



- (51) **International Patent Classification:**
A61K 31/4015 (2006.01) *A61P 25/00* (2006.01)
- (21) **International Application Number:**
PCT/EP2014/068538
- (22) **International Filing Date:**
1 September 2014 (01.09.2014)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
1358352 30 August 2013 (30.08.2013) FR
- (71) **Applicants:** **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (C.N.R.S)** [FR/FR]; 3, rue Michel Ange, F-75016 Paris (FR). **UNIVERSITE D'AUVERGNE CLERMONT I** [FR/FR]; 49 Bd François Mitterrand, F-63000 Clermont Ferrand (FR). **CENTRE HOSPITALIER UNIVERSITAIRE DE CLERMONT-FD** [FR/FR]; 58 rue Montalembert BP 69, F-63003 Clermont Ferrand Cedex (FR).
- (72) **Inventors:** **ESCHALIER, Alain**; 18, rue des Vignettes, F-63400 Chamalieres (FR). **MALLET, Christophe**; 1, place de Regensburg, F-63000 Clermont Ferrand Cedex (FR). **DUBRAY, Claude**; 17, rue de Janolle, F-63122 Ceyrat (FR). **KERCKHOVE, Nicolas**; Appartement 93 Résidence Le Chaix 16B, Avenue Anatole France, F-63130 Royat (FR). **BOURINET, Emmanuel**; 6 allée Albert Dubout, F-34270 St Mathieu De Treviers (FR).

(74) **Agents:** **COLOMBET, Alain** et al.; Lavoix, 62, rue de Bonnel, F-69003 Lyon (FR).

(81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) **Title:** Cav3 CHANNEL BLOCKING AGENT FOR PAIN TREATMENT

(57) **Abstract:** The invention concerns the use of a Cav3, especially Cav3.2, channel blocking agent, ethosuximide in particular, in the treatment or prevention of pain triggered by excess nociception, especially of inflammatory origin. The invention also concerns the use of said agent in the treatment or prevention of inflammatory dysfunctional pain, or in the treatment or prevention of non-inflammatory dysfunctional pain, and the independent or simultaneous use of this agent for anti-inflammatory treatment.



Ca_v3 channel blocking agent for pain treatment

The invention concerns the use of a Ca_v3, especially Ca_v3.2, channel blocking agent, ethosuximide in particular, in the treatment of pain triggered by excess nociception (or nociceptive pain). The invention more particularly concerns the use of said agent in the treatment of inflammatory dysfunctional pain or of a pain of inflammatory type. The invention also concerns the use of this agent for anti-inflammatory treatment whether independently or simultaneously with pain treatment. The invention also concerns the corresponding methods of treatment.

The incidence of chronic pain is high; it has been estimated at 9% in the USA, 27% in Italy and 31.7% in France. Chronic pain is a significant factor for the deterioration of quality of life. It may be associated with co-morbidities such as depression anxiety, insomnia, etc. It is the source of major economic and social cost. Persons suffering from chronic pain are generally more exposed to absenteeism and presenteeism at work. The medical costs incurred for these patients are generally higher.

Faced with this finding the management of chronic pain is not always optimal due to an ageing pharmacopeia and characterised by a benefit/risk ratio that is often unsatisfactory. Numerous patients report that the pain suffered is not under control. The different treatments indicated for chronic pain pathologies, in particular pain of inflammatory type, bone and joint pain (arthrosis and arthritis) or visceral pain are of limited efficacy or have major adverse effects.

It is therefore important to have available a solution to treat this pain which provides an improved benefit/risk ratio. Patient tolerance to the products used is effectively an essential factor.

It is therefore of importance to provide novel solutions to treat pain or to have available alternative solutions to treat pain or to treat pain combined with anti-inflammatory action.

It is important to have available novel solutions allowing anti-inflammatory action. It is advantageous to have available analgesic treatments which exhibit improved efficacy and whose adverse effects are low or attenuated.

It is also advantageous to have available analgesic treatments allowing limited cost of treatment and with improved patient tolerance.

Also advantageously it is important to have available pain control agents which can be 100 % absorbed or else pain control agents having low protein binding and little or no enzyme inducing effect.

Pain control agents with long elimination half-life are particularly advantageous since they generally allow limiting of the number of daily doses, for example to only two daily doses.

Chronic pain, for example inflammatory pain, joint pain and visceral pain, therefore represents a major public health problem and calls for subsequent innovation. This is the objective of the present invention which allows a solution to be provided for all or part of the problems existing in the state of the art.

Several data suggest that targeting voltage-gated Ca^{2+} channels (VGCCs) appears to be a promising new therapeutic approach. Changes in cytosolic $[\text{Ca}^{2+}]$ are principally set by resting membrane potential and steady state Ca^{2+} influx through VGCCs (Knot HJ and Nelson MT, J Physiol 1998;508:199-209 ; Welsh DG et al., J Physiol 2000;527:139-148 ; Welsh DG et al., Circ Res 2002;90:248-250). Moreover, a range of VGCCs has been identified as being involved in transmitter release and prolonged excitatory states of the neuronal membrane (Catterall WA., Cold Spring Harb Perspect Biol 2011;3:a003947). Voltage-gated Ca^{2+} channels are divided into two groups depending on activation threshold, high-voltage (L-, P/Q, N- and R-type) and low-voltage (T-type) activated channels. Three genes (*Cacna1g*, *Cacna1h* and *Cacna1i*) encode T-type channels, giving rise to voltage-sensing, pore-forming $\alpha 1\text{G}$, $\alpha 1\text{H}$ and $\alpha 1\text{I}$ subunits, termed Cav3.1, Cav3.2, and Cav3.3, respectively (Welsh DG et al., Circ Res 2002;90:248-250). Among them, Cav3.2 T-type calcium channels seem particularly involved in pain. Bourinet *et al.* (Bourinet E. et al., EMBO J2005;24:315-324), demonstrated that only oligonucleotides downregulating mRNA encoding Cav3.2 protein, but not Cav3.1 and Cav3.3, present antinociceptive properties by decreasing mechanical hyperalgesia and allodynia in a rat model of mononeuropathy. Moreover, Cav3.2 knockout (Cav3.2 KO) mice were shown to exhibit decreased responses to acute noxious stimuli (Choi S. et al., Genes Brain Behav 2007;6:425-431; Francois A. et al., Pain 2013;154:283-293 ; Kerckhove N. et al., Pain 2014;155:764-772) hypersensitivity in animal models of neuropathic pain (Bourinet et al., EMBO J2005;24:315-324 ; Latham JR. et al., Diabetes 2009;58:2656-2665 ; Messinger RB. et al., Pain 2009;145:184-195). Accordingly, pharmacological blockade of T-type channels reduces mechanical and thermal hypersensitivity induced by peripheral nerve injury in rats (Dogrul A. et al., Pain 2003;105:159-168 and Hamidi GA. Et al., Eur, J Pharmacol 2012;674:260-264).

Despite all of this strong evidence, the role of Cav3.2 channels is still poorly studied in inflammatory-related pain. In this study, the involvement of Cav3.2 channels in inflammatory process and related pain was investigated by using genetic (Cav3.2 KO mouse) and pharmacological (ethosuximide, a T-type channel blocker, and TTA-A2, a novel

selective inhibitor preferentially affecting Cav3.2) tools in inflammatory-related pain models. Cav3.2 calcium channels were demonstrated herein to participate actively to edema development through the activation of macrophages and pro-inflammatory mediator production and to related pain through both neuron and macrophage-expressed channels.

- 5 The present work highlights T-type calcium channels as a potent target for inflammation and related pain to develop future treatments.

The invention provides a compound selected from among a Cav3, preferably Cav3.2, channel blocking agent for use in the treatment or prevention of pain triggered by excess nociception, in the treatment or prevention of inflammatory dysfunctional pain or in the
10 treatment or prevention of non-inflammatory dysfunctional pain. More particularly, the invention provides a compound selected from among a Cav3, preferably Ca_v3.2, channel blocking agent for use thereof in the treatment or prevention of pain of inflammatory type.

The invention also provides a method of treating or preventing pain triggered by excess nociception, inflammatory dysfunctional pain or non-inflammatory dysfunctional pain.
15 More particularly, the invention provides a method for treating or preventing pain of inflammatory type. The method comprises administering an efficient amount of a compound selected from among a Cav3, preferably Ca_v3.2, channel blocking agent, to a patient in need thereof.

The following description of additional and/or preferred features applies to both objects
20 presented supra, say compound for use and method of treatment (treating or preventing).

Advantageously, the Ca_v3, preferably Ca_v3.2, channel blocking agent of the invention is selected from among ethosuximide, levetiracetam, phenobarbital and phenytoin, preferably ethosuximide. The Ca_v3, preferably Ca_v3.2, channel blocking agent may be any other agent of whatever nature that is able to block the existing channel, such as TTA-A2 (2-(4-cyclopropylphenyl)-N-((1R)-1-{5-[(2,2,2-trifluoroethyl)oxo]-pyridin-2-yl}ethyl)acetamide). The
25 efficacy of an agent as a Ca_v3, preferably Ca_v3.2, channel blocking agent may be readily determined without undue experimentation by methodology well known in the art, including the "FLIPR Ca²⁺ Flux Assay" and the "T-type Calcium (Ca²⁺) Antagonist Voltage-Clamp Assay", described by Xia, et al., Assay and Drug Development Tech.,
30 1(5), 637-645 (2003)]. In a typical experiment ion channel function from HEK 293 cells expressing Cav3.2 is recorded to determine the activity of compounds in blocking the calcium current mediated by said channel. The agent may be under the form of a pharmaceutically acceptable salt.

Advantageously, the Ca_v3, preferably Cav3.2, channel blocking agent of the invention can be used in adults, in adolescents, in children and in the elderly.

Particularly advantageously, the Ca_v3, preferably Ca_v3.2, channel blocking agent of the invention, ethosuximide in particular, is used in children.

5 The Ca_v3, preferably Ca_v3.2, channel blocking agent of the invention, ethosuximide in particular, is generally used at a dose ranging from 500 mg/day to 2 g/day.

A gradual dose increase can allow an optimal effective dose to be reached to treat pain.

10 For example a daily dose taken twice a day, for example at mealtimes in the morning and evening, is possible.

Once pain has been brought under control a single daily dose is possible.

Usually the initial daily dose is 500 mg in children aged between 3 and 6 years and 1 g from 6 years upwards.

15 Depending on response, the dosage can be gradually increased from 500 mg after a period of 4 to 7 days up until full pain control. In children the effective dose may vary, in particular between 20 and 30 mg/kg/day; the maximum dosage is generally 1 g per day.

In adults, the effective dosage is generally around 20 mg/kg/day, corresponding to about 1.5 g per day. The maximum dosage is usually 2 g per day.

20 The Ca_v3, preferably Ca_v3.2, channel blocking agent of the invention may be used in the treatment or prevention of pain triggered by excess nociception and, in accordance with the present disclosure, in those pains originated from inflammation.

25 Advantageously, the Ca_v3, preferably Ca_v3.2, channel blocking agent of the invention may be used in the treatment or prevention of acute pain or chronic pain or in the treatment or prevention of skin-derived pain, muscular pain, joint pain, osteo-articular pain, visceral pain, tendon pain, post-operative pain, dental pain, cancer pain or post-trauma pain.

The Ca_v3, preferably Ca_v3.2, channel blocking agent of the invention, preferably ethosuximide, may be used to treat or prevent numerous diseases associated with pain and more particularly inflammatory pain.

30 It is also demonstrated herein that a Ca_v3, preferably Ca_v3.2, channel blocking agent, e.g. ethosuximide, may unexpectedly exert an anti-inflammatory effect and/or an anti-edematous effect. In accordance with this disclosure, this effect is advantageously combined with a favorable effect on related pain, say inflammatory pain.

35 Advantageously the Ca_v3, preferably Ca_v3.2, channel blocking agent of the invention, preferably ethosuximide, may be used in the treatment or prevention of arthritis or in the treatment or prevention of arthrosis, or in the treatment or prevention of an inflammatory

intestinal disease or in the treatment or prevention of irritable bowel syndrome, or the treatment or prevention of migraine or the treatment or prevention of cephalalgia or the treatment or prevention of myalgia or in the treatment or prevention of tendinitis or in the treatment or prevention of dorsal pain or in the treatment or prevention of cervical pain or in the treatment or prevention of lumbago. More particularly, the agent may be used in the treatment or prevention of inflammation and/or edema in these diseases.

The present invention is directed to the use of these agents for, and methods for, treating or preventing inflammation, inflammatory-related disorders, and pain. It comprises the step of administering ethosuximide or another Cav₃, preferably Ca_v3.2, channel blocker, or a pharmaceutically acceptable salt thereof to a subject in need thereof.

For treating or preventing pain and/or the associated disease or disorder, the agent or the pharmaceutical composition comprising the agent can be applied by any accepted mode of administration including topical, oral, and parenteral (such as intravenous, intramuscular, subcutaneous, or rectal).

"Pharmaceutically acceptable salts," as used herein, are salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects. Pharmaceutically acceptable salt forms include various crystalline polymorphs as well as the amorphous form of the different salts. The pharmaceutically acceptable salts can be formed with metal or organic counter-ions and include, but are not limited to, alkali metal salts such as sodium or potassium; alkaline earth metal salts such as magnesium or calcium; and ammonium or tetraalkyl ammonium salts, i.e., NX₄⁺ (wherein X is C1-4).

The agent, or its pharmaceutically acceptable salt in the pharmaceutical composition in general is in an amount of about 0.01-20%, or 0.05-20%, or 0.1-20%, or 0.2-15%, or 0.5-10%, or 1-5% (w/w) for a topical formulation; about 0.1-5% for an injectable formulation, 0.1-5% for a patch formulation, about 1-90% for a tablet formulation, and 1-100% for a capsule formulation.

In one embodiment, the agent is incorporated into any acceptable carrier, including creams, gels, lotions or other types of suspensions that can stabilize the active compound and deliver it to the affected area by topical applications. In another embodiment, the pharmaceutical composition can be in the dosage forms such as tablets, capsules, granules, fine granules, powders, syrups, suppositories, injectable solutions, patches, or the like. The above pharmaceutical composition can be prepared by conventional methods.

Pharmaceutically acceptable carriers, which are inactive ingredients, can be selected by those skilled in the art using conventional criteria. Pharmaceutically acceptable carriers include, but are not limited to, non-aqueous based solutions, suspensions, emulsions,

microemulsions, micellar solutions, gels, and ointments. The pharmaceutically acceptable carriers may also contain ingredients that include, but are not limited to, saline and aqueous electrolyte solutions; ionic and nonionic osmotic agents such as sodium chloride, potassium chloride, glycerol, and dextrose; pH adjusters and buffers such as salts of hydroxide, phosphate, citrate, acetate, borate; and triethylamine; antioxidants such as salts, acids and/or bases of bisulfite, sulfite, metabisulfite, thiosulfite, ascorbic acid, acetyl cysteine, cysteine, glutathione, butylated hydroxyanisole, butylated hydroxytoluene, tocopherols, and ascorbyl palmitate; surfactants such as lecithin, phospholipids, including but not limited to phosphatidylcholine, phosphatidylethanolamine and phosphatidyl inositol; poloxamers and ploxamines, polysorbates such as polysorbate 80, polysorbate 60, and polysorbate 20, polyethers such as polyethylene glycols and polypropylene glycols; polyvinyls such as polyvinyl alcohol and povidone; cellulose derivatives such as methylcellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, carboxymethyl cellulose and hydroxypropyl methylcellulose and their salts; petroleum derivatives such as mineral oil and white petrolatum; fats such as lanolin, peanut oil, palm oil, soybean oil; mono-, di-, and triglycerides; polymers of acrylic acid such as carboxypolymethylene gel, and hydrophobically modified cross-linked acrylate copolymer; polysaccharides such as dextrans and glycosaminoglycans such as sodium hyaluronate. Such pharmaceutically acceptable carriers may be preserved against bacterial contamination using well-known preservatives, these include, but are not limited to, benzalkonium chloride, ethylene diamine tetra-acetic acid and its salts, benzethonium chloride, chlorhexidine, chlorobutanol, methylparaben, thimerosal, and phenylethyl alcohol, or may be formulated as a non-preserved formulation for either single or multiple use.

For example, a tablet formulation or a capsule formulation may contain other excipients that have no bioactivity and no reaction with the agent. Excipients of a tablet may include fillers, binders, lubricants and glidants, disintegrators, wetting agents, and release rate modifiers. Binders promote the adhesion of particles of the formulation and are important for a tablet formulation. Examples of binders include, but not limited to, carboxymethylcellulose, cellulose, ethylcellulose, hydroxypropylmethylcellulose, methylcellulose, karaya gum, starch, starch, and tragacanth gum, poly(acrylic acid), and polyvinylpyrrolidone.

For example, a patch formulation may comprise some inactive ingredients such as 1,3-butylene glycol, dihydroxyaluminum aminoacetate, disodium edetate, D-sorbitol, gelatin, kaolin, methylparaben, polysorbate 80, povidone, propylene glycol, propylparaben, sodium carboxymethylcellulose, sodium polyacrylate, tartaric acid, titanium dioxide, and purified

water. A patch formulation may also contain skin permeability enhancer such as lactate esters (e.g., lauryl lactate) or diethylene glycol monoethylether.

Topical formulations can be in a form of gel, cream, lotion, liquid, emulsion, ointment, spray, solution, and suspension. The inactive ingredients in the topical formulations for example include, but not limited to, lauryl lactate (emollient/permeation enhancer), diethylene glycol monoethylether (emollient/permeation enhancer), DMSO (solubility enhancer), silicone elastomer (rheology/texture modifier), caprylic/capric triglyceride, (emollient), octisalate, (emollient/UV filter), silicone fluid (emollient/diluent), squalene (emollient), sunflower oil (emollient), and silicone dioxide (thickening agent). In one embodiment, lauryl lactate (for example, at about 0.1-10%, or about 0.2-5%, or about 0.5-5%) is included in the topical gel formulation. In another embodiment, diethylene glycol monoethylether is included in the topical gel formulation.

The present invention is particularly directed to a method of treating or preventing inflammation and/or pain of inflammation type. The active compound ethosuximide or another Cav₃, preferably Ca_v3.2, channel blocker can be used as is, or it can be administered in the form of a pharmaceutical composition that additionally contains a pharmaceutically acceptable carrier. The method may comprise the steps of first identifying a subject suffering (or in risk of suffering) from inflammation and/or pain of inflammation type, and administering to the subject said active compound, in an amount effective to treat (or prevent) inflammation and/or pain of inflammation type.

"An effective amount," as used herein, is the amount effective to reduce or abolish (or prevent) pain and/or to treat (prevent) a disease by ameliorating the pathological condition or reducing the symptoms of the disease.

In one embodiment, the method reduces or alleviates the symptoms associated with inflammation. The present invention provides a method to treat or prevent localized manifestations of inflammation characterized by acute or chronic swelling, pain, redness, increased temperature, or loss of function in some cases.

In another embodiment, the present invention provides a method to alleviate the symptoms of pain regardless of the cause of the pain. The general term "pain" treatable by the present method includes pain triggered by excess nociception, in the treatment of inflammatory dysfunctional pain or in the treatment of non-inflammatory dysfunctional pain. It includes traumatic pain, organ pain, and pain associated with diseases. Traumatic pain includes pain resulting from injury, post-surgical pain and inflammatory pain. Organ pain includes ocular, corneal, bone, heart, skin/burn, visceral (kidney, gall bladder, etc.), joint, and muscle pain, including possible inflammatory pain related thereto. Pain associated with

diseases includes pain associated with cancer, AIDS, arthritis, herpes and migraine, including possible inflammatory pain related thereto. Pain, especially inflammatory pain, associated with mental disorders, such as depression or nervous breakdown, are also concerned. The present invention prevents or reduces pain of varying severity, i.e. mild, moderate and severe pain; acute and chronic pain. The present invention is effective in preventing or treating joint pain, muscle pain, tendon pain, and burn pain. In an embodiment, the invention concerns the prevention or the treatment of the inflammation manifestation in these various traumatic pain, organ pain, and pain associated with diseases.

In embodiments, the present invention is useful in treating or preventing inflammation and/or pain associated in a musculoskeletal system or on the skin. The highly innervated, musculoskeletal and skin systems have a high capacity for demonstration of pain. In addition, the musculoskeletal system has a high capacity for tissue swelling, and the skin has a high capacity for redness, swelling, and heat. In musculoskeletal and skin systems.

The present invention provides a method for treating or preventing inflammation and/or pain associated with inflammatory skeletal or muscular diseases or conditions. The method may comprise the steps of identifying a subject in need thereof, and administering to the subject the active compound, in an amount effective to treat or prevent inflammation and/or pain. The skeletal or muscular diseases or conditions include musculoskeletal sprains, musculoskeletal strains, tendonopathy, peripheral radiculopathy, rheumatoid arthritis, polymyalgia rheumatica, juvenile arthritis, gout, ankylosing spondylitis, psoriatic arthritis, systemic lupus erythematosus, costochondritis, tendonitis, bursitis, such as the common lateral epicondylitis (tennis elbow), medial epicondylitis (pitchers elbow) and trochanteric bursitis, temporomandibular joint syndrome, and fibromyalgia.

The present invention provides a method for treating or preventing inflammation and/or pain associated with inflammatory skin diseases such as dermatitis and psoriasis. The method may comprise the steps of identifying a subject in need thereof, and administering to the subject the active compound, in an amount effective to treat inflammation and/or pain.

The pharmaceutical composition of the present invention can be applied by local administration and systemic administration. Local administration includes topical administration. Systemic administration includes oral, parenteral (such as intravenous, intramuscular, subcutaneous or rectal), and other systemic routes of administration. In systemic administration, the active compound first reaches plasma and then distributes into target tissues. Topical administration and oral administration are preferred routes of administration for the present invention.

Dosing of the composition can vary based on the extent of the injury and each patient's individual response.

The Ca_v3, preferably Ca_v3.2, channel blocking agent of the invention can be used over quite variable periods in particular in relation to the type pain being treated and the type of patient.

Advantageously, the Ca_v3, preferably Ca_v3.2, channel blocking agent of the invention can be used over the short term for a time of one of two days. It can also be used over the medium term for a time of 3 days to 3 months. It can also be used over the long term for a time of more than 3 months.

The Ca_v3, preferably Cav3.2, channel blocking agent of the invention can also be used permanently.

The invention also concerns a compound selected from among Ca_v3, preferably Ca_v3.2, channel blocking agents for use in association with another pain treatment agent to treat pain triggered excess nociception, especially of inflammation type

Preferably the Ca_v3, preferably Ca_v3.2, channel blocking agent associated with another pain treating agent for pain triggered by excess nociception is selected from among ethosuximide, levetiracetam, phenobarbital and phenytoin, in particular ethosuximide.

The associated agent can notably be an opioid pain control agent e.g. an agent selected from among codeine, tramadol, morphine, oxycodone, or a non-opioid pain control agent in particular a nonsteroidal anti-inflammatory agent, a pain control agent selected from among paracetamol and nefopam, a local anaesthetising agent.

The invention also provides a compound selected from among Ca_v3, preferably Ca_v3.2, channel blocking agents for independent or simultaneous use thereof in the treatment or prevention of an inflammatory reaction, in particular a post-operative inflammatory reaction.

Preferably, the invention also concerns the independent use of ethosuximide in the treatment or prevention of an inflammatory reaction, in particular a post-operative inflammatory reaction.

Also preferably the invention concerns the simultaneous use of ethosuximide in the treatment or prevention of an inflammatory reaction in particular a post-operative inflammatory reaction, and in the treatment or prevention of pain triggered by excess nociception, in the treatment or prevention of inflammatory dysfunctional pain or in the treatment or prevention of non-inflammatory dysfunctional pain.

The invention also concerns a composition used for one or other treatments of the invention.

The composition of the invention comprises a compound selected from among a Ca_v3 , preferably $\text{Ca}_v3.2$, channel blocking agent for use thereof in the treatment or prevention of pain triggered by excess nociception, in the treatment or prevention of inflammatory dysfunctional pain or in the treatment or prevention of non-inflammatory dysfunctional pain, and at least one pharmaceutical excipient.

Advantageously the composition of the invention comprises a compound selected from among a Ca_v3 , preferably $\text{Ca}_v3.2$, channel blocking agent for use thereof in the treatment or prevention of pain triggered by excess nociception.

Also advantageously the Ca_v3 , preferably $\text{Ca}_v3.2$, channel blocking agent of the invention is selected from among ethosuximide, levetiracetam, phenobarbital and phenytoin, preferably ethosuximide.

The composition of the invention may also comprise another agent for treating or preventing pain triggered by excess nociception.

In the composition of the invention, the associated agent may in particular be an opioid pain control agent e.g. an agent selected from among codeine, tramadol, morphine, oxycodone, or a non-opioid pain control agent in particular a non-steroidal anti-inflammatory agent, a pain control agent selected from among paracetamol and nefopam, a local anaesthetising agent.

The present invention is useful in treating a mammal subject, such as humans, sports animals such as horses, and pets such as dogs. The present invention is particularly useful in treating humans.

The different aspects and advantageous properties of the invention can be illustrated by the following examples and the related figures. These examples do not limit the scope of this invention.

Figure 1 : The $\text{Cav}3.2$ calcium channels are involved in symptoms of tactile allodynia and hyperalgesia, pro-inflammatory mediator production and edema development associated with inflammation. (A) Carrageenan-induced mechanical allodynia, (B) mechanical hyperalgesia and (C) edema. The areas under the curve of A, B and C are represented in (D), (E) and (F), respectively. (G, H, I and J) IL-6 , $\text{TNF-}\alpha$, $\text{IL-1}\beta$ and PGE_2 in edema, (K) IL-6 in serum. Tests were performed on $\text{Cav}3.2$ knockout mice ($\text{Cav}3.2^{-/-}$) and their wild type littermates ($\text{Cav}3.2^{+/+}$). Serum and edema were sampled 4 hours after carrageenan injection. Data are shown as mean ($n = 8$) $\pm$ SEM. (A and B) $*P < 0.05$, compared to respective vehicle groups, (C) $^{\$}P < 0.05$ and (G-K) $*P < 0.05$, carrageenan $\text{Cav}3.2^{-/-}$ group compared to carrageenan $\text{Cav}3.2^{+/+}$ group.

Figure 2: Cav3.2 channels specifically expressed by hematopoietic cells are involved in the development of edema, pro-inflammatory mediator release and related pain. The assessment of edema development, pro-inflammatory mediator production and hypersensitivity was performed before (baseline, white histogram, for pain test) and 4 hours after carrageenan injection (black histogram, for pain test). (A) Edema development, (B) Il-6 in serum, (C, D and E) Il-6, Il-1 β and PGE₂ in edema, (F) carrageenan-induced mechanical allodynia, (G) mechanical hyperalgesia. Tests were performed on chimeric mice, 8 weeks after their irradiation and bone marrow transplantation. Behavioral test, serum and edema sample were performed from chimeric mice 4 hours after carrageenan injection. Donor: mouse bone marrow donor, Recipient: transplanted mouse; KO: Cav3.2 knock-out mice (Cav3.2^{-/-}) and WT: littermates mice (Cav3.2^{+/+}). Data are shown as mean (n = 6-8) $\pm$ SEM. *P < 0.05 (compared to baseline for pain test); ^{\$}P < 0.05.

Figure 3: Cav3.2 channels are involved in pro-inflammatory mediator production by bone marrow derived macrophages (BMDM) and their activation in response to LPS. (A-C) Il-6, Il-1 β and TNF- α production, (D) representative percentage of BMDM responding to LPS, (E) representative recording of LPS-induced calcium signals in single Cav3.2^{+/+} and Cav3.2^{-/-} BMDM, (F) immunohistochemistry of BMDM (actin [phalloidin toxin] = typically appear in red and nucleus [DAPI] = typically appear in blue), scale bar: 20 μ m, (G) representative BMDM cell area. All tests were performed 24 hours after LPS (20 ng/ml) injection (or during 5 minutes for calcium imaging experiment). (D and F) The number of analyzed cells is shown at the bottom of the histograms. Data are shown as mean (n = 8 mice for mediators, n=3 mice for morphology and n = 4 mice for calcium imaging) $\pm$ SEM. *P < 0.05.

Figure 4: Pharmacological inhibition of T-type calcium channels produces anti-allodynia, anti-hyperalgesia and anti-inflammatory effect in sub-chronic inflammatory pain. (A, D and G) Carrageenan-induced mechanical allodynia, (B, E and H) mechanical hyperalgesia and (C, F and I) edema. Mechanical allodynia and hyperalgesia were assessed 4 hours after carrageenan injection and 30 minutes after drugs administration (TTA-A2 = 1 mg/kg per os or 0.1 mg/kg subcutaneous intraplantar; ethosuximide [ETX] = 200 mg/kg intraperitoneal; vehicle). Edema development was assessed 2 hours after drugs administration. Data are shown as mean (n = 8) $\pm$ SEM. *P < 0.05.

Figure 5: Preventive pharmacological inhibition of T-type calcium channels produces anti-allodynia, anti-hyperalgesia and anti-inflammatory effect in chronic inflammatory pain. (A) Animals receive repeated administration (3 times daily from D0 to D6 and once on D7 after CFA injection) of the Cav3.2 inhibitor (Ethosuximide [ETX], 200 mg/kg, intraperitoneal) or vehicle. Edema development was assessed before (D0) and 1, 2,

3, 4, 5, 6 and 7 days after peri-articular injection of CFA. Assessment of allodynia and hyperalgesia was performed on the last day (D7) 30 minutes after the last ethosuximide treatment, (B) time course of edema development, (C) mechanical allodynia and (D) mechanical hyperalgesia. Data are shown as mean ($n = 8$) $\pm$ SEM. * $P < 0.05$, compared to vehicle groups.

Figure 6: Curative pharmacological inhibition of T-type calcium channels produces anti- allodynia, anti-hyperalgesia and anti-inflammatory effect in chronic inflammatory pain. (A) Animals receive repeated administration (3 times daily, from D7 to D13 and once on day 14 after CFA injection) of the Cav3.2 inhibitor (Ethosuximide [ETX], 200 mg/kg, intraperitoneal) or vehicle. Edema development was performed before (D0) and 7, 8, 9, 10, 11, 12, 13 and 14 days after peri-articular injection of CFA. Assessment of allodynia and hyperalgesia was performed on the last day (D14) 30 minutes after the last ethosuximide treatment, (B) time course of edema development, (C) mechanical allodynia and (D) mechanical hyperalgesia. Data are shown as mean ($n = 8$) $\pm$ SEM. * $P < 0.05$, compared to vehicle groups.

Figure 7: Cav3.2 calcium channel are expressed by bone marrow-derived macrophages. RT-q-PCR in bone marrow-derived macrophages (BMDM) of Cav3.2^{-/-} and Cav3.2^{+/+} mice. Data are shown as mean ($n=3$) $\pm$ SEM. * $P < 0.05$.

Figure 8: Representation of chimeric mice generation. WT and Cav3.2 KO mice (recipients) are irradiated with a total dose of 10 Gy. In these irradiated mice, 10^7 total bone marrow (BM) cells retrieved from un-irradiated WT or Cav3.2 KO mouse (donors) are transplanted by i.v injection immediately after irradiation. This procedure led us to obtain four experimental groups of irradiated mice including chimeric mice [WT mice transplanted with BM cells from Cav3.2 KO (Recipient WT/Donor KO); Cav3.2 KO mice transferred with BM cells from WT donors (Recipient KO/Donor WT)] and control mice (not represented) [WT mice transferred with BM cells from WT donors (Recipient WT/Donor WT); Cav3.2 KO mice transferred with BM cells from KO donors (Recipient KO/Donor KO)].

Figure 9: Cav3.2 knockout mice develop less mechanical hypersensitivity and edema in chronic inflammatory pain. (A) CFA-induced mechanical allodynia, (B) mechanical hyperalgesia and (C) edema. Tests were performed at 7 day after peri-articular injection of CFA on Cav3.2 knockout mice (Cav3.2^{-/-}) and their wild type littermates (Cav3.2^{+/+}). Data are shown as mean ($n = 8$) $\pm$ SEM. * $P < 0.05$.

Example 1

Protocols:

Knock-out mice $Ca_v3.2$ ($Ca_v3.2^{-/-}$) and their wild littermates ($Ca_v3.2^{+/+}$), initially generated by Chen (Chen *et al.*, Abnormal coronary function in mice deficient in α_{1H} T-type Ca^{2+} channels, Science, vol. 302, 2003), were bred at Université d'Auvergne (Clermont-Ferrand, France) under controlled environmental conditions (21-22 °C, 55 % humidity) and kept under a 12h/12h light/dark cycle. Food and water were available *ad libitum*. For all the experiments male mice weighing 20 to 25g were used and euthanized with CO_2 .

The following molecules were used: ethosuximide (Sigma-Aldrich, St Louis, USA) and carrageenan (Sigma-Aldrich, St Louis, USA). Solutions were prepared extemporaneously in NaCl (0.9 %). The doses used were: ethosuximide (100, 200 and 300 mg/kg, i.p.) and carrageenan (3 % solution, 20 μ l per intraplantar application).

The nociception assays conducted included tests using chemical, thermal and mechanical stimuli.

Heat sensitivity was measured using the paw immersion test in water at the nociceptive temperature of 46°C. The animal was held manually and the paw immersed in a hot water bath until withdrawal of the paw by the animal or until a cut-off limit set at 30 s. Pain threshold was defined as the mean of the first two latency periods not differing by more than one second. The animals were accustomed to being held one week before the start of the experiment.

Mechanical sensitivity was measured using pressured applied by a von Frey filament (Bioseb, France) of 1.4 g force under the animal paws. The animals were placed in boxes (85 x 35 mm having an opaque separation between the mice, a wire mesh floor to access the arch of the paw) 60 minutes before the test, for mouse habituation. The filaments were pressed perpendicularly against the underside of the right hind paw of the mice until filament bending. This operation was repeated five times for a time of 3 seconds. When the pressure applied corresponded to the threshold of the animal's touch sensitivity, it reacted by withdrawing or licking the paw, the equivalent of reflex flicking. The pain response score (0 to 5) was then retained as the value of mechanical sensitivity.

Chemical sensitivity was measured using the formalin test. The mice were accustomed to the Plexiglas chamber L (30 cm x 30 cm x 30 cm) for at least 30 minutes before the test. 20 μ l of formalin (2.5 % formalin in saline solution) were injected via intraplantar route into the hind paw. The behaviour of spontaneous pain (licking) was recorded over two typical nociceptive phases: 0 to 5 min (phase 1) and 15 to 40 min (phase 2) after formalin injection. Sub-acute inflammation of the paw was induced by intraplantar injection of 20 μ l physiological saline solution containing 3 % λ -carrageenan (Sigma-Aldrich, France). The baseline pain thresholds were calculated before injection of carrageenan.

Behavioural assays were conducted 4 h after inducing inflammation. For monoarthritis 5 µl of Complete Freund's Adjuvant (CFA, Difco Laboratories, Detroit, United States) were injected on both sides of the left ankle joint of the mice under short anaesthesia (halothane/N₂O/O₂).

The paw withdrawal thresholds subsequent to thermal and mechanical stimulation were determined before the start of the test then 7 days after injection of CFA or vehicle composition.

The development of paw oedema induced by the intraplantar injection of carrageenan and formalin was measured using a calliper 4 hours and 40 minutes respectively after the inducing of inflammation.

The results were analysed statistically. Data are expressed as a mean ± SEM; they were analysed using SigmaStats 3.5 software. Data were tested for variance normality and equality. Measurements were compared using the two way ANOVA test or Kruskal-Wallis test for data not having normal distribution. *Post hoc* comparisons were carried out using the Bonferroni method. Values of *p* < 0.05 were considered to have statistical significance.

The analgesic efficacy of ethosuximide on inflammatory pain was evaluated in 3 models of inflammatory pain in mice.

Ethosuximide displayed an analgesic effect in the 3 models: formalin model (Table 1), carrageenan model (Table 2) and CFA model (Table 3).

The anti-inflammatory properties of ethosuximide were evaluated in mice. Systemic administering induced a reduction in the volume of the oedema induced by inflammation found in the formalin model (Table 1) and carrageenan model (Table 2).

Ethosuximide dose (mg/kg)	Duration of pain responses (s)		Size of oedema (% increase)
	Phase 1	Phase 2	
0 (vehicle)	93.3	242.5	67.2
100	69.5	219.3	61.4
200	29.6 ^{**}	137.4 ^{**}	55.5 [*]
300	0.3 ^{***}	36.7 ^{***}	45 ^{**}

Table 1: Ethosuximide induces an analgesic effect on acute and inflammatory pain and an anti-oedematous effect (formalin model)

A dose effect of ethosuximide (Etx, 100, 200 and 300 mg/kg, i.p.) was used to evaluate the minimum effective dose to induce an analgesic and anti-oedematous effect in the formalin model. The test was performed 20 min after administering ethosuximide. a) Formalin, phase 1 (0 - 5 min; acute pain); b) Formalin, phase 2 (15 - 40 min; inflammatory pain); c) Measurement of oedematous development. The data are given as a mean ± SEM (n = 6-8); *: *p* < 0.05; **: *p* < 0.01; ***: *p* < 0.001 in comparison with the vehicle group (Table 1).

Ethosuximide dose (mg/kg)	Number of pain responses		Size of oedema (% increase)
	Allodynia	Hyperalgesia	
0 (vehicle)	3.38 / 5	4.88 / 5	69.28
200	0.38 / 5 ^{***}	2.88 / 5 ^{**}	42.95 ^{**}

Table 2: Effect of ethosuximide on mechanical inflammatory pain and oedematous development (carrageenan model)

The ethosuximide dose (Etx, 200 mg/kg, i.p.) was used to evaluate its anti-allodynia and anti-hyperalgesia effect using the von Frey test and anti-oedematous effect in the carrageenan model. The assays were performed 4h after inducing inflammation. a) Allodynia (pressure of 0.04 g); b) Hyperalgesia (pressure of 1.4 g); c) Measurement of oedematous development. The data are given as a mean $\pm$ SEM (n = 6-8); **: p < 0.01; ***: p < 0.001 in comparison with the vehicle group (Table 2).

Ethosuximide dose (mg/kg)	Number of pain responses	
	Allodynia	Hyperalgesia
0 (vehicle)	1.17 / 5	3.83 / 5
200	0.33 / 5 [*]	1.83 / 5 [*]

Table 3: Effect of ethosuximide on mechanical inflammatory pain (CFA model)

The ethosuximide dose (Etx, 200 mg/kg, i.p.) was used to evaluate its anti-allodynia and anti-hyperalgesia effect using the von Frey test, and anti-oedematous effect in the monoarthritis model (CFA). The assays were performed 7 days after inducing inflammation. a) Allodynia (pressure of 0.04 g); b) Hyperalgesia (pressure of 1.4 g). Data are expressed as a mean $\pm$ SEM (n = 6-8); *: p < 0.05 in comparison with the vehicle group (Table 3).

Example 2

Experiments were carried out using male C57BL6/J mice (20-24 g). Cav3.2 knock-out mice (Cav3.2^{-/-}) and their wild-type littermates (Cav3.2^{+/+}) were bred and maintained at the University of Auvergne (Clermont-Ferrand, France). Cav3.2^{-/-} mice were originally generated by Chen CC et al. supra. Mice were housed under controlled environmental conditions (21-22°C; 55% humidity) and kept under a 12/12 hours light/dark cycle. Food and water were available *ad libitum*. For all experiments, mice were euthanized by cervical dislocation or CO₂.

λ -carrageenan and ethosuximide were obtained from Sigma-Aldrich (France). TTA-A2 as provided by Merck Laboratories (Pennsylvania, USA). Complete Freund's adjuvant (CFA) was from Difco Laboratories (Detroit, USA). TTA- A2 was administered by subcutaneous intraplantar (20 μ l, 0.1 mg/kg) or oral (1 mg/kg) route and ethosuximide was administered by intraperitoneal (200 mg/kg) route to a volume of 10 ml/kg.

Carrageenan and monoarthritic model. On the day of the experiment, subacute paw

inflammation was induced by an intraplantar (left hindpaw) subcutaneous injection of physiologic serum - 3% λ -carrageenan (20 μ l). Comparisons of the reaction threshold and edema development were performed before (baseline) and 2, 4, 6, 8 and 24 hours or 4 hours after inflammation induction for Cav3.2^{-/-} mice and TTA-A2 and ethosuximide experiments, respectively, to ensure mice exhibited related-pain behavior (Morris CJ., Methods Mol Biol Clifton NJ 2003;225:115-121).

For monoarthritic model, 5 μ l of CFA were injected at the two sides of the left ankle joint under brief anesthesia (halothane/N₂O/O₂). Comparisons of the reaction threshold and edema development were performed before (baseline) and 7 or 14 days after the inflammation induction to ensure mice exhibited related-pain behavior (Lolignier S. et al., PLoS One 2011;PloS one:e23083).

Edema measurement. Paw edema induced by intraplantar carrageenan and peri-articular CFA injection was measured at 4 hours or every day (from D0 to D7 or D7 to D14), respectively, after inflammation induction, using a caliper. Assessment of edema was performed before (baseline) and 2 hours after drugs administration.

Assessment of mechanical allodynia and hyperalgesia. Mice were acclimated to the testing environment before baseline testing. On the behavior testing day, mice were placed in individual compartments on top of a wire surface and allowed to acclimatize for one hour before testing. Mechanical allodynia and hyperalgesia were assessed with the 0.04 and 1.4 grams calibrated von Frey filaments (Bioseb, France). The latter was pressed perpendicularly five times against the mid paw and held for 3 seconds. A positive response was noted if the paw was withdrawn and a pain score (from 0 to 5) response was determined. Assessment of mechanical allodynia and hyperalgesia were performed before (baseline) and 30 minutes after drugs administration.

Bone-marrow derived macrophage and edematous tissue culture. Bone-marrow derived macrophage (BMDM) cells were prepared and cultured as previously described (Carvalho FA. et al., Mucosal Immunol 2011;4:102-111). Briefly, mouse femurs were washed out and flushed with DMEM and bone-marrow cells were plated at an initial density of 10⁶ cells/ml. Then, cells were cultured and differentiated into BMDM using DMEM supplemented with 10% FBS, 1 % glutamine, 1 % antibiotic (neomycin) and 50% L929 supernatants containing macrophage-stimulating factor (M-CSF). For *in vitro* experiments, BMDMs were cultured for at least 5 days before LPS stimulation by in serum-free BMDM culture medium.

To perform edematous tissue cultures, edema (30 mg tissue / mouse) was withdrawn 4 hours after carrageenan injection and cultured in DMEM supplemented with 10% heat-inactivated FBS, 1 % glutamine and 1 % antibiotic. After 24 hours, the medium was harvested and supernatant

used to quantify pro-inflammatory factors by ELISA.

ELISA assay. Cytokine production (IL-1 β , IL-6, TNF- α) and PGE₂ were assessed by ELISA assays 4 hours after carrageenan hindpaw injection in serum and 24 hours after edema culture. All ELISA kits were Duoset kits (R&D Systems, Minneapolis, MN, USA) and assays performed according to the manufacturer protocol. The minimum detectable doses were 15.6 pg/ml for IL-1 β , 15.6 pg/ml for IL-6, 31 pg/ml for TNF- α , and 15.6 pg/ml for PGE₂.

Immunocytochemistry. Mouse macrophages Cav3.2^{-/-} and Cav3.2^{+/+} were activated by contact with LPS (20 ng/ml) for 24 hours. Alexa Fluor[®] 546 Phalloidin antibody from Invitrogen was used at the indicated dilution of 5/200. BMDM plated on cover slips were fixed for 5 minutes with 4% paraformaldehyde in PBS. After three washes in PBS, cells were incubated 1 hour with PBS-BSA 3% solution for saturation. Cells were permeabilized in PBS containing 0.3% Triton X-100 for 15 minutes at room temperature and, after washes, were incubated 20 minutes at room temperature with Phalloidin toxin in PBS-BSA 3% solution. After three washes and DAPI staining, BMDM plated cover slips were mounted and observed. Image acquisitions were performed with Axio Scope A.1 (Zeiss).

Calcium imaging. Mouse macrophages Cav3.2^{-/-} and Cav3.2^{+/+} were activated by contact with LPS (20 ng/ml) during 5 minutes. The involvement of Cav3.2 channels on intracellular Ca²⁺ concentration was evaluated by recording the changes in cytoplasmic Ca²⁺ concentration using the radiometric fluorescent probe Fura-2 in BMDMs purified from Cav3.2^{-/-} and Cav3.2^{+/+} mice. Cells were loaded with 2 μ M of Fura-2 acetoxymethyl ester (Fura-2/AM, Invitrogen), 0.5% BSA in the recording saline solution (135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM Glucose, 10 mM Hepes, pH7.4). After one hour of incubation at room temperature, cells were washed three times and then stimulated with indicated concentration of LPS. The Metafluor Imaging system (Molecular Devices) was used for fluorescence acquisition and analysis of individual cells. Fluorescence was excited by illumination through x20 water immersion objective with a light wavelength switch provided by a DG4 filter wheel and detected with a CCD camera under the Metafluor software control. Pairs of images were acquired every 2 seconds.

Generation of chimeric mice. The objective was to determine the role of Cav3.2 channels expressed by hematopoietic cells in inflammation and related pain. For that, bone marrow (BM) chimeric mice generation was performed by: 1) irradiating WT and Cav3.2 KO mice (recipient) with a total dose of 10 Gy (1 Gy/min) by the X-RAD 320 irradiator (320kV, 45mAmp, Precision X-RAY Inc. Brandford-CT) performed at the PAVIRMA platform (University Blaise Pascal, Clermont-Ferrand, France); 2) transferring in these irradiated mice, by i.v injection, 100 μ l Dulbecco's Modified Eagle Medium (DMEM) with 10⁷ total BM cells retrieved

from un-irradiated WT or Cav3.2 KO mouse (donor) immediately after irradiation (Figure 8). This procedure led to four experimental groups of irradiated mice including controls [WT mice transferred with BM cells from WT donors (Recipient WT/Donor WT); Cav3.2 KO mice transferred with BM cells from KO donors (Recipient KO/Donor KO)]; chimeric animals [WT mice transferred with BM cells from Cav3.2 KO (Recipient WT/Donor KO); and Cav3.2 KO mice transferred with BM cells from WT donors (Recipient KO/Donor WT)]. During 2 weeks after the irradiation, mice received drinking water complemented with 2 mg/ml of neomycin. At 8 weeks after the BM transplantation, chimeric mice were used for experiments.

RT-PCR and quantitative RT-PCR. RNA was isolated using RNeasy mini kit (Qiagen) according to the manufacturer instructions, including a 15 min DNase incubation step. Purified RNA was quantified by measuring the 260 nm absorbance (A₂₆₀) with the Epoch® spectrophotometer (Biotek) and quality was assessed by analyzing the A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ ratios. Integrity of RNA samples was confirmed by electrophoresis on a 1.5% agarose gel. Before quantitative PCR analysis, 1 mg of total RNA was submitted to reverse transcription with MultiScribe™ Reverse Transcriptase (Applied Biosystems) in a 20 µl volume, using the supplier's procedure. PCR amplifications were performed using a Mastercycler ep realplex (Eppendorf). All samples were run in triplicate in a final volume of 6.3 µl containing 3 µl of 1/40 diluted cDNA, 0.5 µM primers, 3 mM MgCl₂ and LightCycler Fast-Start SYBR Green reaction mix (Roche), according to manufacturer protocol. Prior to PCR, a 5 minute enzyme activation step was done at 95°C. The PCR protocol consisted of 10 s denaturation at 95°C, 10 s at annealing temperature (62°C) and 10 s elongation at 72°C for 60 cycles. Optimal annealing temperature was initially determined by gradient PCR. The primers sequences used were the following: SEQ ID NO:1 ACACAACGTGAGCCTCTCTG (forward) and SEQ ID NO:2 AGCAGTGTGACCAGGATTCG (reverse) for Cav3.2 (NM_021415.4, 8,240 bp) and SEQ ID NO:3 TCCAGGCTTTGGGCATCA (forward) and SEQ ID NO:4 CTTTATCAGCTGCACATCACTCAGA (reverse) for 36B4 (NM_007475.5, 1,360 bp). Amplification specificity was assessed by melting curve analysis and PCR products were run on a 1.5% agarose gel to confirm amplicons sizes. Primers were designed on intron-flanking sequences to prevent genomic contamination. Cav3.2 and 36B4 cDNA relative amounts were calculated function of the samples cycle threshold (Ct) using a standard concentration curve constructed with a serial dilution from 1/10 to 1/640 of a total cDNA mix. For each sample, the relative amount of Cav3.2 cDNA was normalized by the 36B4 cDNA amount.

Statistics. Data are expressed as mean ± SEM and recorded using

SigmaStats 3.5 software. Data was tested for normality and for equal variance. Simple measurements were compared by Student test (independent sample) or by Mann-Whitney test in case of data that were not normally or equal variance distributed. Multiple measurements were compared by one-way or two-way ANOVA or by Kruskal-Wallis or Friedman tests in case of data that were not normally or equal variance distributed. The post hoc comparisons were performed by the Bonferroni method. Values of $P < 0.05$ were considered statistically significant.

Results

Cav3.2 calcium channels contribute to pain, edema development and production of pro-inflammatory mediators. Symptoms of mechanical allodynia and hyperalgesia associated with carrageenan-induced inflammation were assessed by using the von Frey test. *Cav3.2* KO mice (*Cav3.2*^{-/-}), unlike their wild type (WT) littermates (*Cav3.2*^{+/+}), did not develop allodynia (Figure 1 A and 1 D) or hyperalgesia (Figure 1B and 1E). To evaluate the inflammatory state, edema development and the production of pro-inflammatory mediators were determined. An edema was elicited in *Cav3.2*^{-/-} mice but its size was significantly decreased by $33.1 \pm 2.0\%$ regarding area under the time-course curves of edema thickness compared to *Cav3.2*^{+/+} mice (Figure 1C and 1F). In addition to reduced edema development and painful sensitivity, *Cav3.2*^{-/-} mice also exhibited a strongly decreased production of pro-inflammatory mediators (IL-6, IL-1 β , TNF- α and PGE₂) in the inflamed paw and a decrease in IL-6 serum levels (Figure 1 G-K).

Edema development is dependent on expression of Cav3.2 channels in macrophages. Edema development and pro-inflammatory mediators produced during inflammation are mainly dependent on macrophages recruitment and their activation on the inflammatory site. Before investigating the role of *Cav3.2* channels in macrophages, we showed, using quantitative RT-PCR, a *cacna1h* (the gene encoding *Cav3.2* channel) mRNA production on cell culture of bone marrow derived macrophages (BMDM) and so, for the first time, the possible expression of voltage-dependent calcium channel on macrophages (Figure 7).

To demonstrate the specific involvement of immune cells expressing *Cav3.2* in edema development, chimeric mice were generated. These mice were either disabled for the gene encoding the *Cav3.2* channel only in the immune system (they were Recipient WT mice which received hematopoietic cells from Donor *Cav3.2* KO mice) or invalidated in the entire body except for the immune system (Recipient *Cav3.2* KO mice and Donor WT mice) (Figure 8). The inflammatory status of these chimeric mice in inflammatory conditions was assessed 4 hours after intraplantar injection of carrageenan. The absence of *Cav3.2* channels specifically in the immune system induced a less edema development, similarly to

the situation observed with the global Cav3.2 KO mice ($43.4 \pm 4.7\%$ and $48.6 \pm 3.0\%$, respectively). Adversely, edema was restored in the case of Cav3.2 KO mice receiving WT hematopoietic cells (Figure 2A). Consistently, the absence of Cav3.2 channels only in hematopoietic cells (Recipient WT Donor KO) significantly reduced the production of pro-inflammatory mediators, as previously observed in total Cav3.2 KO mice and conversely (Figure 2B-E). Concerning the painful behavior, the absence of Cav3.2 channels only in hematopoietic cells did not modify mechanical allodynia and hyperalgesia in recipient WT mice (Recipient WT Donor KO), while the presence of Cav3.2 channels exclusively in hematopoietic cells more or less partially restored mechanical allodynia and hyperalgesia in recipient Cav3.2 KO mice (Recipient KO Donor WT) (Figure 2F and G).

Cav3.2 calcium channels are essential for macrophage activation. Since previous studies have demonstrated the involvement of Cav3.2 channels in the production and exocytosis of catecholamines (Carbone E. et al., Cell Calcium 2006;40:147-154) and in regard of the present results, we hypothesized that Cav3.2 channels expressed in macrophages play a central role in their activation during the inflammatory process. To demonstrate this role, *in vitro* studies were performed on Cav3.2^{-/-} and Cav3.2^{+/+} mouse BMDM culture. Following inflammatory-like stimulation (20 ng/ml of LPS during 24 hours), Cav3.2^{-/-} BMDM produced a less amount of pro-inflammatory mediators (IL-6, IL-1 β and TNF- α) compared to Cav3.2^{+/+} BMDM (Figure 3A-C). Since calcium was shown to be a major factor in the macrophage activation (Zhou X., Yang W., Li J., J Biol Chem 2006;90:1830-31347), the study of intracellular calcium influx, by calcium imaging, was performed on the same condition. Results showed that $30.2 \pm 5.6\%$ (156/515) of Cav3.2^{+/+} BMDM cells and only $6.7 \pm 2.1\%$ (26/446) of Cav3.2^{-/-} BMDM cells exhibited an intracellular calcium influx in response to LPS stimulation (Figure 3D and 3E). Cav3.2 channels were thus involved in calcium influx in LPS- stimulated macrophages. In addition, the study of the macrophage morphology performed by phalloidin-immunofluorescence labelling of actin, revealed swelling morphology for Cav3.2^{+/+} BMDM (cells area without LPS = $283 \pm 15 \mu\text{m}^2$; with LPS = $643 \pm 22 \mu\text{m}^2$), supposing strong cytoplasmic production of inflammatory mediators. In contrast, Cav3.2^{-/-} BMDM showed no morphological signs of activation (cells area without LPS = $307 \pm 12 \mu\text{m}^2$; with LPS = $260 \pm 10 \mu\text{m}^2$) in response to LPS stimulation (Figure 3F and G).

Pharmacological blockade of T-type calcium channels exhibits anti-allodynia, anti-hyperalgesia and anti-inflammatory effect in sub-acute and chronic inflammatory-related pain. The analgesic and anti-inflammatory effects of the TTA-A2 (T-type calcium channel inhibitor, preferentially Cav3.2 isoform) (Francois A et al., Pain 2013;154:283-293 ; Kerckhove N et al., Pain 2014;155:764-772), and ethosuximide (T-type calcium channel blocker and marketed

antiepileptic), were first evaluated using sub-acute carrageenan-induced paw inflammation. As previously observed with Cav3.2 KO mice, pharmacological blockade of T-type calcium channels, using systemic administration of TTA-A2 or ethosuximide (1 mg/kg *per os*, 200 mg/kg i.p, respectively), induced an anti-hyperalgesic and anti-allodynic effect (Figure 4A-B and D-E).
5 In addition, an anti-edematous property of these inhibitors was observed (Figure 4C and F). The same result was obtained by intraplantar injection of TTA-A2 (0.1 mg/kg i.p. Figure 4G-I) confirming an involvement of Cav3.2 channels (more widely T-type calcium channels) in inflammation and related pain.

To confirm previous results, inflammation and related pain parameters were assessed in
10 another model, a chronic inflammatory one, using peri-articular complete Freund's adjuvant (CFA) injection. Due to the chronicity of the model and in order to assess the maintenance over time of the clinical used ethosuximide efficacy, the effect of repeated treatments was investigated. Repeated injections (3 times daily) of ethosuximide (200 mg/kg i.p.) for 7 days "preventively" (Figure 5A) or curatively (Figure 6A), induced an anti-edematous (Figures 5B and 6B), anti-
15 allodynic (Figures 5C and 6C) and anti-hyperalgesic (Figures 5D and 6D) effect. In addition, to support previous result, Cav3.2^{-/-} mice developed less edema and did not display mechanical hypersensitivity symptoms at 7 days after CFA injection (Figure 9A-C).

Discussion

Using *ex vivo* and *in vivo* methods coupled with genetic strategies, this work
20 demonstrated, for the first time, the involvement of Cav3.2 channels in inflammation and inflammatory-related pain by modulating macrophage activation and inflammatory mediator release. This discovery was reinforced by the efficiency of the T-type blocker ethosuximide and the specific Cav3.2 inhibitor TTA-A2 on both inflammation and pain, which opens new clinical perspectives for the treatment of inflammatory-related pain.

Total genetic deletion of Cav3.2 reduced edema induced by carrageenan paw injection or CFA peri-articular injection and carrageenan-induced systemic IL-6 and tissue IL-6, TNF- α , IL-10 and PGE₂ production. Moreover, suspecting the involvement of Cav3.2 channels on macrophages in the inflammatory process, it is herein demonstrated: 1) the presence of Cav3.2 transcripts on BMDM from Cav3.2^{+/-} mice and 2) the ability of these
30 channels to participate to macrophages activation. These results are in accordance with recent studies which demonstrated the role of calcium channels and extracellular calcium in macrophage activation by LPS (Zhou X., Yang W., Li J., J Biol Chem 2006;281:31337-31347) and the potential role of Cav3.2 channels in exocytose processes (Carbone E. et al., Cell Calcium 2006;40:147-154 ; Giannippoli A. et al., Biophys J 2006;90:1830-
35 1841 ; Carabelli V. et al., Eur Biophys J EBJ 2007;36:753-762 ; Mahapatra S. et al.,

Cell Calcium 2012;51:321-330). Thus, expression of functional Cav3.2 calcium channels on macrophages could explain the decrease of pro-inflammatory mediator production by BMDM from *Cav3.2^{-/-}* mice, resulting in a tissue (edema) and systemic reduced inflammatory marker secretion. Although macrophages are non-excitabile, the importance of several voltage gated ion channel in these cells as well as other immune cells (lymphocytes, monocytes, etc.) is emerging (Kis-Toth K. et al., J Immunol Baltim Md 1950 2011;187:1273-1280). In that respect, the ability of T-type calcium channels to generate a steady state window current upon little variations of membrane potential could actively participate to macrophage activation, as observed for in differentiation processes of other cell types (Lory P., Bidaud I., Chemin J., Cell Calcium 2006;40:135-146). Finally, using chimeric mice, it is herein demonstrated that the unique absence of Cav3.2 channels in hematopoietic cells (progenitors of macrophages) reduced edema development and pro-inflammatory mediator release. Interestingly, this reduction was close to that observed in total Cav3.2 KO mice, suggesting that Cav3.2 channels expressed by hematopoietic cells were involved in the inflammatory process. Accordingly, transplantation of hematopoietic cells expressing Cav3.2 channels in total Cav3.2 KO animals completely restored edema development and inflammatory pro-mediators release. Thus, this chimeric mouse experiment demonstrated that inflammation induced by carrageenan and CFA needed Cav3.2 channels expressed in hematopoietic cells, even if their deletion did not totally suppress the inflammatory process.

Cav3.2 channels may be involved in carrageenan-induced hypersensitivity to noxious or non-noxious stimuli, owing to their neuronal [by modulating nociceptive influx (Bender KJ. et al., J Physiol 2012;590:109-118 ; Todorovic SM., Jevtovic-Todorovic V., CNS Neurol Disord Drug Targets 2006;5:639-653)] or macrophagic (by participating to inflammation, as shown previously) location. Total deletion of Cav3.2 induced a marked reduction of allodynia and hyperalgesia suggesting an important tonic involvement of these channels in hypersensitivity, and thus confirming previous other studies (Bourinet E. et al., EMBO J 2005;24:315-324 ; Kerckhove N et al., Pain 2014;155:764-772 ; Todorovic SM, Jevtovic-Todorovic. V, Pflüg Arch Eur J Physiol 2013;465:921-927). Regarding the respective role of their locations, deletion of channels only in the hematopoietic cells did not alter mechanical hyperalgesia and allodynia, demonstrating that the tonic involvement of Cav3.2 channels in hypersensitivity is mainly due to their presence in sensory neurons. This observation of an unchanged level of pain, in WT mice with absence of Cav3.2 in hematopoietic cells, despite a decreased edema, can be explained by the potential ability of the residual inflammation and pro-inflammatory mediators to activate nociceptors and induce

hyperalgesia/allodynia. Furthermore, the existence of a decoupling of pain and edema has been shown by several studies in the model of inflammation induced by carrageenan (Jing JN. Et al., J Vet Pharmacol Ther 2009;32:1-17) and formalin (Lee IO., Jeong YS., J Korean Med Sci 2002;17:81-85). Interestingly, transfer of Cav3.2 hematopoietic cells in Cav3.2 KO mice restored edema, partially hyperalgesia and allodynia demonstrating that Cav3.2 expressed by hematopoietic cells could, in a lesser extent, participate to the inflammatory-related pain by inducing inflammation. Taken together, these results demonstrate that Cav3.2 channels are markedly involved in inflammatory pain from their neuronal expression in sensory neurons. However, we cannot exclude a role of Cav3.2 expressed by immune system but their involvement in inflammatory pain is minimal.

In line with the pathophysiological role of both Cav3.2 expressed by immune and neuronal systems demonstrated in the present study, systemic administration of ethosuximide reduced edema, hyperalgesia and allodynia in the carrageenan and CFA models. Interestingly, repeated systemic injection of ethosuximide induced a curative anti-hyperalgesic and anti-edematous effect, but was also able to limit development of edema when administered at the same time of CFA injection, these effects being maintained over time. Working more specifically on Cav3.2 channels, we also demonstrated an analgesic and anti-inflammatory effect of systemic administration of TTA-A2, a specific inhibitor of Cav3.2 channels. This result validates the concept of Cav3.2 channels as a novel target for inflammation and related pain relief. Moreover, the demonstrated analgesic and anti-edematous effect observed after intraplantar administration of a low dose of TTA-A2 confirmed that peripheral (potentially on immune cells and primary afferent fibers) Cav3.2 channels are involved in the pathophysiology of inflammation and related pain and expands the array of potential clinical use of T-type calcium channel inhibitors.

Claims

1. Ethosuximide for use in the treatment or prevention of pain of inflammatory type and/or in
5 the treatment of inflammation.
2. Ethosuximide for the use according to claim 1 for use in the treatment or prevention of
acute or chronic pain by excess nociception.
- 10 3. Ethosuximide for the use according to claim 1 or 2, for use in the treatment or prevention
of arthritis or in the treatment or prevention of arthrosis or in the treatment or prevention of an
intestinal inflammatory disease or in the treatment or prevention of irritable bowel syndrome
or in the treatment or prevention of tendinitis.
- 15 4. Ethosuximide for the use according to claim 1 or 2, for use in the treatment or prevention
of inflammation in arthritis, arthrosis, intestinal inflammatory disease, irritable bowel
syndrome, migraine, cephalalgia, myalgia, tendinitis, dorsal pain, cervical pain or lumbago.
- 20 5. Ethosuximide for the use according to any one of claims 1 to 4, for use in the short term
for a time of one of two days; over the medium term for a time of 3 days to 3 months; over
the long term for a time exceeding 3 months; or permanently.
6. Ethosuximide for the use according to any one of claims 1 to 5, for use in adults, in
adolescents, in children, or in the elderly.
- 25 7. Ethosuximide for the use according to any one of claims 1 to 6, for use in association with
another agent for treating or preventing pain triggered by excess nociception, in particular an
opioid pain control agent for example an agent selected from among codeine, tramadol,
morphine, oxycodone, or a non-opioid pain control agent in particular a non-steroidal anti-
30 inflammatory agent, a pain control agent selected from among paracetamol and nefopam, a
local anaesthetising agent.
8. Ethosuximide for the use according to any one of claims 1 to 7, for use at a dosage
ranging from 500 mg/day to 2 g/jour.

9. Ethosuximide for the use according to any one of claims 1 to 8, for simultaneous use in the treatment or prevention of an inflammatory reaction and of a pain of inflammatory type.

5 10. A method for treating or preventing pain of inflammatory type and/or in the treatment or prevention of inflammation in a patient in need thereof, comprising administering an effective amount of ethosuximide.

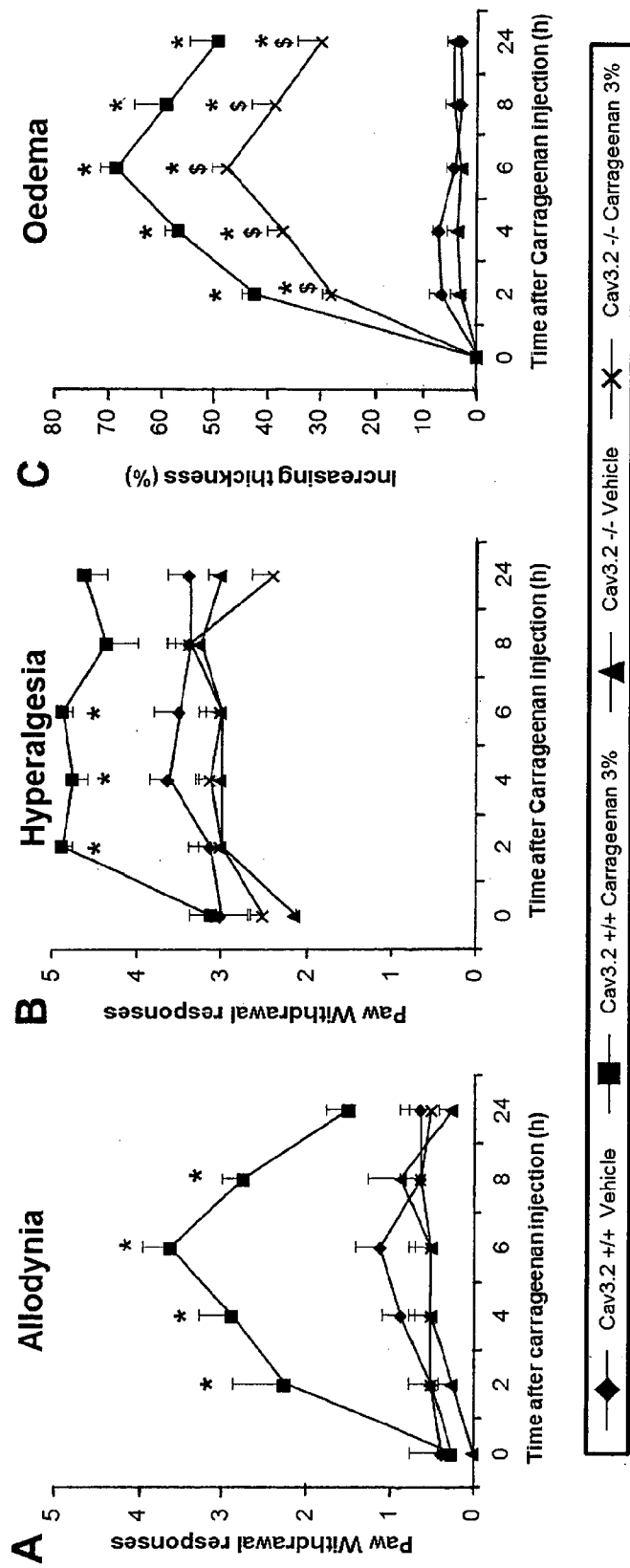


FIG.1

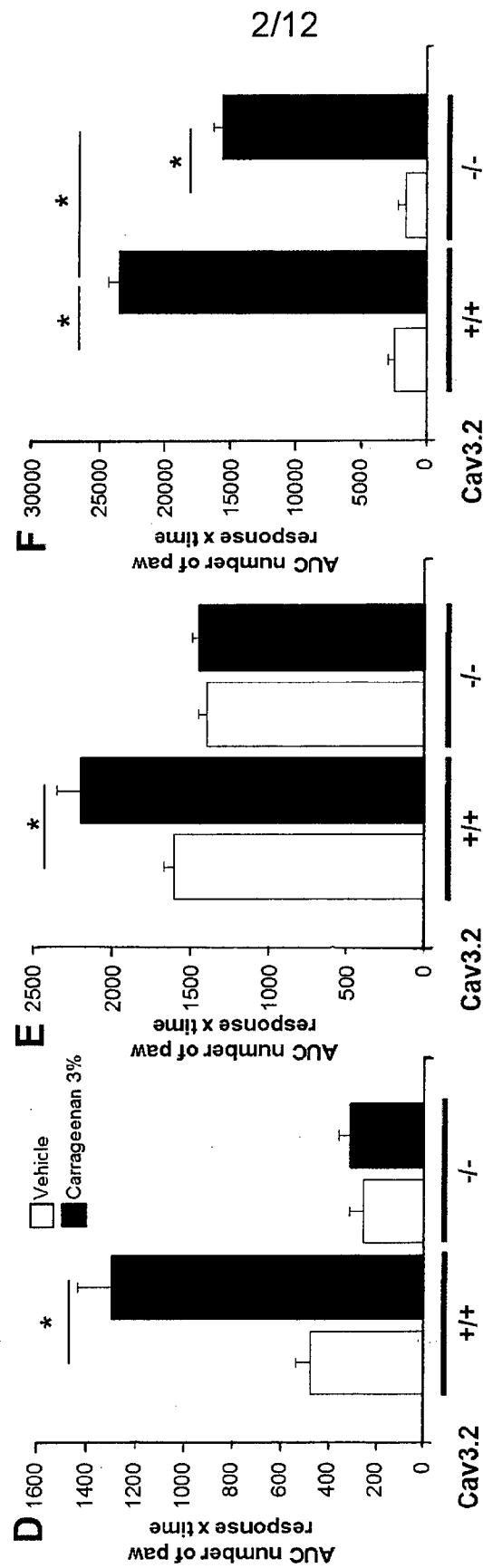


FIG.1 following

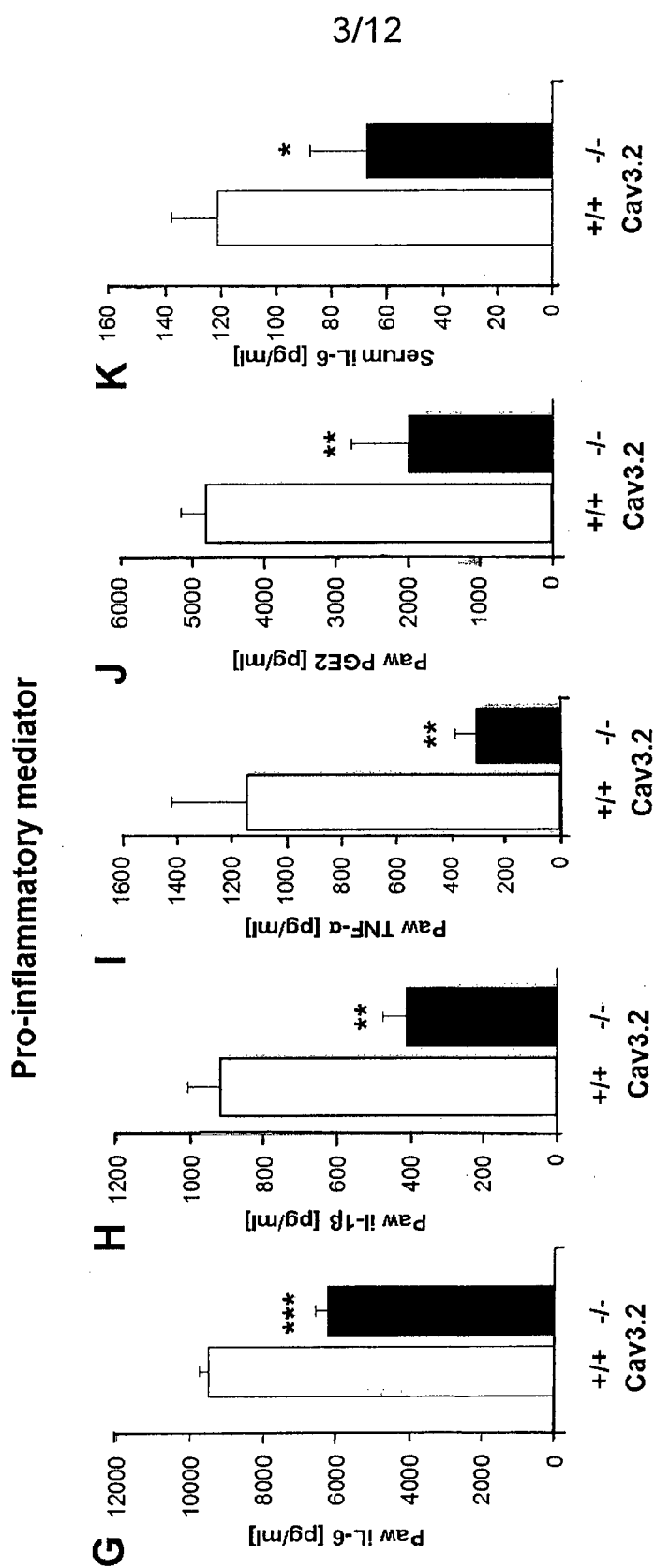
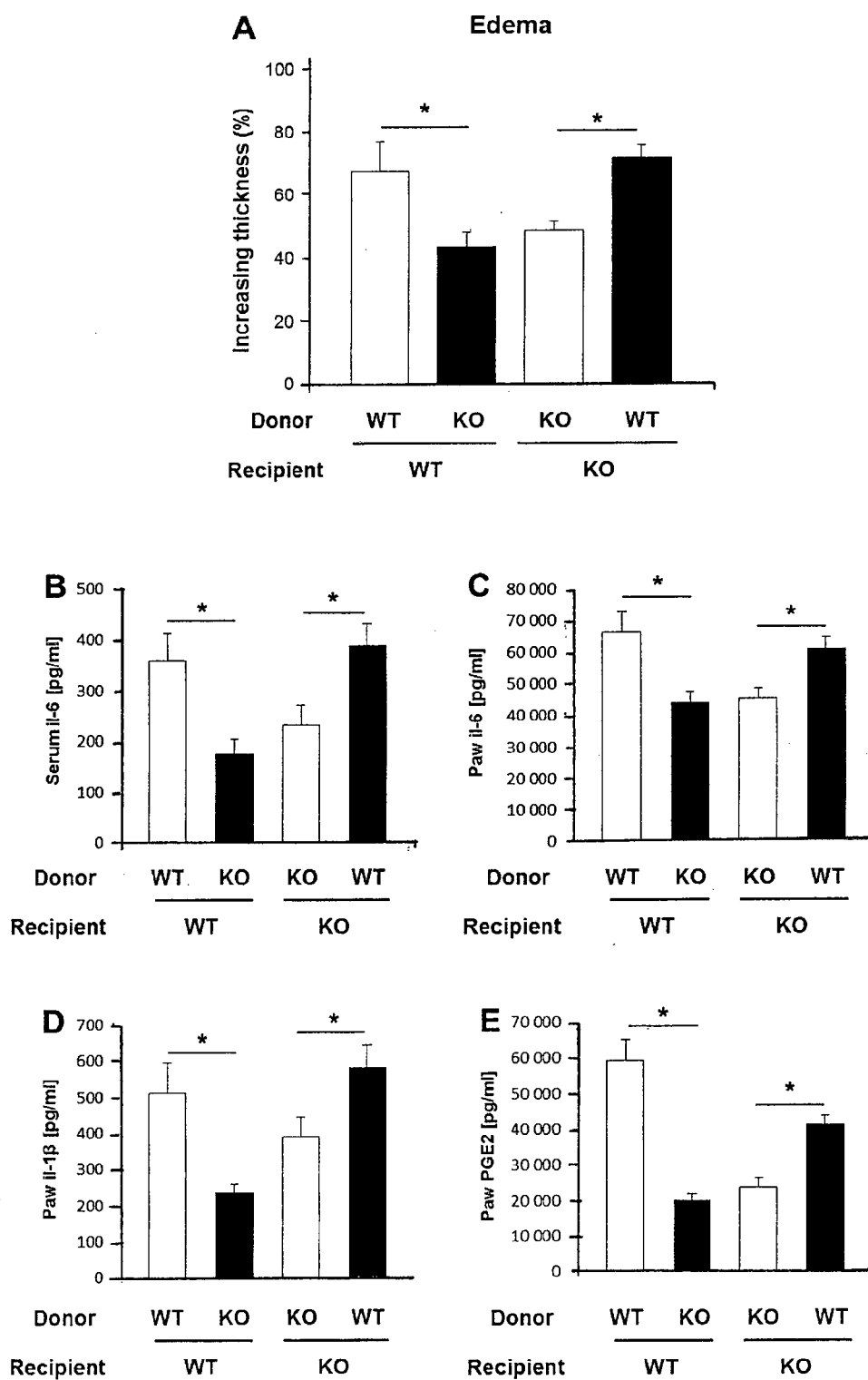
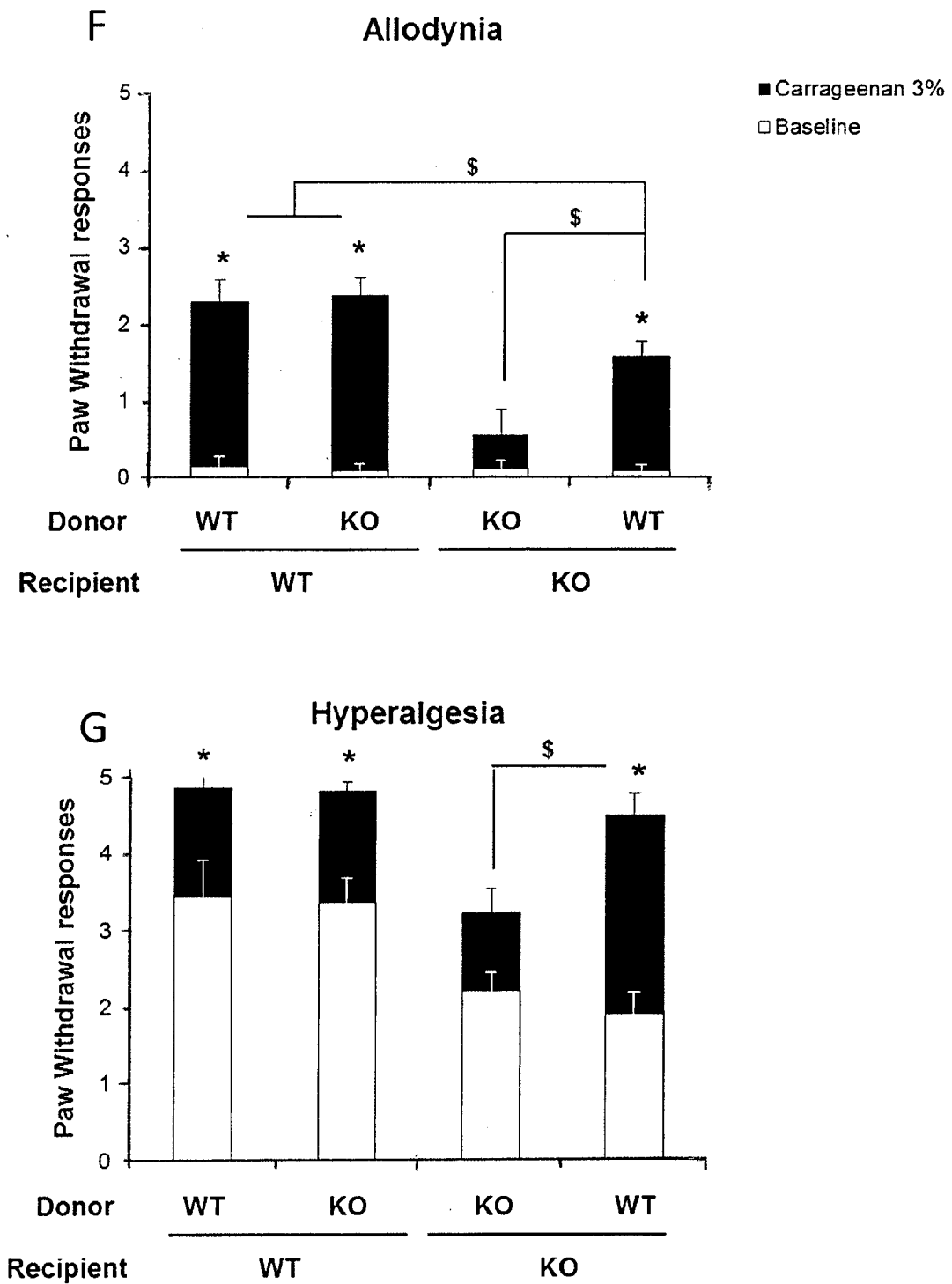


FIG.1 following

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**FIG.2**

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**FIG.2 following**

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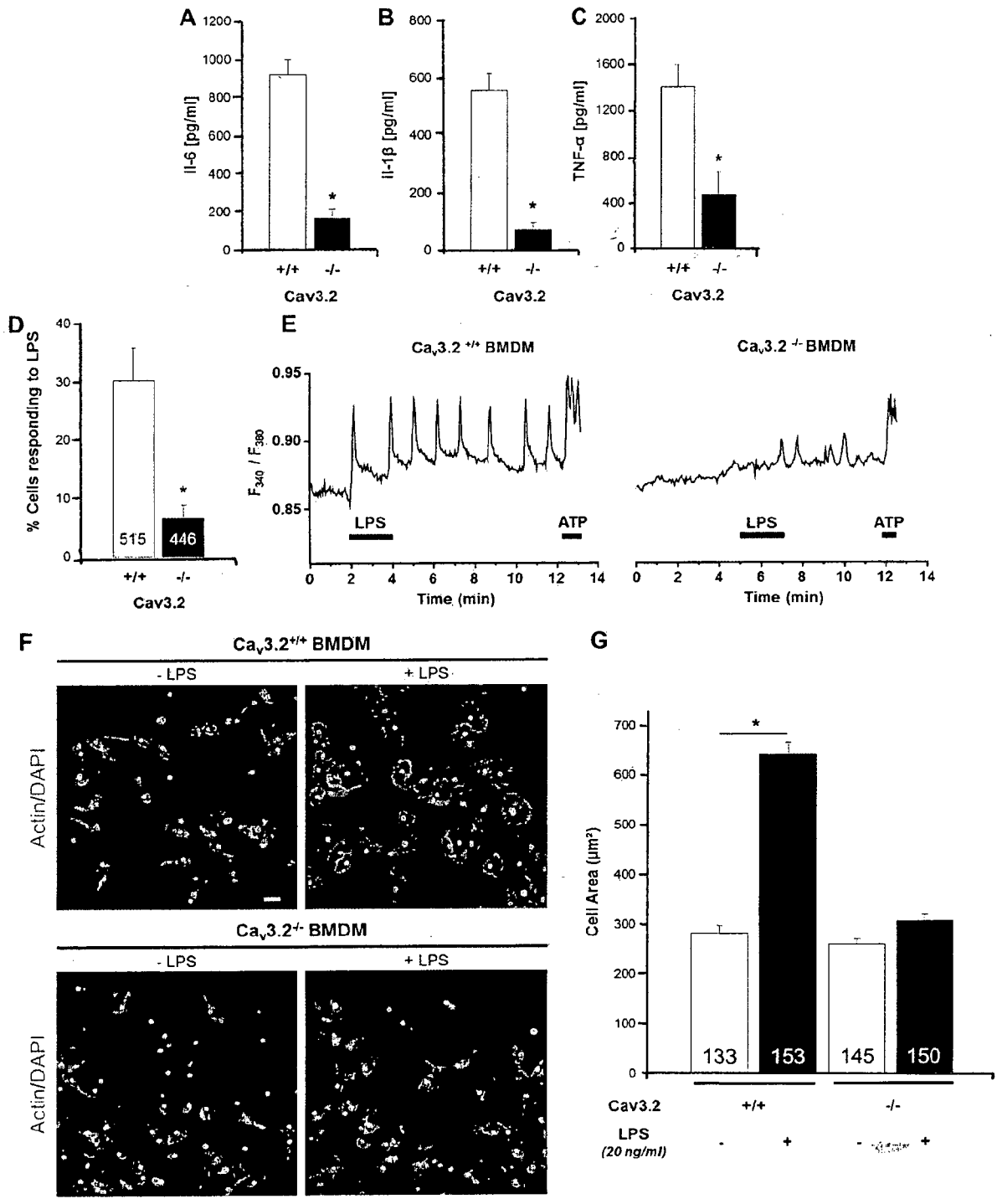
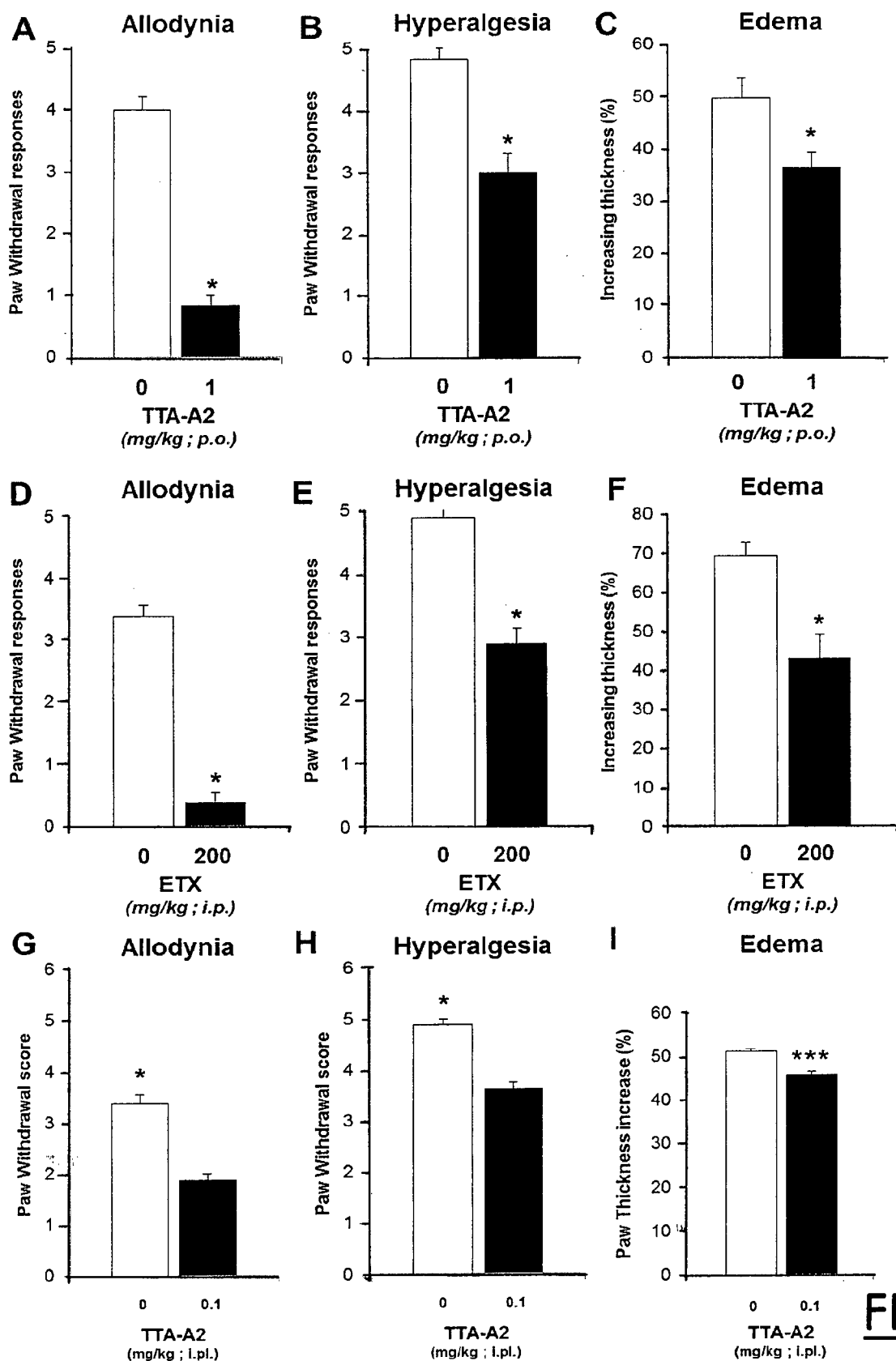
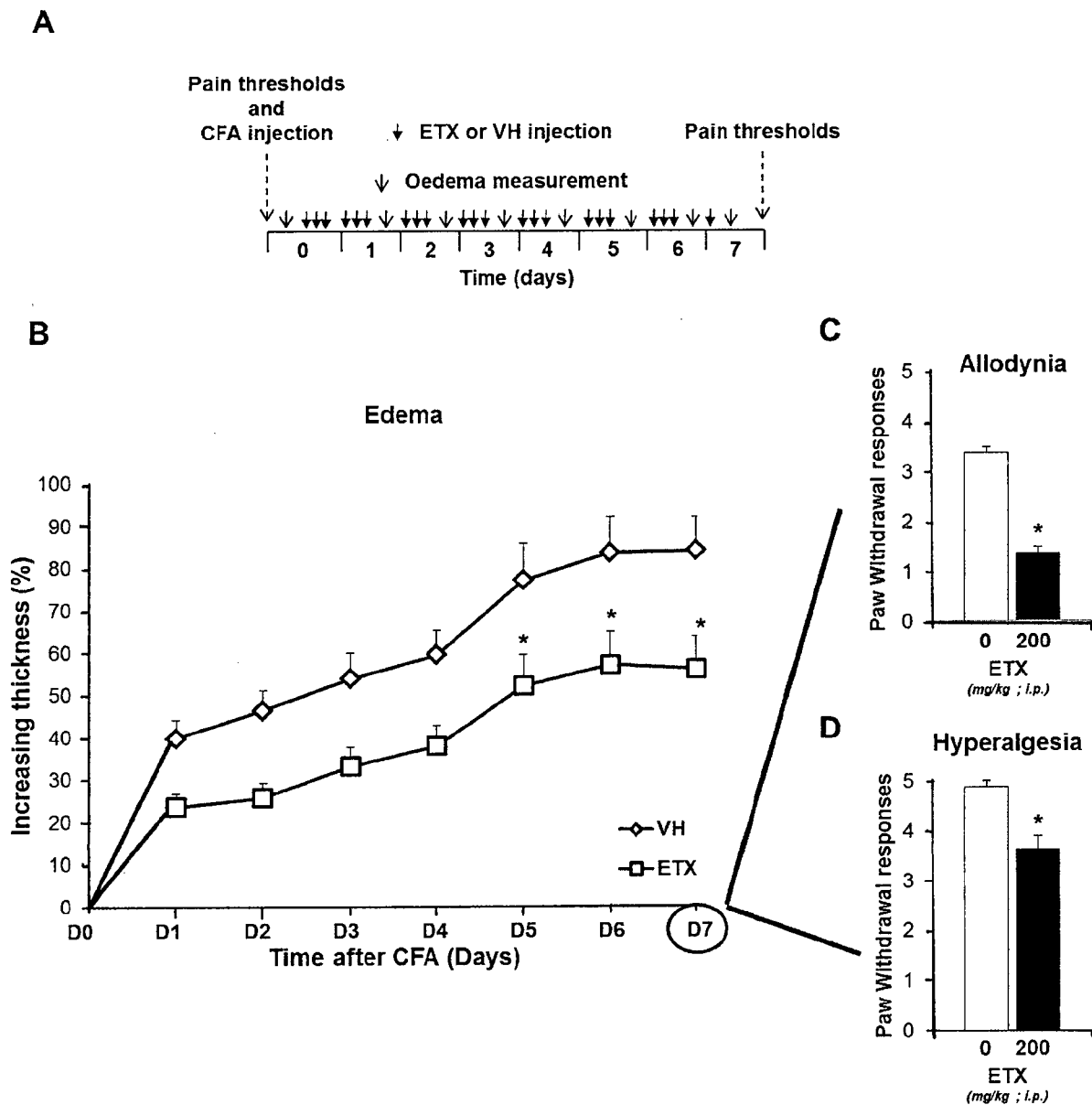


FIG.3

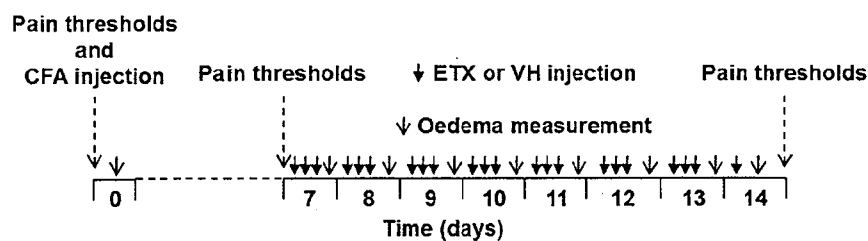
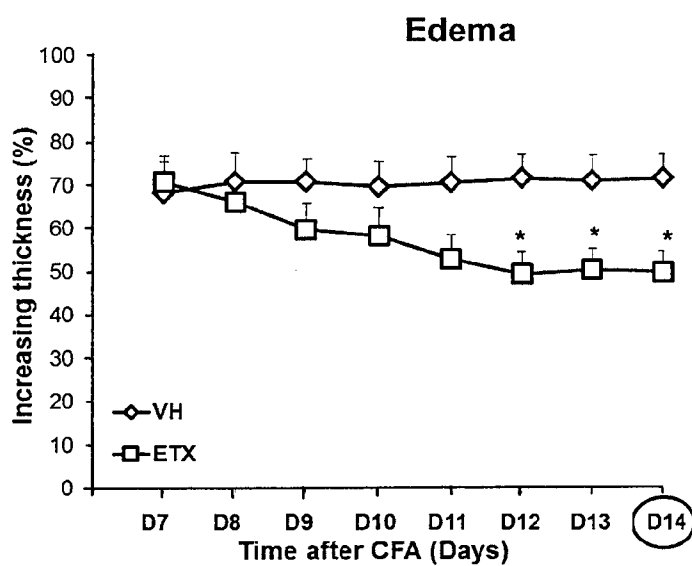
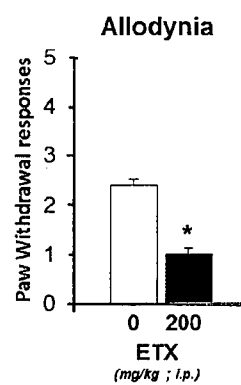
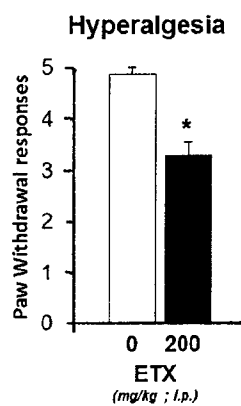
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**FIG.4**

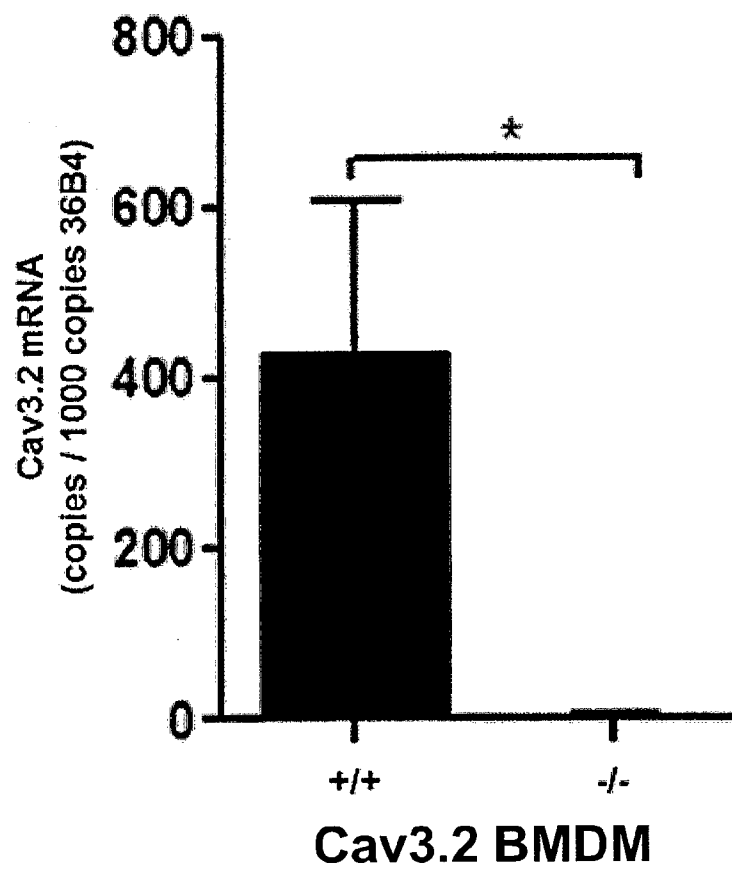
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**FIG.5**

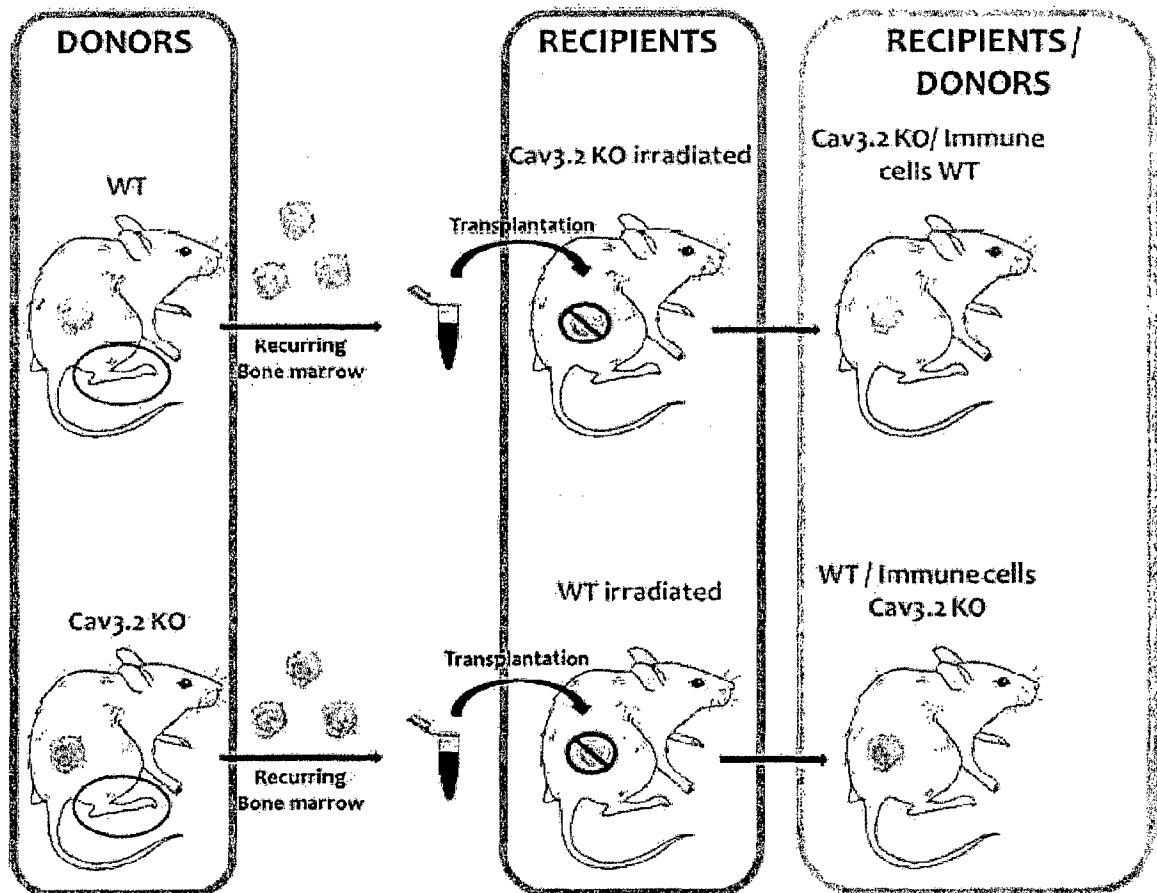
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A**B****C****D****FIG.6**

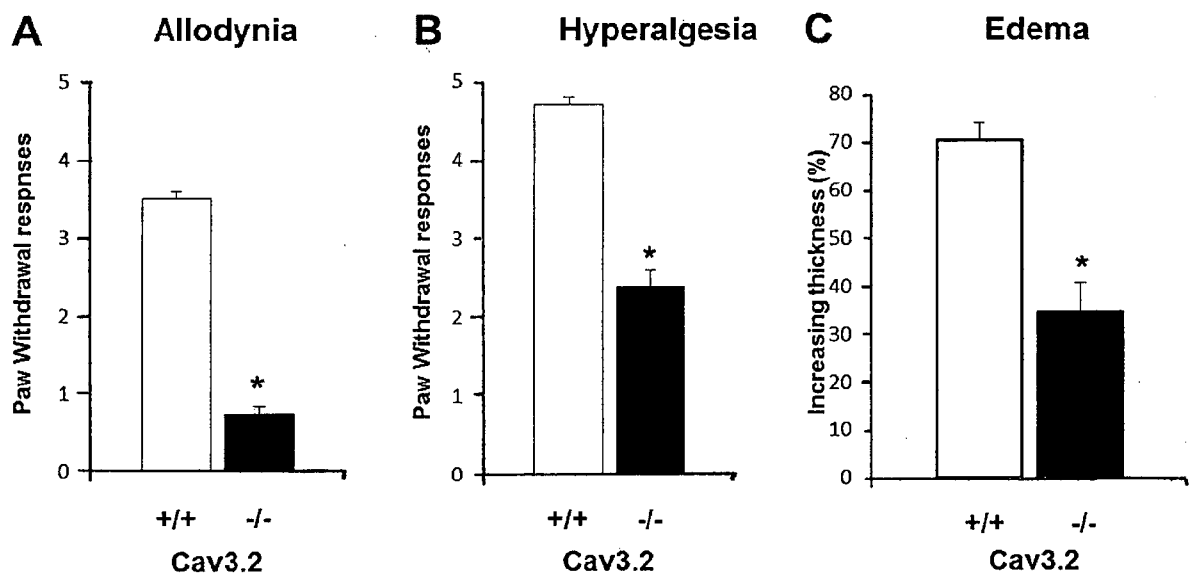
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FIG. 7

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**FIG.8**

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**FIG.9**

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/068538

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/4015 A61P25/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/033460 A2 (SCHERING CORP [US]; HARRIS JOEL M [US]; NEUSTADT BERNARD R [US]; SOROT) 20 March 2008 (2008-03-20)	1-7,9,10
Y	page 9, line 14 - page 10, line 14 page 10, line 20 - page 11, line 16 page 11, line 17 - line 34 page 6, line 4 - line 7 ----- -/--	8



Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

31 October 2014

Date of mailing of the international search report

18/11/2014

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Uryga-Polowy, V

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/068538

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BARTON M E ET AL: "The antihyperalgesic effects of the T-type calcium channel blockers ethosuximide, trimethadione, and mibefradil", EUROPEAN JOURNAL OF PHARMACOLOGY, ELSEVIER SCIENCE, NL, vol. 521, no. 1-3, 3 October 2005 (2005-10-03), pages 79-85, XP027695650, ISSN: 0014-2999 [retrieved on 2005-10-03] abstract page 83 - page 84 -----	1,2,5,6, 10
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Y	paragraph [0079] claims 1, 5, 13-15 -----	8
Y	WO 2008/079727 A2 (ALLERGAN INC [US]; GIL DANIEL W [US]; DONELLO JOHN E [US]) 3 July 2008 (2008-07-03) page 6 -----	8

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