors, but our experiments are directed towards the functional modulation of TRPV1.

pH 6.2 induced sensitization of TRPV1 excitability in rat nociceptors was evaluated using MEA technique. A continuous protocol was used where three repetitive

pulses of 500 nM capsaicin were applied. Consecutive application of capsaicin led to desensitization in the number of spikes evoked and desensitized responses could be significantly recovered with pH 6.2 sensitization (pH 6.2 was applied between P2 and P3) as shown in **figure- 67**.

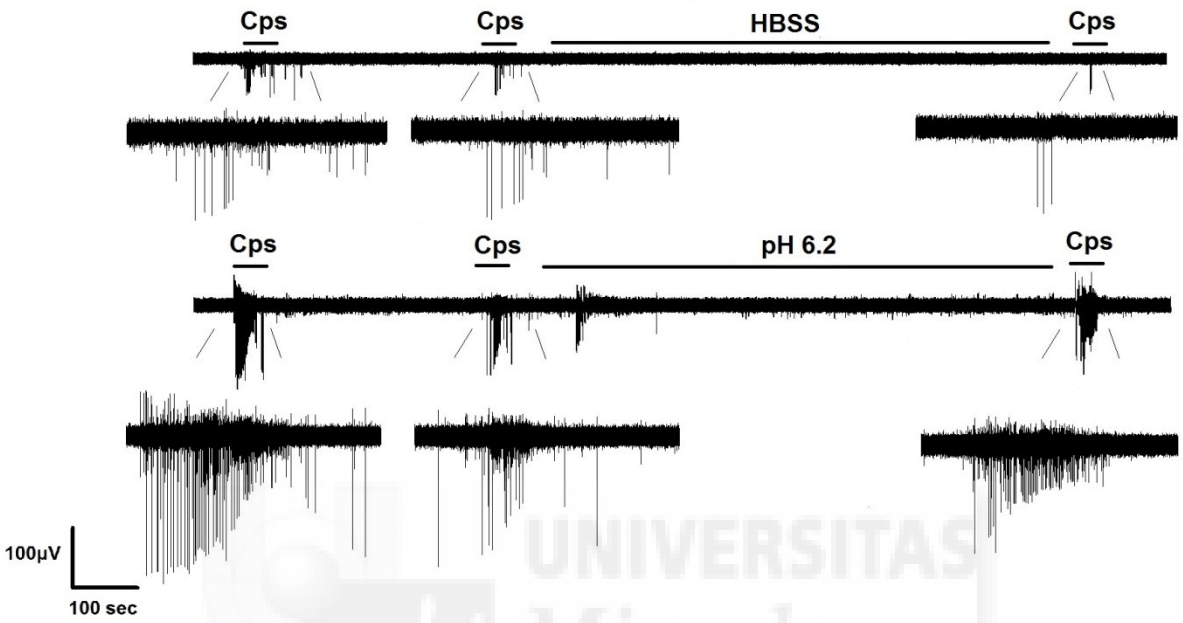

Figure- 67: pH 6.2 potentiates TRPV1- mediated nociceptor excitability. Representative traces of potentiation of capsaicin (500 nM, 15 s) responses elicited by applying the standard external solution with a reduction in pH to 6.2 between the second (P2) and third (P3) vanilloid pulse, in neonatal rat DRG neurons. Neurons treated with the pH 6.2 (bottom) had significant potentiation of TRPV1 compared to vehicle (top).

We observed a significantly higher potentiation of TRPV1 evoked neuronal spikes in pH 6.2 treated group compared to control group (***- $P<0.001$). This confirms that pH 6.2 alone potentiates TRPV1 evoked neuronal spikes as shown in **figure- 68**.

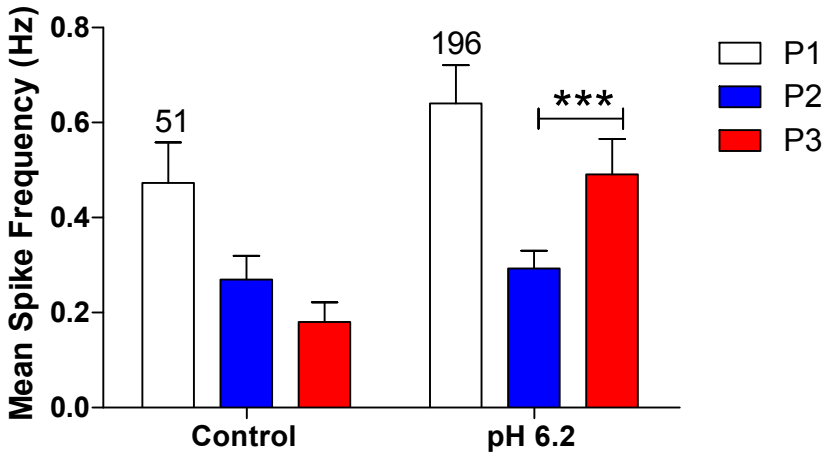

Figure- 68: pH 6.2 induces inflammatory sensitization on TRPV1 mediated neuronal firing in rat DRG neurons. Effect of pH 6.2 on TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, and P3) in each group and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (***- $p<0.001$).

Blockage of neuronal exocytosis reduces ATP- BK pH 6.2 induced TRPV1 excitability in rat peptidergic nocieptors

From our earlier experiment, we did not see a blockage effect of DD04107 on ATP-BK pH 6.2 mediated TRPV1 potentiation. This could be due to its activity on differential subpopulation of DRG neurons. We wanted to know if this mechanism of TRPV1 potentiation by ATP- BK pH 6.2 is common for all subpopulations of DRG neurons. From our previous experiments with ATP and BK, we found that potentiation of TRPV1 in peptidergic neurons is primarily due to the recruitment of new functional TRPV1 channels from the vesicles to the membrane and it is abrogated by DD04107. This corroborates that the distinct signalling pathways are involved in sensitization of TRPV1 in both peptidergic and nonpeptidergic nociceptors. Hence we wanted to further elucidate whether ablation of nonpeptidergic neurons from the DRG neuronal culture could enhance the potential blockade effect of DD04107 on ATP- BK pH 6.2 induced potentiation of TRPV1 in peptidergic nociceptors. For that we used IB4 SAP toxin which removes completely all $IB4^+$ neurons, leaving only $IB4^-$ neurons. The experiments were done 48 hours after toxin treatment.

ATP- BK pH 6.2 induced sensitization of TRPV1 excitability in peptidergic nociceptors was evaluated using MEA technique. A continuous protocol was used where three repetitive pulses of 500 nM capsaicin was applied. Consecutive application of capsaicin led to desensitization in the number of spikes evoked as shown in **figure- 69 (Vehicle & DD04107)** under control conditions and desensitized responses could be significantly recovered with ATP- BK pH 6.2 sensitization (10 µM ATP- 1 µM BK- pH 6.2 was applied between P2 and P3) as shown in **figure- 70 (Vehicle)**. ATP-BK pH 6.2 induced TRPV1 sensitization of excitability was blocked by DD04107, this sensitization was mainly due to the insertion of new TRPV1 channels into the plasma membrane of peptidergic nociceptors as shown in **figure- 70 (DD04107)**.

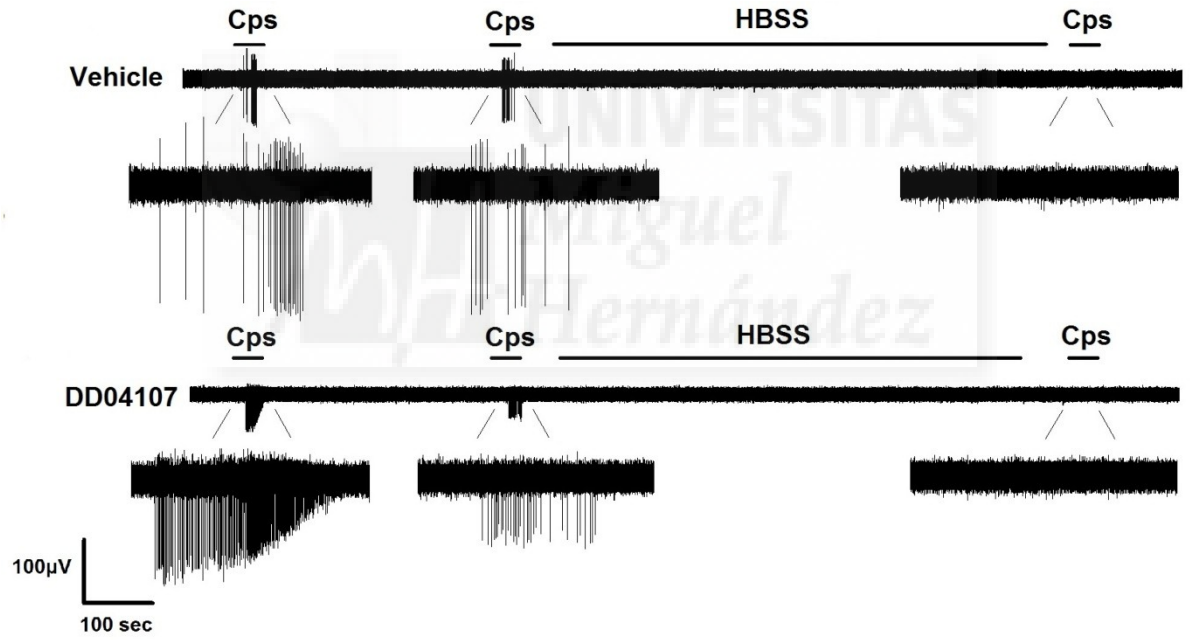

Figure- 69: Representative MEA recordings of capsaicin induced action potentials and desensitization in IB4 saporin treated nociceptors. Application of capsaicin (500 nM, 15 s) on neonatal rat DRG neurons induced TRPV1 evoked neuronal excitability in both vehicle and DD04107 treated groups (1st pulse- P1) in IB4 Saporin treated nociceptors. Repeated exposure of DRG neurons to capsaicin (2nd pulse (P2) and 3rd pulse (P3)) evoked fewer action potentials compared to the 1st pulse due to the desensitization of TRPV1 channel activity.

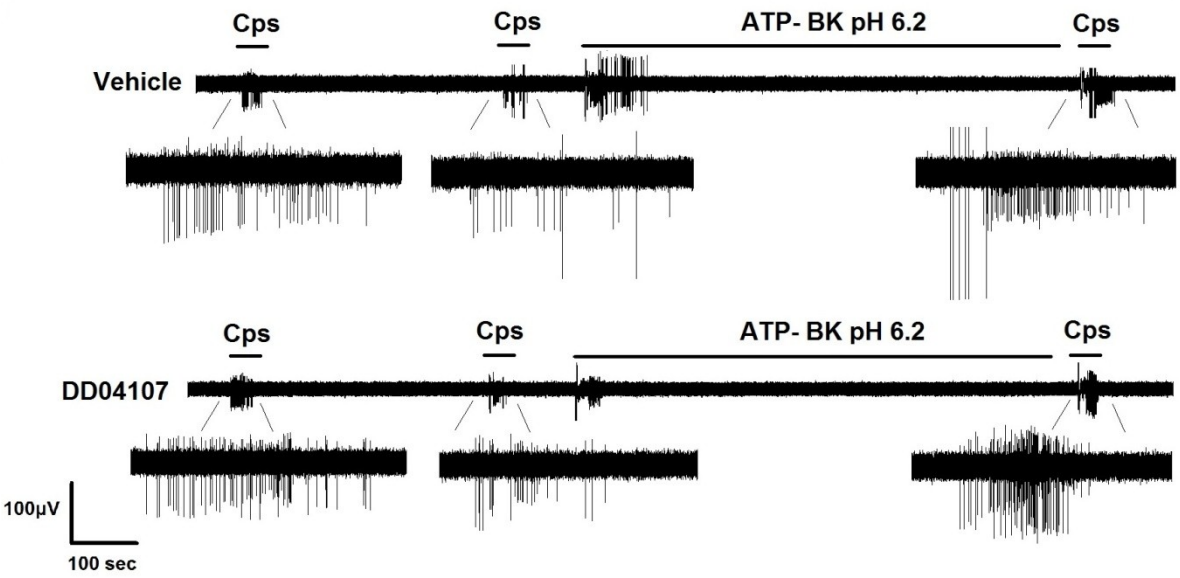

Figure- 70: DD04107 abrogates, ATP-BK pH 6.2 sensitized TRPV1- mediated nociceptor excitability in peptidergic rat DRG neurons. Representative traces of potentiation of capsaicin (500 nM, 15 s) responses elicited by applying the cocktail of 10 µM ATP- 1 µM BK with less pH 6.2 between the second (P2) and third (P3) vanilloid pulse (top), in peptidergic neonatal rat DRG neurons. Preincubation of the neurons with DD04107 (bottom) also had potentiation of TRPV1 but it was less significant compared to Vehicle (top).

Though we saw a less significant potentiation of ATP- BK pH 6.2 induced TRPV1 activity in DD04107 treated group (*- $p<0.05$) compared to Vehicle group (***- $p<0.001$), we still observed potentiation of TRPV1 evoked excitability as shown in **figure- 71**. Hence it was important to compare ATP- BK pH 6.2 induced fold increase in the potentiation of TRPV1 between Vehicle and DD04107 treated groups.

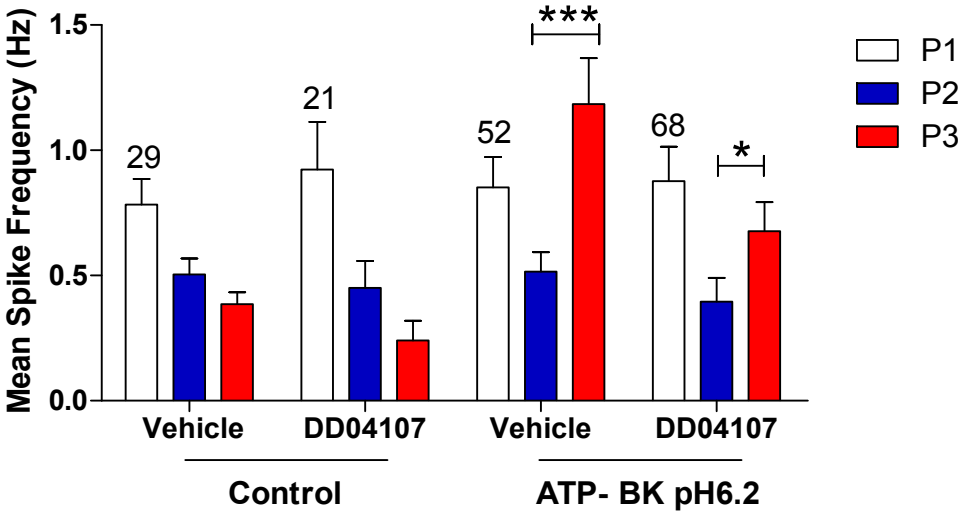

Figure- 71: DD04107 abolishes ATP, BK –pH 6.2 induced inflammatory sensitization of TRPV1 mediated neuronal firing in IB4 saporin treated rat DRG neurons. Effect of 20 µM DD04107 on 10 µM ATP, 1 µM BK pH 6.2 mediated potentiation of TRPV1 evoked neuronal firing activity was determined by comparison of all the three pulses (P1, P2, P3) in each group and represented as Mean spike frequency (Hz). Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by one way ANOVA repeated measures with Bonferroni's post hoc test (*- $p<0.05$, ***- $p<0.001$).

We calculated the ratio of third pulse to second pulse (P3/P2) in both Vehicle and DD04107 treated groups exposed to ATP- BK pH 6.2. Then, the impact of DD04107 on blocking the potentiation of TRPV1 channel by ATP- BK pH 6.2 treated neurons was compared between Vehicle and DD04107 groups as shown in **figure- 72 (A)**. We observed a significant blockage of ATP- BK pH 6.2 induced TRPV1 potentiation on peptidergic DRG neurons pre incubated with DD04107 (2±0.3) compared to the Vehicle (2.8±0.3) (*- $p<0.05$). Experiments from neonatal rat DRG neurons exposed to Saporin alone (which does not eliminate nonpeptidergic neurons and served as a control) did not show any blockage effect of DD04107, in addition, neurons treated with DD04107 (6.2±1.9) showed significantly higher potentiation of TRPV1 evoked neuronal activity compared to the Vehicle (2±0.8) (*- $p<0.05$) as shown in **figure- 72 (B)**.

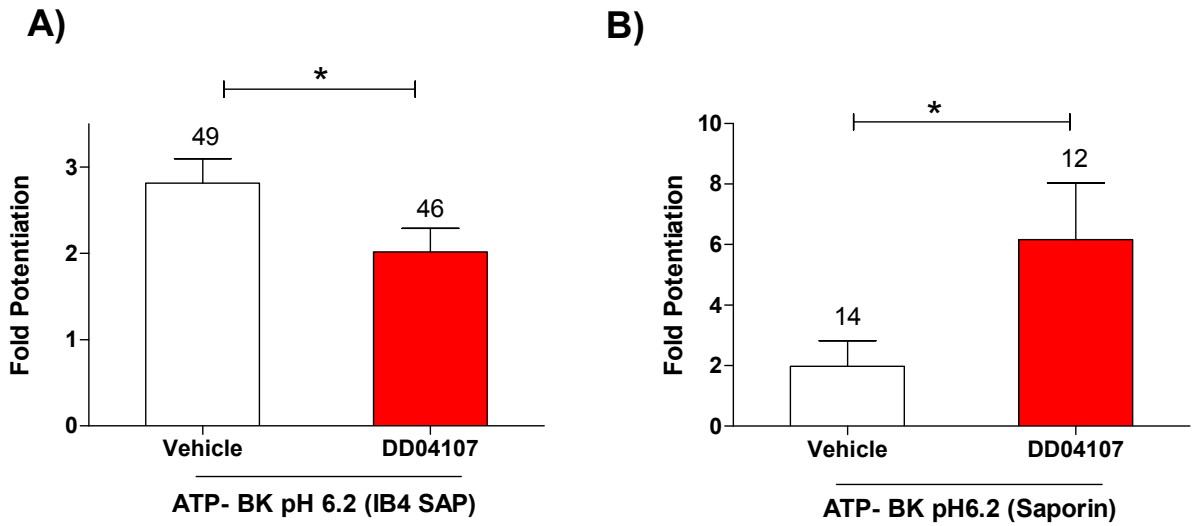

Figure- 72: ATP- BK at pH 6.2 mediated TRPV1 sensitization requires channel recruitment in a subset of nociceptors in IB4 Saporin treated (A) but not in Saporin treated (B) rat nociceptors. Effect of 20 µM DD04107 on 10 µM ATP – 1 µM BK with pH 6.2 mediated potentiation of TRPV1 activity was determined by comparison of capsaicin-mediated neuronal spikes before and after incubation of ATP- BK pH 6.2 (ratio P3/P2) of the groups Vehicle and DD04107 in both IB4 Saporin and Saporin treated neonatal rat DRG neurons . Data are expressed as mean ± SEM. The numbers above the bars represent the total number of electrodes that responded. Number of cultures = 3. Statistical analysis was performed by Unpaired Student's t- test (*- $p<0.05$).

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DISCUSSION

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DISCUSSION

Ion channels play an important role in controlling physiological activity. Activation of ion channels lead to alteration in the membrane potential. During pathophysiological conditions an up regulation or down regulation of the ion channel activity is detected and represented as an increase or decrease in the number of action potentials. Similarly, a considerable increase in the excitability of neurons represents the underlying mechanism in sensitization of nociceptors (324). During tissue injury, a plethora of algogen factors are released from damaged nociceptors and surrounding immune cells. These inflammatory mediators will act on their receptors on sensory neurons, thus leading to modulation of ion channel gating, thereby provoking an enhanced pain sensation (325). Inflammation can sensitize nociceptors through short term modifications induced by either a decrease in excitability threshold of the ion channels or activation of phosphorylation mechanisms (326). One such primary ion channel involved in sensitization of nociceptor is TRPV1, a thermoTRP channel which is primarily involved in heat sensation (194). Many studies on inflammatory sensitization of TRPV1 suggest its significant role in nociceptive pain signalling pathway (124, 202).

Earlier studies have reported that inflammatory sensitization of TRPV1 could involve differential mechanism from the recruitment of new TRPV1 channels to the plasma membrane through regulated exocytosis (304) to the modulation of channel gating through phosphorylation (327). Experiments using blockers of regulated exocytosis BoNTA and DD04107 revealed that these blockers potently reduce increased surface expression of TRPV1 induced by algogens. Hence the nociceptors exhibit reduced TRPV1 activity confirming that exocytotic mobilization of TRPV1 is a key mechanism in sensitizing a subset of nociceptors upon an inflammatory insult. A previous study from our group reported that some inflammatory mediators like ATP, NGF and IGF-1 can sensitize TRPV1 through exocytosis of new channels, whereas BK, Artemin and IL-1β sensitize TRPV1 through phosphorylation mechanism (312). Due to their higher heterogeneity, which subpopulation of nociceptor is involved in TRPV1 exocytosis and phosphorylation mechanisms remains unclear.

Therefore, the principal objective of this work is to unravel the underlying mechanism behind inflammatory sensitization of TRPV1 in nociceptors. For this purpose, we have studied the mechanism of two inflammatory mediators ATP and BK on TRPV1 sensitization in peptidergic and nonpeptidergic nociceptor subpopulations.

We have found that:

1. Both ATP and BK sensitize TRPV1 through exocytosis in peptidergic nociceptors.
2. αCGRP is essential for both ATP and BK induced exocytotic mobilization of TRPV1 in peptidergic nociceptors.

Regulated exocytosis of TRPV1 in nociceptors was examined by using a hexamer lipopeptide DD04107 (EEMQRR), an inhibitor of neuronal exocytosis. This peptide is patterned after the N-terminus of SNAP 25 protein which inhibits SNARE complex formation thereby specifically blocking regulated exocytosis (328). In addition, peptide DD04107 was found to exhibit long lasting anti nociceptive potential in distinct animal pain models *in vivo* (313). From experimental conditions *(in vitro)*, we have confirmed that the peptide does not interfere neither with the basal electrical properties of the nociceptors nor the initial TRPV1 current densities in both peptidergic and nonpeptidergic subpopulations.

TRPV1 MODULATION BY ATP

Initially, we explored the mechanism of ATP mediated TRPV1 potentiation. ATP, the prominent cytosolic content of keratinocytes and sensory neurons, is released upon tissue damage and excites nearby nociceptors (75). ATP evoked excitatory action potentials in nociceptors are due to the activation of purinergic P2X and P2Y receptors (315). In nociceptors, the purinergic receptor families P2X3, P2Y1 and P2Y2 receptors are widely expressed. Although most of the $TRPV1^{+}$ sensory neurons express P2X3 receptor, they do not contribute for TRPV1 sensitization (329). P2Y1 was found to be strongly colocalized with IB4, whereas it was weakly colocalized with TRPV1. Initial studies on ATP induced TRPV1 sensitization revealed that P2Y1 receptor was involved in sensitizing TRPV1 currents through PLC- DAG/ IP_3 pathway induced by $G\alpha_{q/11}$ signalling protein (329). To further validate the role of P2Y1 receptors on TRPV1 sensitization, experiments were done in P2Y1 knockout mice ($P2Y1^{-/-}$). ATP induced TRPV1 sensitization was preserved in $P2Y1^{-/-}$

mice, suggesting a lack of P2Y1 receptor involvement on TRPV1 potentiation. In sensory neurons, P2Y2 receptor was strongly expressed with TRPV1 suggesting that P2Y2 can functionally interact with TRPV1 (172) thus contributing for TRPV1 evoked thermal hyperalgesia. Furthermore, pharmacological activation of P2Y2 receptors in sensory neurons exhibited robust TRPV1 sensitization (277). Other studies on P2Y2 receptors also corroborated their potential role in TRPV1 sensitization and nociception**.** During CFA induced inflammation, P2Y2 was the only receptor found to be elevated, thus contributing to significant ATP responses in sensory neurons leading to enhanced thermal hyperalgesia (330). Another study on kidney projecting sensory neurons validated the significant role of P2Y2 receptors on ATP induced TRPV1 sensitization (331). Thus ATP induced TRPV1 potentiation seems to be chiefly mediated by P2Y2, although we cannot exclude the possible involvement of P2Y1 receptors on sensitizing TRPV1 channel activity. Moreover, activation of P2Y2 receptor stimulates pertusis toxin insensitive $G\alpha_{q/11}$ protein which associates with PLC- β3 catalysing PIP2 and activating IP_3 and DAG- PKC pathways (332). $G\alpha_{q/11}$ induced PLC-β3 activation is crucial for ATP mediated pain signalling since PLC-β3 is primarily expressed in DRG neurons among other isoforms of PLC β (333).

From our experiments on neonatal rat DRG neurons, we observed a high impact of inhibiting neuronal exocytosis on lessening ATP induced sensitization of TRPV1 evoked neuronal spikes. This suggests a major role of exocytotic mobilization of new TRPV1 channels in a subset of the whole neuronal population. Furthermore, targeted differentiation of peptidergic and nonpeptidergic neurons using IB4 marker revealed that two distinct mechanism of ATP induced TRPV1 sensitization is involved. Inhibitor of neuronal exocytosis – DD04107 completely abolished TRPV1 sensitization in peptidergic neurons but had no effect on nonpeptidergic neurons. Additionally, TRPV1 sensitization in peptidergic neurons is PKC independent, whereas in nonpeptidergic neurons ATP induced TRPV1 sensitization is PKC dependent. This confirms the exocytotic release of new vesicles as a prime mechanism in peptidergic and phosphorylation mechanism in nonpeptidergic subpopulations upon ATP induced TRPV1 sensitization.

Since we found that in peptidergic nociceptors TRPV1 was sensitized mainly through exocytosis, we hypothesized that the inflammatory recruitment of TRPV1 channel could occur through release of TRPV1 channel from the vesicles containing neuropeptides CGRP

and SP. Many studies suggested the potential role of these neuropeptides in sorting receptors/ ion channels into large dense core vesicles (LDCVs) (334-336). The presence of TRPV1 along with CGRP and SP suggests their possible involvement in sorting TRPV1 into the LDCVs and driving its regulated exocytosis. We performed experiments on wild type and $\alpha CGRP^{-/-}xTac1^{-/-}$ (DKO) mice in order to know the potential role of neuropeptides on ATP induced TRPV1 sensitization. In adult wild type mice, TRPV1 expression is primarily restricted to peptidergic nociceptors (199). Notably, experiments from adult wild type mice exhibited a strong sensitization of TRPV1 evoked spikes induced by ATP. Such sensitization was completely abolished by inhibiting neuronal exocytosis thus corroborating that ATP induced TRPV1 exocytosis is specific for peptidergic nociceptors. Nociceptors from DKO mice also exhibited ATP induced sensitization of TRPV1 evoked neuronal spikes which was insensitive to the neuronal exocytosis inhibitor, suggesting a significant role of neuropeptides on TRPV1 release through exocytosis. Furthermore, experiments from single knockout animal (Tac1 and αCGRP) revealed that αCGRP but not SP is essential for ATP induced regulated exocytosis of TRPV1. Since the released neuropeptides can also sensitize TRPV1 in primary sensory neurons, we did not exclude the possibility that the released neuropeptides could act through autocrine signalling mechanism. Indeed, earlier studies revealed that SP released upon TRPV1 activation sensitizes the receptor through both autocrine and paracrine mechanism. SP binds to NK1 receptor and activates PKC_{ε} which induces phosphorylation of TRPV1. NK1 receptor strongly colocalises with TRPV1 in unmyelinated small diameter neurons (337). SP induced TRPV1 sensitization further enhances capsaicin evoked SP release from cultured rat DRG neurons (286). In addition, activation of NK2 receptor in afferent neurons also leads to PKC induced phosphorylation of TRPV1 leading to sensitization of capsaicin activated currents (287). Though, no direct evidence of CGRP involvement on sensitizing TRPV1 channel was reported, a vital role of CGRP on sensitizing nociceptors has been extensively described (305). From our experiments we pharmacologically blocked both CGRP and NK1 receptors *in vitro* using specific antagonists and found that no autocrine signalling mechanism of neuropeptides was involved in sensitization of TRPV1. This validates the prime role of TRPV1 release in ATP induced sensitization process.

It has been reported that, akin to the activation of TRPV1, a strong depolarization stimuli can also induce CGRP release. All these stimuli require external Ca^{2+} ions to release CGRP. Studies on trigeminal sensory neurons showed that an elevated K^{+} concentration can

induce increase in CGRP release which is Ca^{2+} dependent. We tested if depolarization of neurons could mimic a similar mechanism of ATP induced TRPV1 exocytosis, since depolarization is also an important underlying signal for dense core vesicle exocytosis (338). Experiments on wild type mice showed that depolarization of the neurons with elevated external K^+ concentration [40 mM] did not induce sensitization of TRPV1 evoked spikes. Hence, we confirmed that TRPV1 exocytosis is a specific mechanism for ATP induced inflammatory signalling.

TRPV1 MODULATION BY BK

Bradykinin is another endogenous pain causing potent algogenic nonapeptide released during tissue damage (318). BK belongs to kinin family whose production is enhanced in tissues during pathophysiological conditions leading to activation of kallikrein (339). BK is released from mast cells and basophils (340). Bradykinin exerts its action through B1 and B2 receptors. Both B1 and B2 are metabotropic receptors which trigger second messenger signalling (341).

B2 receptor expression is ubiquitous and constitutive, whereas B1 receptor is expressed only in inflammatory conditions. Although in DRG and TG neurons constitutive B1 receptor expression was found to be inconsistent, few studies have identified the constitutive expression of B1 receptor. B1 receptor was found to be expressed preferentially in $IB4^+$, but not in CGRP containing C fibers of dorsal root ganglion (321). Recent studies from TG neurons disclosed that B1 and B2 receptors were functionally expressed, although immunoreactivity of B1 was weak when compared to B2 (342).

Prior to the identification and cloning of TRPV1 channel, studies on DRG neurons reported that a novel heat activated current was observed. Sensitization of this heat activated current was observed upon exposure to bradykinin, which was mediated by PKC (76). Furthermore, bradykinin increased the percentage of neurons responding to capsaicin (343). After the identification of TRPV1 in sensory neurons, studies revealed that BK exposure led to lowering of temperature threshold to activate TRPV1 channel. The reduction in temperature was up to 24 ^oC which is under the normal physiological temperature thus leading to activation of the channel at room temperature. All these mechanisms were PKC

dependent, specifically through PKC_{ε} (278, 344). In addition, interaction of TRPV1 and PKC_{ε} with AKAP 79/150 protein was found to be important for bradykinin induced sensitization of TRPV1 (319).

The amino acid positions S502 and S800 of TRPV1 are the putative phosphorylation sites responsible for PKC mediated sensitization of capsaicin responses in sensory neurons. Of these phosphorylation sites, S800 was identified as a major contributor for bradykinin induced hypersensitivity of TRPV1 in sensory neurons (345).

Earlier studies from our group also reported that BK can sensitize TRPV1 through phosphorylation mechanism and not through exocytosis (312). Numerous studies on nociceptors have reported the possible involvement of BK in trafficking and exocytosis. Likewise BK induced trafficking of opioid receptors in nociceptors was extensively studied (316, 346). BK has also been involved in exocytotic release of CGRP in primary sensory neurons (305). Another study on DRG neurons showed that acute incubation (10 minutes) of BK (1 μM) markedly increased the release of CGRP in a concentration dependent manner (317). In our experiments we tested whether Bradykinin (1 μM), which is coupled to $G\alpha_{q/11}$ protein signalling, can induce exocytosis of TRPV1 similar to ATP in peptidergic subpopulations.

Experiments on BK mediated TRPV1 potentiation from neonatal rat DRG neurons revealed that BK can induce TRPV1 exocytosis similar to ATP. A high impact of the peptide in blocking TRPV1 sensitization from whole neuronal population was observed, suggesting a major role of exocytotic mobilization of new TRPV1 channels in a subset of nociceptors. In addition, targeted differentiation of peptidergic and nonpeptidergic neurons using IB4 marker revealed that a different mechanism is involved in BK induced TRPV1 sensitization. Inhibitor of neuronal exocytosis (DD04107) completely abolished TRPV1 sensitization through regulated exocytosis in peptidergic neurons but had no effect on nonpeptidergic neurons. Additionally, no significant TRPV1 sensitization was observed in nonpeptidergic neurons by BK. This could be due to the differential expression of BK receptors in nociceptor subpopulations, where B2 is specifically expressed in peptidergic and B1 is specifically expressed in nonpeptidergic. Furthermore, a small population of $IB4^{+}$ neurons showed positive immunoreactivity for B2 receptors (342), which suggests that the observed TRPV1

sensitization by BK in nonpeptidergic nociceptors could be due to the expression of B2 receptor. Since it is known that BK induces phosphorylation of TRPV1, we studied if exocytosis of TRPV1 is the prime mechanism involved in peptidergic nociceptors similar to ATP. We observed that in peptidergic neurons BK induced TRPV1 sensitization is also PKC dependent, suggesting that a dual mechanism of TRPV1 sensitization (exocytotic release of new vesicles and phosphorylation) is involved in peptidergic subpopulation. Also PKC was reported as one of the major activator for regulated exocytosis of TRPV1 (304).

Though BK induced TRPV1 sensitization is primarily mediated by B2 receptor, contribution of B1 receptor on TRPV1 sensitization cannot be ruled out. By pharmacologically activating BK receptors we found that B2 receptor is predominantly involved in BK induced TRPV1 sensitization with a less contribution from B1 receptor. Specific agonist for B2 receptor mediated TRPV1 sensitization was similar to BK activation.

Since we found that BK induced TRPV1 sensitization in peptidergic nociceptors is primarily mediated through regulated exocytosis, we extended our studies to identify the possible involvement of αCGRP on BK induced TRPV1 sensitization.

Notably, experiments from adult wildtype mice exhibited strong sensitization of TRPV1 induced by BK. Such sensitization was completely abolished by inhibiting neuronal exocytosis corroborating that BK induced TRPV1 exocytosis is specific for peptidergic nociceptors. Conversely, studies on nociceptors from single knockout mice revealed that αCGRP but not SP is essential for BK induced TRPV1 sensitization. These results corroborate with our ATP experiments, suggesting that both ATP and BK activate $G\alpha_{q/11}$ signalling protein and induce TRPV1 exocytosis in peptidergic nociceptors. Recently *in vivo* studies to understand the role of $G\alpha_{q/11}$ signalling protein on nociceptor sensitization induced by algogens were performed using $G\alpha_q^{-/-}$, $G\alpha_{11}^{-/-}$ and $G\alpha_{q/11}^{-/-}$ knockout mice models. The results showed that both ATP and BK induced thermal hyperalgesia was preserved in $G\alpha_{11}^{-/-}$ deficient mice, whereas it was completely abolished in $G\alpha_q^{-/-}$ and $G\alpha_{q/11}^{-/-}$ deficient mice. This confirms that the major common signalling mechanism involved in ATP and BK induced nociceptor sensitization is mediated through $G\alpha_q$ protein and provokes thermal hyperalgesia, where TRPV1 could play a significant role (347). This study supports our hypothesis that both ATP and BK induce their effect on nociceptors through $G\alpha_{q/11}$ protein with similar mechanism.

FACILITATORY EFFECT OF ATP- BK ON TRPV1 MODULATION

Furthermore, we studied the combinatorial effect of ATP and BK on facilitating TRPV1 sensitization under acidic pH conditions. This is to mimic the tissue acidosis microenvironment during inflammatory conditions. Algogens are known to interact with protons and facilitate their effects in different animal models. Bradykinin functions as a co-operative algogen with other inflammatory mediators to evoke pain sensation (348). In addition, combinatorial applications of BK with other inflammatory mediators were found to significantly increase the release of CGRP in DRG neurons. This release of CGRP could not be enhanced further by acidification. Exposure of DRG neurons to acidic pH also induced CGRP release which was lower compared to inflammatory mediators. Both, low pH and inflammatory mediators induced CGRP release, are dependent of external Ca^{2+}. Also the inflammatory mediators were proposed to act as endogenous activators for TRPV1 since TRPV1 antagonist Capsazepine (CPZ) inhibited inflammatory mediators induced release of CGRP in DRG neurons (349).

From our experiments on neonatal rat DRG neurons, combined application of both ATP and BK significantly increased TRPV1 evoked neuronal spikes which were abrogated by blocking neuronal exocytosis. This validates the major role of TRPV1 exocytosis similar to what we have observed earlier in our individual experiments with ATP and BK. In addition, we increased the strength of inflammatory mediators by decreasing the extracellular pH to 6.2, to mimic the acidic microenvironment conditions observed during tissue injury. We found that combined application of ATP and BK at pH 6.2 significantly increased TRPV1 evoked neuronal spikes which were insensitive to neuronal exocytosis inhibitor. This could be due to the decrease in extracellular pH, in fact numerous studies have reported that acidic pH 6.1 can potentiate TRPV1 currents by strongly reducing the EC_{50} of capsaicin from μM to nM concentrations (323). We proposed that the reduced pH alone could sensitize TRPV1 evoked spikes and we confirmed from our experiments that exposure of TRPV1 desensitized neurons to pH 6.2, led to significant sensitization of TRPV1. From our results we confirmed that DD04107 significantly abrogates TRPV1 sensitization in peptidergic subpopulations and the reduction of pH could enhance its sensitizing effect of TRPV1 channel in other subpopulations of primary sensory neurons. In rat DRG neurons TRPV1 is expressed in both C fibers (peptidergic and nonpeptidergic) and Aδ fibers. We proposed that

the pH 6.2 induced TRPV1 modulation is mainly occurring in nonpeptidergic and Aδ fibers. Then DD04107 could inhibit ATP and BK at pH 6.2 induced TRPV1 sensitization in peptidergic subpopulations. This was proved by using IB4 saporin toxin which selectively eliminates $IB4^+$ neurons (350). Studies reported that injection of IB4 saporin toxin eliminated acute and chronic pain conditions *in vivo* (351, 352). We found that ATP, BK and pH 6.2 induced TRPV1 sensitization on remaining $IB4^-$ neurons was significantly inhibited by blocking neuronal exocytosis. As a control, in neurons treated with saporin toxin alone we did not observe any significant blockage of TRPV1 sensitization by DD04107. This result corroborates the significant role of DD04107 on alleviating inflammatory sensitization of TRPV1 in peptidergic nociceptors.

Hence in conclusion, this study reveals the involvement of distinct signalling mechanism in TRPV1 sensitization induced by algogens in peptidergic and nonpeptidergic nociceptors. Furthermore, it substantiates the potential therapeutic role of DD04107 to alleviate inflammatory pain induced by sensitization of TRPV1.

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CONCLUSIONS

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- Inflammatory mediators (ATP/ BK) induce enhanced excitability in peptidergic nociceptors, while in nonpeptidergic nociceptors induce a moderate effect.

- TRPV1 sensitization induced by inflammatory mediators (ATP/ BK) occurs primarily through exocytotic recruitment of channels from large dense core vesicles (LDCVs) to the plasma membrane in peptidergic nociceptors however, in nonpeptidergic nociceptors TRPV1 is sensitized through differential mechanism which also includes phosphorylation of the channel.

- Deletion of αCGRP but not Tac1 expression impairs inflammatory TRPV1 recruitment to the surface of peptidergic nociceptors, leading to abrogation of TRPV1 sensitization. This corroborates the potential role of αCGRP in promoting regulated exocytosis of TRPV1.

- TRPV1 is co- trafficked with αCGRP in LDCVs in peptidergic nociceptors.

- Inhibition of LDCV exocytosis is a valuable therapeutic strategy to treat pain, as it concurrently reduces the release of pro- inflammatory peptides and the membrane recruitment of TRPV1 channels.

- DD04107 abolishes TRPV1 sensitization induced by an inflammatory microenvironment (*in vitro*), thus substantiating the therapeutic potential of DD04107 to alleviate inflammatory pain induced by sensitization of TRPV1.

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- Los mediadores inflamatorios (ATP/ BK) provocan una excitabilidad mayor en neuronas peptidérgicas mientras que en neuronas no peptidérgicas producen un efecto moderado.

- La sensibilización de TRPV1 inducida por los mediadores inflamatorios (ATP/ BK) en neuronas peptidérgicas se produce principalmente a través de exocitosis regulada del canal desde vesículas de centro denso (LDCVs) a la membrana plasmática. En el caso de nociceptores no peptidérgicos la sensibilización del TRPV1 es causada principalmente por fosforilación del canal.

- El silenciamiento del gen αCGRP pero no de Tac1, provoca una disminución de TRPV1 en la membrana neuronal suprimiendo completamente la sensibilización inflamatoria del canal, lo que indica el papel potencial de este neuropéptido en promover la exocitosis de TRPV1.

- TRPV1 es transportado a la membrana neuronal en LDCVs junto con el neuropéptido αCGRP en nociceptores peptidérgicos.

- La inhibición de la exocitosis de LDCVs puede ser una estrategia terapéutica muy eficaz en el tratamiento del dolor ya que reduce la liberación de péptidos pro-inflamatorios y la presencia de TRPV1 en la membrana neuronal.

- DD04107 suprime la sensibilización de TRPV1 inducida por el microambiente inflamatorio *(in vitro)*, demostrando el potencial papel terapéutico del péptido DD04107 en la disminución del dolor inflamatorio inducido por la sensibilización de TRPV1.

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MATERIALS AND METHODS

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All procedures were approved by the Institutional Animal and Ethical Committee of the University Miguel Hernández de Elche, in accordance with the guidelines of the Economic European Community, the National Institutes of Health, and the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Animals. Animals were kept in a controlled environment (21-23 ºC, 12h light/dark cycle), and had food and water available *ad libitum*. Neonatal Wistar rats and wild type C57BL/6J mice were purchased from in house bred stock (originally from Harlan Laboratories). αCGRP-deficient mice (B6;129-Calca^{tm}) were kindly provided by Dr. J.P. Changeaux. Tac1-deficient mice (B6.Cg-Tac1^{tm1Bbm}/J) were purchased from The Jackson Laboratory. Double knockout mice αCGRP and Tac1 genes were in house generated by crossing homozygous αCGRP and Tac1 null mice to obtain first heterozygous and later homozygous deficient mice (B6; B6; 129-Calca^{tm}Tac1^{tm1Bbm} /J).

Primary culture of sensory neurons. DRG from neonatal Wistar rats (3-5 days old) or adult male 12-15 week-aged mice (strains: C57BL/6J, αCGRP$^{-/-}$, Tac1$^{-/-}$, and αCGRP$^{-/-}$xTac1$^{-/-}$ mice), cultured following previously described protocols with some modification (290, 353). Neonatal rat ganglia were digested with 0.25% (w/v) collagenase (type IA) in DMEM-glutamax (Invitrogen) with 1% penicillin-streptomycin (P/S) (5000 U/mL, Invitrogen) for 1 h (37 ºC, 5% CO_2). Isolated mouse DRG were incubated with 0.67% (w/v) collagenase type XI and 3% (w/v) dispase (Gibco) in INC mix medium (in mM): 155 NaCl, 1.5 K_2HPO_4, 5.6 HEPES, 4.8 NaHEPES, 5 glucose) for 1 h (37 ºC, 5% CO_2). After digestion, rat and mouse DRG were mechanically dissociated using a glass Pasteur pipette. Single cell suspension was passed through a 100 µM cell strainer, and washed with DMEM glutamax plus 10% fetal bovine serum (FBS) (Invitrogen) and 1% P/S. Cells were seeded at the required density for each experiment on 12 mm cover-glass slides, or microelectrode array chambers previously coated with poly-L-lysine (8.33 µg/ml) and laminin (5 µg/ml). After 2 h, medium was replaced with DMEM glutamax, 10% FBS and 1% P/S, supplemented with mouse 2.5s NGF 50 ng/mL (Promega), and 1.25 µg/mL cytosine arabinoside when required (37 ºC, 5% CO_2). If it is not specified, all experiments were made 48 h after cell seeding. To deplete Isolectin IB4^{+} neurons, rat DRG were cultured in the presence of 10 nM IB4-saporin or saporin as control (Advanced Targeting Systems) for 48 h.

Patch clamp recordings. Whole-cell voltage clamp was made in neurons seeded on coverslips, placed in RC-25 chamber and connected to a external perfusion system at RT. HBSS external solution (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 5 D-glucose, 20 mannitol, pH 7.4 (adjusted with NaOH). Internal pipette solution (in mM): 144 KCl, 2 $MgCl_2$, 10 HEPES, 5 EGTA, pH 7.2 (adjusted with KOH). For the experiments with less pH (pH 6.2) HEPES was replaced with MES. Membrane currents were acquired by using EPC10 HEKA Patch amplifier (HEKA Electronics). Cells were held at a resting membrane potential of -60 mV, and at a sampling rate of 2.5 Hz. Patch glass pipettes with O.D 1.5 mm x I.D. 1.17 mm (Harvard Instruments) made with Sutter Pippete puller Equipment (Sutter Instruments) with 3-6 MΩ resistance. Data acquisition and offline analysis with PatchMaster software (HEKA Electronics). Rat DRG neurons labelled with IB4-alexa 568 (10 μg/ml, 10 min, RT) in external solution (Molecular Probes, Invitrogen), followed by two 5 min-washes, and visually identified through x20 air objective (Aixiovert 200 inverted microscope, Carl Zeiss), with an excitation filter ET545 and an emision filter ET605 (CHR-49004, Laser 2000 SAD). Neuronal viabiliy determined through typical neuronal Na^+-K^+ currents. TRPV1 desensitization was induced by three repetitive 10s-pulse of 1 μM capsaicin using perfusion system (2 mL/min flux). 10 μM ATP or 1 μM BK was applied for 8 min between P2 and P3 pulse. Potentiation of TRPV1-mediated currents was calculated as ratio P3/P2 current peak. Capsaicin dose-response was determined in wild type and $\alpha CGRP^{-/-}xTac1^{-/-}$ neurons with resting membrane potential below -40 mV.

Electrical properties. Electrical properties of the neuron were determined 2 minutes after establishing whole cell access using current clamp mode. For current-clamp recording, cells were held at 0 pA. Cells were assessed for the presence of spontaneous activity for 1 min and processed to measure electrogenic properties. Both $IB4^-$ and $IB4^+$ nociceptors were processed for electrogenic properties before ATP and BK application. Firing threshold was measured first by injecting a series of 100 ms depolarizing current in 10 pA steps from 0 pA to elicit the first action potential. To further examine the neurons firing properties, a depolarizing current injection in 100 ms, 40 pA ($IB4^-$) and 100 pA ($IB4^+$) was delivered to elicit an action potential which was analysed for the following intrinsic membrane properties: threshold potential (mV), amplitude of action potential (mV), duration of action potential (ms), overshoot of action potential (mV). 10 μM ATP and 1 μM BK was applied to $IB4^-$ and $IB4^+$ nociceptors for 4 minutes to observe both ATP and BK induced spontaneous neuronal

excitabilities. RMP was checked before and after ATP, BK application. Mean depolarization was calculated by selecting the maximum depolarized voltage observed upon ATP and BK application in $IB4^-$ and $IB4^+$ nociceptors. ATP and BK induced changes in electrically evoked action potentials were measured before (Vehicle) and during ATP and BK application. Nociceptors ($IB4^-$ and $IB4^+$) were injected with 100 pA for 100 ms, 300 pA for 1 sec to measure electrically evoked action potentials.

Microelectrode Array (MEA). Extracellular recordings were made using multiple electrode planar arrays of 60-electrode thin MEA chips, with 30 µm diameter electrodes and, 200 µm inter-electrode spacing with an integrated reference electrode (Multichannel Systems GmbH). The electrical activity of primary sensory neuron was recorded by the MEA1060 System (Multi Channel Systems GmbH, http://www.multichannelsystems.com), and MC_Rack software version 4.3.0 at a sampling rate of 25 kHz. TRPV1-mediated neuronal firing activity was evoked by three repetitive 15s-applications of 500 nM capsaicin, using continuous perfusion system (2 mL/min flux). 10 µM ATP or 1 µM BK in external solution was perfused between P2 and P3 for 8 min. For the experiments on combined application of ATP- BK on TRPV1 sensitization, 10 µM ATP- 1 µM BK was perfused between P2 and P3 for 8 min. For the experiments on bradykinin receptor agonists, selective bradykinin receptor agonists for BK1 and BK2 was applied between P2 and P3 for 8 min. Data were analyzed using MC_RACK spike sorter and Neuroexplorer Software (Nex Technologies). An evoked spike was defined when the amplitude of the neuronal electrical activity overcame a threshold set at -20 µV. The recorded signals were then processed to extract mean spike frequency.

Immunocytochemistry. For immunocytochemistry, cultured neonatal DRG seeded on coverslips washed with PBS, were fixed with 4% w/v paraformaldehyde for 20 min, washed, permeabilized with 0.3% Triton X-100 for 5 min at RT, and then blocked with 5% NGS for 1 h at RT. Primary antibody was incubated in 5% NGS in PBS overnight at 4 ºC. Once washed with PBS-Tween 0.05%, samples were incubated with secondary antibodies in blocking solution (1 h, RT). Samples were mounted with Mowiol® (Calbiochem). Primary antibody: rabbit anti-TRPV1 (Alomone, 1.6 µg/mL). Secondary antibody: Isolectin B4-Alexa 568 (Molecular Probes, 10 µg/mL). Samples visualized with an inverted confocal microscope (Zeiss LSM 5 Pascal, Carl Zeiss). Number of positive stained neurons determined by

capturing at least 5 images per sample, and then analysed with Zen lite 2012 software (Zeiss) or Image J software (NIH), under blinded conditions.

Chemicals. DD04107 (BCN Peptides) freshly prepared at 20 mM in H_2O. For microelectrode array, cells were incubated with compound 20 µM DD04107 in HBSS for 1 h (37 ºC, 5% CO_2). For electrophysiological recordings, non-palmitoylated DD04107 (BCN Peptides) at 100 µM concentration was prepared in standard internal solution from 10 mM stock solution in H_2O. Peptide or vehicle were applied through the patch pipette 10 min before recording. ATP was dissolved in H_2O at 10 mM and further diluted in HBSS at 10 µM. The CGRP receptor 1 antagonist $CGRP_{8-37}$ (Tocris Bioscience, R&D Systems) dissolved in 30% acetonitrile (v/v) at 100 µM and further diluted in HBSS at 250 nM. The neurokinin receptor 1 antagonist CP96345 (Tocris Bioscience, R&D Systems) dissolved in DMSO at 20 mM and further diluted in HBSS at 10 µM. Both antagonists applied with ATP. BIM was dissolved in DMSO at 1mM and further diluted in HBSS at 1 µM. BK was dissolved in H_2O at 1 mM and further diluted in HBSS at 1 µM. The BK1 receptor agonist- Sar-[D- Phe^8]-des-Arg^9-Bradykinin, BK2 receptor agonist- [$Phe^8\psi$(CH-NH)-Arg^9]-Bradykinin (Tocris Bioscience, R&D Systems) was dissolved in H_2O at 1 mM and further diluted in HBSS at 1 µM. When not specified, all chemicals were obtained from Sigma-Aldrich.

Data analysis. All data are expressed as mean ± SEM, with *n* as number of registered cells and *N* as the number of experiments. The percentage of sensitized neurons was calculated considering those cells that exhibited a fold potentiation above 1.1. Statistical analysis was made using 1-way ANOVA or 2-ways ANOVA as required followed by Bonferroni's *post-hoc* test using Graph Pad Prism 5.0 (Graph-Pad). For the experiments on characterization of nociceptors using patch clamp, paired and unpaired student's t-test was used. MEA data were analysed by Bonferroni's *post-hoc* test as paired values through comparison of the responses of each electrode along time. Fold potentiation (ratio P3/P2) was analyzed by unpaired student's t- test $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

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ANNEX

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1. Devesa I, Ferrándiz-Huertas C, **Mathivanan S**, Wolf C, Luján R, Changeux JP, Ferrer-Montiel A. (2014) αCGRP is essential for algesic exocytotic mobilization of TRPV1 channels in peptidergic nociceptors. **PNAS** 111(51):18345-50

2. **Mathivanan S**, Devesa I, Changeux JP and Ferrer- Montiel A. Bradykinin induces TRPV1 exocytotic recruitment in peptidergic nociceptors. **(Manuscript submitted)**.

3. Ferrandiz-Huertas C, **Mathivanan S**, Wolf CJ, Devesa I, Ferrer-Montiel A.(2014) Trafficking of ThermoTRP Channels. **Membranes (Basel)** 4(3):525-64.

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ACKNOWLEDGEMENTS

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Acknowledgements

First and foremost, I would like to thank my supervisor Prof. Antonio Ferrer Montiel for giving me an opportunity to do my doctoral thesis in his laboratory, for his constant support and encouragement, for introducing me to the field of pain research and electrophysiology. Also for permitting me to collaborate with different projects of the lab apart from my thesis work.

I am extremely thankful to Dr. Isabel Devesa for her continuous support and being a backbone throughout my thesis with her valuable suggestions.

Thanks to the PhD program committee of IBMC- UMH for helping me to organise my work every six months.

Thanks to the people from Secretaria of IBMC (May, Raquel, Javier and Carmen) for assisting me with the paper works without any hesitation whenever I was in need.

I never thought of leaving my country for my career and to be honest I had no idea about Spain and the people. But here I am completing my six years of stay in Spain. I am just thinking to rewind my clock to 6 years back in my life to start my experience again in Spain. I can't even imagine how I ended up in Spain without having any idea about the people and their customs especially the language. Amidst my nervousness of stepping into a new country I should say the warm reception I have had here I am sure I will not see in any other country.

If your work environment is good you can really achieve what you have aimed for. I feel I am gifted to work with these lovely people of my lab for supporting me all these years, for tolerating and taking care of me. Just words cannot describe my experience with these lovely colleagues/ friends. I am thankful to all our present and past group members Luci, Roberto S/P, Aaron, Roberto torre, Maria grazia, Isa, Cloti, Gema, Maite, Paco, Vero, Ainara, Christoph, Nuria, Pier, Jan, Antonio zafra, Monica, Gregorio, Asia, Carlo, Carmen, Efren, Laura, Magdalena, Jose, Eva. In other words I can say thanks to Sensory Neurobiology Group just to see the reaction of Prof. De la Torre ☺. Also thanks to Angeles, Lourdes and Estefania from IBMC for the wonderful time. Although I felt missing my home during initial days later all these people made me to feel happy. Now Spain has become my second home

(It was so crazier for me to get angry when Spain lost in the league matches of FIFA 2014 and the people around me were looking like why this Indian is so angry than us).

I am thankful to Luci, Roberto S/P and Aaron for helping me to settle down in Elche. I must say they were the first people who made me to feel comfortable and helping with all the paper works and house hunting (well I was lucky to find a house in just 4 days of my arrival and staying there for all these years).

I would like to remember the wonderful moments I have had in Spain. They are always the sweet memories. Right from Ola que ace? Just potato, Brave potatoes, Ey man, My Mobile in La boda de Gema (the best wedding party I have enjoyed in my lifetime), El Karma, El Chiringuito, La morcilla (my morcilla rankings in Elche), jamon Serrano, How to make Mojitho class from prof. De la torre, our Wednesday movies and dinner, after work drinks on Fridays, Sangria, chori- chori, my swimming classes, those chungo moments, evening meeting with my friend George in the parque, more fiestas and more Resaca !!! It is really hard to leave my second home but I have to move on with these memories. There is always space to meet again, the world is so small and there are more flights. Who knows Ryanair will start a flight service from India to Europe in future.

How can I miss my wonderful Spanish Sobrinos and Sobrinas? A special thanks to them for their love and for considering me as an Indian Tio. For sure I am going to miss them tooooooo...

My heartfelt thanks to my friends Henrique, Cheo, Silvia, Laura, Alicia, Pablo, Estefania, Rayco, Ana, Espartaco, Silvia in Elche for having wonderful time.

A Special thanks to Maria grazia, Cloti, Maite and to my sister Ammu for helping me in thesis correction.

Thanks to the amable people of Spain for treating me well and making me to feel good at all times.

A special thanks to Dr. Rema, Dr. Soumya, Dr.Sayali from National Brain Research Centre, India for their support and helping me to get an initial research experience.

Acknowledgements

Thanks to all my seniors for their guidance in my research career.

Thanks to Dr.Sadiq, Dr. Gowthaman, Mr. Arulkumaran, and Dr. Preetham for introducing me into life sciences.

Thanks to my friends Balaji, Sivaraj, Ethiraj, Thamizh and Narendar for their continuous support and for being with me always.

And last but not least thanks to Appa, Amma, Ammu, Chinna, Chitti, Chittappa, Vivi, Somu and to my grandma for their unconditional love and endless support.

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