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(54) Title: MEANS AND METHODS FOR TREATING NEUROPATHIC PAIN

(57) Abstract: The present invention relates CNGB1 inhibitors for use in a method for the prophylaxis and/or treatment of neuropathic pain. CNG channels containing the CNGB1b subunit are expressed in dorsal root ganglia (DRG) neurons and are upregulated after peripheral nerve injury. CNGB deficient mice (CNGB -/-) developed less mechanical hypersensitivity compared to wild-type animals after peripheral nerve injury and after intrathecal delivery of a cAMP analog. Moreover, peripheral nerve injury-induced mechanical hypersensitivity could be reduced by treatment with a CNGB1 inhibitor.



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MEANS AND METHODS FOR TREATING NEUROPATHIC PAIN

TECHNICAL FIELD OF THE INVENTION

5 **[001]** The present invention relates to an inhibitor of CNGB1 for use in a method of treating of neuropathic pain.

BACKGROUND ART

[002] Neuropathic pain is a major clinical health problem, affecting millions of people worldwide. As patients are often resistant to currently available treatments and regarding to their significant systemic side effects, it is important to elucidate the molecular mechanisms of neuropathic pain processing to obtain new insights in potential future therapies (Basbaum et al., 2009; Johannes et al., 2010; Ji et al., 2014). Recent studies implicated specific cyclic nucleotide-dependent signaling pathways in neuropathic pain processing. For example, genetic deletion of cAMP-producing adenylyl cyclases (AC) or cGMP-producing guanylyl cyclases (GC), and administration of AC or GC inhibitors, ameliorated neuropathic pain behaviors in rodents (Kim et al., 2007; Schmidtke et al., 2008b; Xu et al., 2008; Pierre et al., 2009; Schmidtke et al., 2009; Wang et al., 2011). Conversely, delivery of cAMP and cGMP analogs induced pain hypersensitivity and enhanced the hyperexcitability of injured dorsal root ganglia (DRG) neurons (Song et al., 2006; Schmidtke et al., 2008b; Heine et al., 2011; Huang et al., 2012; Kallenborn-Gerhardt et al., 2014).

[003] Specific mechanisms by which cAMP and cGMP contribute to pain processing are poorly understood. So far identified targets of pain-relevant cAMP signaling include cAMP-dependent protein kinase (PKA), cAMP response element-binding protein (CREB), exchange proteins activated by cAMP (Epac), and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Ma et al., 2003; Hucho et al., 2005; Song et al., 2005; Hagiwara et al., 2009; Pierre et al., 2009; Eijkelkamp et al., 2010; Emery et al., 2011; Cordeiro Matos et al., 2015; Gu et al., 2016; Singhmar et al., 2016). In addition, many cGMP-dependent effects during pain processing are mediated by activation of cGMP-dependent protein kinase I (PKG-I, synonym cGKI) (Tegeder et al., 2004; Schmidtke et al., 2009; Luo et al., 2012; Solorzano et al., 2015).

30 **[004]** Cyclic nucleotide-gated (CNG) channels are tetrameric protein complexes that are activated by direct binding of intracellular cAMP and/or cGMP. Six distinct subunits (CNGA1-4, CNGB1 and CNGB3) have been identified in mammals, and their diversity is further increased by splice variants. Under physiological conditions, CNG channels carry inward Na^+ and Ca^{2+} currents, and as nonselective cation channels they also conduct monovalent cations such as K^+ ,

Na⁺, Li⁺, Rb⁺ and Cs⁺. CNG channels are highly expressed in olfactory sensory neurons and retinal photoreceptors, where they play a key role in olfactory and visual signal transduction (Kaupp and Seifert, 2002; Biel and Michalakis, 2007, 2009; Podda and Grassi, 2014). However, CNG channels are also expressed at lower levels in other tissues and their specific contribution to physiological and pathophysiological processes is being increasingly recognized (Podda and Grassi, 2014). Previously, it has been shown that CNG channels containing the CNGA3 subunit are targets for cGMP and specifically modulate the processing of inflammatory pain (Heine et al., 2011). However, CNGA3 channels were not involved in the processing of neuropathic pain caused by peripheral nerve injury.

[005] Thus far, available treatments for neuropathic pain include anticonvulsants (such as gabapentin and pregabalin), tricyclic antidepressants, serotonin-noradrenaline reuptake inhibitors, opioids, and topical therapies. However, more than half of neuropathic pain patients report inadequate pain relief, and currently available medications are often associated with severe side effects. Therefore, there is a large unmet therapeutic need for effective and safe treatment of neuropathic pain (Nightingale, Nat Rev Drug Discov 2012). Accordingly, the technical problem underlying the present application is to comply with this need.

SUMMARY OF THE INVENTION

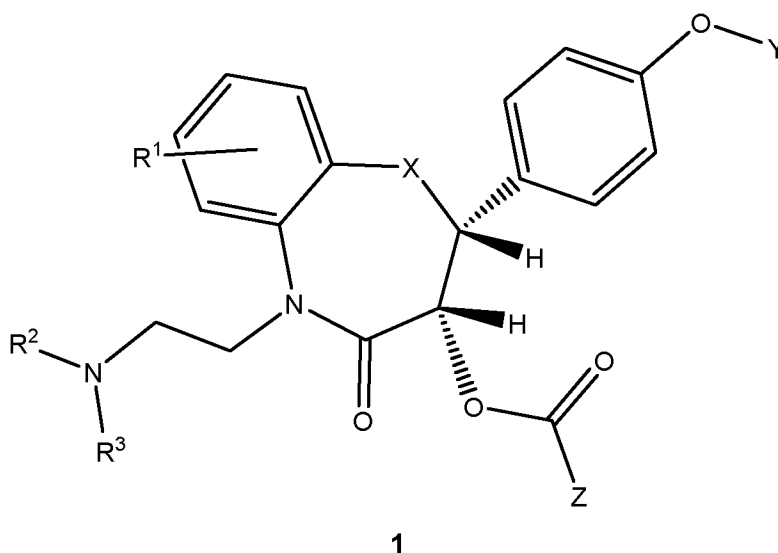
[006] The technical problem is solved by the embodiments reflected in the claims, described in the description, and illustrated in the Examples and Figures.

[007] The inventors surprisingly found that the CNGA2, CNGA4 and CNGB1 subunits of CNG channels are upregulated in models of neuropathic pain (Fig. 1). Furthermore, they found that a knockout of the CNBG1 subunit (Fig. 4A) or inhibition of the CNGB1 subunit (Fig. 4B) is able to reduce neuropathic pain caused by peripheral nerve injury. Additionally, the inventors surprisingly found that the cAMP-induced allodynia is reduced after CNGB1 knockout (Fig. 6). Therefore, the inventors found a way to treat neuropathic pain by inhibiting CNGB1.

[008] Accordingly, the invention relates to an inhibitor of CNGB1 for use in a method for the prophylaxis and/or treatment of neuropathic pain in a subject.

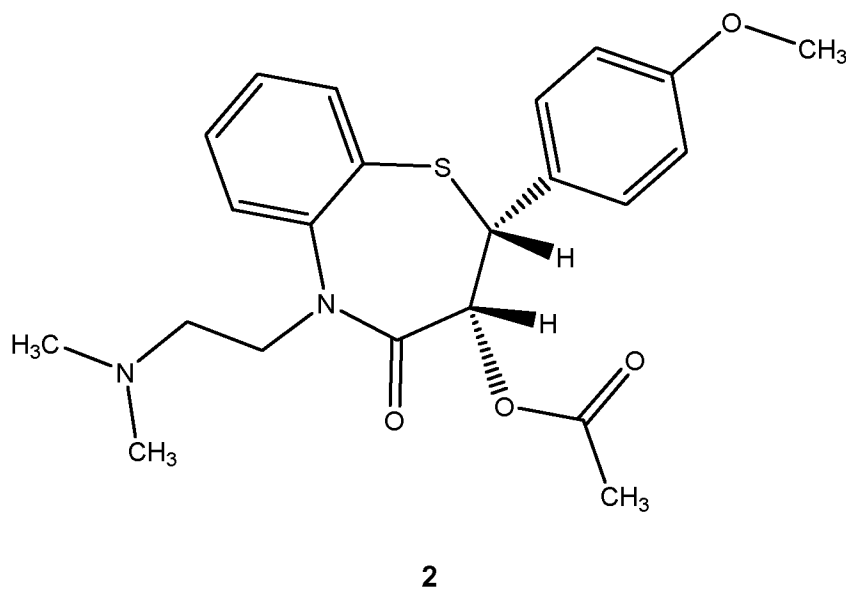
[009] The inhibitor for the use according to the inhibition is preferably selected from the group consisting of peptides, proteins, antibodies, nucleic acids and small molecules. Preferably, the small molecule is a benzodiazepine derivative or a cyclic nucleotide derivative.

[0010] Preferably, the benzodiazepine derivative is characterized by structure 1,



wherein X is O or S; Y, R², R³, Z are each independently selected from the group consisting of hydrogen, (C₁-C₆)alkyl, (C₂-C₆)alkenyl and (C₂-C₆)alkynyl; and R₁ is selected from the group consisting of hydrogen, a nitro group, halogen, -O-(C₁-C₆)alkyl, -S-(C₁-C₆)alkyl and a cyano group.

[0011] Preferably, the small molecule is L-cis-diltiazem ((2R,3R)-(-)-cis-3-Acetoxy-5-(2-dimethylaminoethyl)-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one) characterized by structure **2**:



[0012] The present invention also relates to an inhibitor for the use in a method for the prophylaxis and/or treatment of neuropathic pain in a subject, wherein neuropathic pain is caused by a lesion or disease of the somatosensory nervous system.

[0013] The neuropathic pain, or the lesion or disease of the somatosensory nervous system may affect a sensory neuron.

[0014] Preferably, the lesion or disease of the somatosensory nervous system is caused by a trauma, compression of a nerve, a surgery, a viral infection, pharmacotherapy, and/or antiretroviral treatment.

[0015] The viral infection causing the lesion or disease of the somatosensory nervous system may be mediated by a virus selected from the group consisting of herpes virus, human immunodeficiency virus (HIV), and papilloma virus.

[0016] The pharmacotherapy causing the lesion or disease of the somatosensory nervous system may comprise an anti-cancer agent, including but not limited to cisplatin, oxaliplatin, paclitaxel, docetaxel, nocodazole, thalidomide, thiouracile, vincristine, vindesine, vinblastine, and vinorelbine.

[0017] The antiretroviral treatment causing the lesion or disease of the somatosensory nervous system may comprise an anti-retroviral agent, and the anti-retroviral agent is preferably selected from the group consisting of reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors, CCR5 antagonists or fusion inhibitors.

[0018] The lesion or disease of the somatosensory nervous system causing neuropathic pain may be associated with sciatica, hereditary neuropathy (including but not limited to Friedreich ataxia, familial amyloid polyneuropathy, Tangier disease, Morbus Charcot-Marie-Tooth types 2B or 5, Fabry disease), metabolic disorders (including but not limited to diabetes, renal insufficiency and hypothyroidism), vitamin deficiencies (including but not limited to vitamin B12 deficiency, vitamin B6 deficiency, and vitamin E deficiency), toxic and iatrogenic neuropathies (including but not limited to alcoholism, vitamin B6 intoxication, hexacarbon intoxication, amiodarone, chloramphenicol, disulfiram, isoniazide, gold, lithium, metronidazole, misonidazole, nitrofurantoin), infectious neuropathies (including but not limited to leprosy and Lyme disease), auto-immune neuropathies (including but not limited to Guillain-Barre syndrome, chronic inflammatory de-myelinating polyneuropathy, monoclonal gammopathy of undetermined significance, and polyneuropathy), trigeminal neuralgia, entrapment syndromes (including but not limited to Carpel tunnel), post-traumatic neuralgia, phantom limb pain, multiple sclerosis pain, complex regional pain syndromes (including but not limited to reflex sympathetic dystrophy and causalgia), neoplasia, vasculitic/angiopathic neuropathy, and/or idiopathic neuropathy.

[0019] The neuropathic pain, which is treated or prevented by an inhibitor of CNGB1, may be allodynia, hyperalgesia and/or spontaneous pain.

[0020] Preferably, the subject to be prevented or treated by way of an inhibitor of CNGB1 is human.

[0021] The present invention also relates to the inhibitor for the use of according to the invention, wherein the inhibitor is used in combination with one or more additional neuropathic pain reducing agent(s).

[0022] The present invention also relates to the inhibitor for the use of according to the invention, wherein the inhibitor is administered to the subject orally, topically, transdermally, transmucosally, subcutaneously, intramuscularly and/or intravenously.

BRIEF DESCRIPTION OF THE FIGURES

The figures show:

[0023] Fig. 1 depicts the expression of CNG channel subunits in dorsal root ganglia after spared nerve injury (SNI). Quantitative RT-PCR revealed that CNGA2, CNGA4 and CNGB1 mRNA was significantly up-regulated after SNI, whereas CNGA3 mRNA was down-regulated. CNGA1 and CNGB3 mRNA was not detected. $n = 3-4$ mice per time point. Data are presented as mean $\pm$ SEM. mRNA levels of naive mice were set to 1. *, #, +, °: $p < 0.05$ compared with naive mice for CNGA2, CNGA3, CNGA4 and CNGB1, respectively.

[0024] Fig. 2 depicts the cellular distribution of CNGB1 in dorsal root ganglia. A-D, Fluorescent *in situ* hybridization of CNGB1 mRNA (red) combined with immunostaining for markers (green) and DAPI staining (blue) detected CNGB1 in NF200-positive (A and B) and Peripherin-positive (C and D) DRG neurons. Scale bars: A and C = 25 μ m, B and D = 10 μ m.

[0025] Fig. 3 depicts the basal characterization of CNGB1^{-/-} mice. A, Quantitative RT-PCR revealed that there is no compensatory upregulation of CNG channel subunits in DRGs of CNGB1^{-/-} mice as compared to WT. Depicted are the mRNA levels of CNG subunits relative to mRNA levels of CNGB1, which were set to 1.0 ($n = 4$; n.d., not detected). B, Immunostaining of DRGs for TRPV1, NF200 and substance P (SubP) and binding of isolectin B4 (IB4) revealed a similar distribution of DRG neuron subpopulations in CNGB1^{-/-} and WT mice ($n = 3$). Data are presented as mean $\pm$ SEM.

[0026] Fig. 4 depicts that neuropathic pain behavior is ameliorated in CNGB1^{-/-} mice and after CNGB1 inhibition. A and B, Time course of mechanical hindpaw sensitivity in CNGB1^{-/-} and WT mice following spared nerve injury (SNI, $n = 10-11$, A) and chronic constriction injury (CCI, $n = 8-9$, B). Note that CNGB1^{-/-} mice displayed reduced neuropathic pain behaviors as compared to WT mice in both models of peripheral nerve injury. C, SNI-induced mechanical hypersensitivity in C57BL/6N mice is reduced after intraplantar injection of L-cis-diltiazem. Injection of drugs (L-cis-diltiazem or vehicle) was performed 14 days after SNI surgery ($n = 5-6$). Data are presented as mean $\pm$ SEM. * $p < 0.05$, comparing CNGB1^{-/-} and WT mice or comparing L-cis-diltiazem- and vehicle-treated mice.

[0027] Fig. 5 depicts that inflammatory pain behavior is not affected by CNGB1 deficiency. Time course of mechanical hindpaw sensitivity in CNGB1^{-/-} and WT mice after CFA injection into a hindpaw was similar between CNGB1^{-/-} and WT mice ($n = 7-8$). Data are presented as mean $\pm$ SEM.

- 5 **[0028] Fig. 6** depicts the cAMP- and cGMP-induced nociceptive behavior in CNGB1^{-/-} mice. Time course of mechanical hindpaw sensitivity after intrathecal administration of a cAMP analog (Sp-8-Br-cAMPS; 20 nmol; $n = 7-8$; A), a cGMP analog (8-pCPT-cGMP; 10 nmol; $n = 6-7$; B), or saline ($n = 3$; C). Data indicate that the extent of cAMP-induced allodynia is reduced in CNGB1^{-/-} mice. Data are presented as mean $\pm$ SEM. * $p < 0.05$, comparing CNGB1^{-/-} and WT mice.

10

DETAILED DESCRIPTION OF THE INVENTION

[0029] The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art.

- 15 **[0030]** The present invention is based on the surprising finding that CNGB1 is upregulated in neuropathic pain (Example 2, Fig. 1) and that a knockout of CNGB1 leads to ameliorated neuropathic pain (Example 4, Fig. 3). In addition, also inhibition of CNGB1 reduced neuropathic pain (Example 5, Fig. 4). Further, the inventors could proof that cAMP and not cGMP is involved in pain processing (Example 7, Fig. 6).

- 20 **[0031]** In particular, it was shown that CNGB1 is expressed in DRG neurons and that its expression is induced upon peripheral nerve injury. Mice deficient for CNGB1 or treated with a CNGB1 inhibitor demonstrated reduced pain behaviors in two different models of after peripheral nerve injury. Moreover, mechanical hindpaw hypersensitivity induced by i.t. administration of a cAMP analog was significantly less pronounced in the absence of CNGB1.

- 25 These data suggest that CNG channels containing CNGB1 contribute to neuropathic pain processing in a cAMP-dependent manner. Interestingly, the pain-relevant functions of CNGB1 are corroborated by the following findings: (i) CNGB1 is expressed in DRG neurons and its expression is induced after peripheral nerve injury. (ii) CNGB1^{-/-} mice showed reduced neuropathic pain behavior in models of peripheral nerve injury. The relevance of CNGB1 for
30 neuropathic pain processing is further supported by the ameliorated mechanical hypersensitivity in C57BL/6N mice after delivery of L-cis-diltiazem, a CNG channel inhibitor that only acts on CNGB1- or CNGB3-positive channel complexes (Chen et al., 1993; Gerstner et al., 2000; Michalakakis et al., 2011). Given the lack of CNGB3 expression in DRG neurons this finding strengthens the role of CNGB1 in neuropathic pain. (iii) CNGB1^{-/-} mice demonstrated reduced
35 hindpaw hypersensitivity after i.t. delivery of a cAMP analog. Altogether, these data show that

cAMP-dependent mechanical hindpaw hypersensitivity depends on CNGB1-containing CNG channels and make a role of such ion channels in neuropathic pain processing plausible.

[0032] The above being said, the present invention relates to an inhibitor for use in a method for prophylaxis and/or treatment of neuropathic pain.

5 **[0033]** Cyclic nucleotide-gated channel Beta 1, also known as CNGB1, is a human gene encoding an ion channel protein. There are at least 3 splice variants known: CNGB1a, CNGB1b (also known as glutamic acid-rich protein 2 (GARP2)) and CNGB1c. CNGB1 as used herein comprises all splice variants of CNGB1.

10 **[0034]** The term “prophylaxis” as used herein, refers to any medical or public health procedure, whose purpose is to prevent a medical condition described herein. As used herein, the terms “prevent”, “prevention” and “preventing” refer to the reduction in the risk of acquiring or developing a given condition, namely neuropathic pain as described herein. Also meant by “prophylaxis” is the reduction or inhibition of the recurrence of neuropathic pain in a subject.

15 **[0035]** In general “therapy”, “treatment” or “treating” seeks remediation of a health problem such as neuropathic pain, usually following a diagnosis. In the medical field, this term is synonymous with treatment of a disease or disorder. Therefore, in this context, a therapy also includes the administration of an inhibitor of CNGB1. Likewise, a “therapeutic effect” relieves to some extent one or more of the symptoms of the abnormal condition, such as neuropathic pain.

20 **[0036]** The term “CNGB1 inhibitor” as used herein, refers to a compound able to reduce the current of ions conducted by a CNG complex comprising CNGB1 by 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 % or 90 % in comparison to a mock or control agent or treatment. It is within the ability of the skilled person to determine if a compound is an inhibitor of a CNG complex comprising CNGB1. For example, the following methods can be used to determine, if a compound is an inhibitor of CNGB1:

25 **[0037]** CNGB1-inhibiting properties of novel compounds can be determined *in vitro*: CNGB1-inhibiting properties of novel compounds may be characterized using Ca^{2+} -imaging experiments in CNGB1-expressing HEK293 cells (HEK-CNGB1). For this purpose, a stably transfected cell line expressing CNGB1b together with CNGA2 may be used, because the CNGB1b splice variant is expressed in sensory neurons and CNGB1b forms functionally active CNG channels together with CNGA2. The PiggyBac system (System Bioscience) may be used, which allows
30 an easy insertion of the vector in the target genome. PiggyBac vectors are available with different expression cassettes encoding fluorescent marker proteins, and antibiotic resistance genes (e.g. puromycin) for positive selection of correct cell clones. In addition, a HEK293 cell line with stable expression of CNGA2 only may also be generated using the same approach
35 and may be used in parallel together with naive HEK293 control cells.

[0038] Ca^{2+} imaging experiments in CNGB1b/CNGA2-containing HEK293 and control cells may be carried out by loading the cells with Fura-2 and Pluronic F127 (Bio Trend) for 45 min. After incubation of the novel CNGB1b-inhibiting compounds for 30-60 min, the cells are stimulated with cell-permeable cAMP and cGMP analogs and Ca^{2+} influx is measured.

- 5 **[0039]** After initial screening with Ca^{2+} -imaging, one may characterize the CNGB1b-inhibiting properties of promising novel compounds using patch-clamp experiments in the above described cell lines. The major aim of these experiments is to analyze how the novel compounds affect the electrophysiological CNG channel functions as well as kinetic properties of CNG channel currents.
- 10 **[0040]** Additionally, the CNGB1-inhibiting properties of novel compounds may be analyzed *ex vivo*: Potential CNGB1 inhibitors may be further characterized by Ca^{2+} -imaging in primary cell culture of sensory neurons from CNGB1^{-/-} and WT mice. Primary cell culture may be prepared using an established protocol (Lu et al., Pain 2014). Briefly, DRGs from CNGB1^{-/-} and WT are excised and transferred to DMEM medium containing gentamicin. Following treatment with
- 15 collagenase and protease, ganglia are dissociated using a Pasteur pipette. Isolated cells are then seeded in Neurobasal medium supplemented with B27 on poly-L-lysine coated glass cover slides. Then Ca^{2+} -imaging will be performed.

[0041] The inhibitor as used according to the invention may be selected from the group consisting of peptides, proteins, antibodies, nucleic acids and small molecules.

- 20 **[0042]** A “peptide” in general is a polymer of 50 or less, such as 40, 30, 20, 10 or 5, amino acids linked by peptide bonds. A “protein” refers to a polymer of more than 50 amino acids linked by peptide bonds. Peptides and proteins may interact with the CNGB1 subunit of CNG channels and thereby inhibit the activity of CNGB1 leading to ameliorated symptoms of neuropathic pain.

- [0043]** The term “antibody” refers to antibodies or antibody fragments comprising
- 25 immunoglobulin molecules and immunologically active portions of immunoglobulin (Ig) molecules, i.e., molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen. Such antibodies or fragments include polyclonal antibodies from any native source, and native or recombinant monoclonal antibodies of classes IgG, IgM, IgA, IgD, and IgE, hybrid derivatives, and fragments of antibodies including Fab, Fab' and F(ab')₂,
- 30 humanized or human antibodies, recombinant or synthetic constructs containing the complementarity determining regions of an antibody, an Fc antibody fragment thereof, a single chain Fv antibody fragment (scFv), a synthetic antibody or chimeric antibody construct which shares sufficient complementarity-defining regions (CDR) to retain functionally equivalent binding characteristics of an antibody that binds a desired cell surface receptor, and a binding
- 35 fragment produced by phage display. Certain classes have subclasses as well, such as IgG1, IgG2, and others. Furthermore, in humans, the light chain can be a kappa chain or a lambda

chain. Monoclonal antibodies are antibodies that are made by identical immune cells that are all clones of a unique parent cell. Antibodies may bind to an epitope on CNGB1 and thereby either block binding of cAMP to CNGB1, i.e. inhibiting its activity. Further, the antibody might stabilize an inactive conformation of CNGB1, thereby preventing its action.

- 5 **[0044]** Inhibitors of CNGB1 may also be muteins or other binding molecules such as DARPins, nucleic acid aptamers, affimers, affibodies, affilins, affitins (nanofitins), alphabodies, anticalins, avimers, fynomers, Kunitz domain peptides or monobodies.

[0045] "Nucleic acids" as used herein, relate to nucleic acids that make use of RNA interference (RNAi). RNA interference is a biological process in which RNA molecules inhibit gene
10 expression or translation, by neutralizing targeted mRNA molecules. In the context of the present invention, the nucleic acid is targeted against proteins of the CNG channel, preferably targeted against CNGB1, thereby reducing its expression. The reduced expression of CNGB1 thereby reduces the availability of CNGB1. With less available CNGB1, the ionic current conducted by the CNG channel comprising CNGB1 is reduced, leading to an inhibition of the
15 pain response. Typically, a nucleotide as used herein has a length of 10, 20, 30, 40 or 50 nucleotides.

[0046] A "small molecule" is an organic chemical compound with a molecular weight of 900 Dalton or less, preferably 500 Dalton or less. In the context of the present invention, the small molecule is an inhibitor of CNGB1 for use in a method for the prophylaxis and/or
20 treatment of neuropathic pain in a subject. In general, any small molecule may be an inhibitor if an assay as described in the definition of an inhibitor shows a reduced activity of CNGB1.

[0047] In a preferred embodiment the inhibitor of CNGB1 is a benzodiazepine derivative, which is preferably able to reduce the current of ions conducted by a CNG complex comprising CNGB1. "Derivatives" as used herein, are compounds structurally related to, and derivable
25 from, the parent structure, such as salts, esters, and the like. Benzodiazepine derivatives include chemical modifications, for instance different or additional side groups. The derivatives intended for the use according to the present invention are preferably pharmaceutically acceptable, i.e. capable of eliciting the desired therapeutic effect without causing any undesirable local or systemic effects in the recipient.

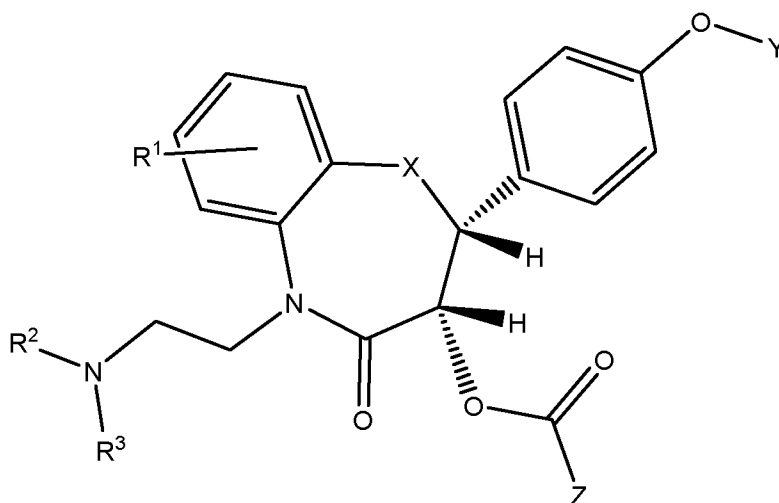
30 **[0048]** As used herein and throughout the entire description, the term "alkyl" refers to a monoradical of a saturated straight or branched hydrocarbon. Preferably, the alkyl group comprises from 1 to 6 carbon atoms, i.e., 1, 2, 3, 4, 5 or 6 carbon atoms, preferably 1 to 3 carbon atoms. In some embodiments, the alkyl group employed contains 1-5 carbon atoms ((C₁-C₅)alkyl). In some embodiments, the alkyl group employed contains 1-4 carbon atoms ((C₁-
35 C₄)alkyl). In some embodiments, the alkyl group employed contains 1-3 carbon atoms ((C₁-C₃)alkyl). In some embodiments, the alkyl group employed contains 1-2 carbon atoms ((C₁-

C₂)alkyl). In some embodiments, the alkyl group employed is methyl. Examples of alkyl radicals include, but are not limited to, methyl, ethyl, propyl, iso-propyl, butyl, iso-butyl, tert-butyl, n-pentyl, iso-pentyl, sec-pentyl, neo-pentyl, 1,2-dimethyl-propyl, iso-amyl, n-hexyl, iso-hexyl, sec-hexyl and the like.

5 **[0049]** As used herein and throughout the entire description, the term "alkenyl" refers to a monoradical of an unsaturated straight or branched hydrocarbon having at least one carbon-carbon double bond. Preferably, the alkenyl group comprises from 2 to 6 carbon atoms, i.e., 2, 3, 4, 5, or 6 carbon atoms, more preferably 2 to 3 carbon atoms. In some embodiments, the alkenyl group contains 2-6 carbons ((C₂-C₆)alkenyl). In some embodiments, the alkenyl group
10 contains 2-5 carbons ((C₂-C₅)alkenyl). In some embodiments, the alkenyl group contains 2-4 carbons ((C₂-C₄)alkenyl). In some embodiments, the alkenyl group contains 2-3 carbons ((C₂-C₃)alkenyl). In some embodiments, the alkenyl group contains 2 carbons ((C₂)alkenyl). The carbon-carbon double bond(s) may be in cis (Z) or trans (E) configuration. Exemplary alkenyl groups include vinyl, 1-propenyl, 2-propenyl (i.e., allyl), 1-butenyl, 2-butenyl, 3-butenyl, 1-pentenyl, 2-pentenyl, 3-pentenyl, 4-pentenyl, 1-hexenyl, 2-hexenyl, 3-hexenyl, 4-hexenyl, 5-hexenyl, and the like.

[0050] The term "alkynyl" refers to a monoradical of an unsaturated straight or branched hydrocarbon having at least one carbon-carbon triple bond. Preferably, the alkynyl group comprises from 2 to 6 carbon atoms, i.e., 2, 3, 4, 5, or 6 carbon atoms, more preferably 2 to 3
20 carbon atoms. Exemplary alkynyl groups include ethynyl, 1-propynyl, 2-propynyl, 1-butylnyl, 2-butylnyl, 3-butylnyl, 1-pentylnyl, 2-pentylnyl, 3-pentylnyl, 4-pentylnyl, 1-hexynyl, 2-hexynyl, 3-hexynyl, 4-hexynyl, 5-hexynyl, and the like.

[0051] Derivatives of benzodiazepines may be derived from structure 1. According to the invention, the benzodiazepine derivative is preferably characterized by the following structure 1:



1

wherein X is O or S; Y, R², R³ and Z are each independently selected from the group consisting of hydrogen, (C₁-C₆)alkyl, (C₂-C₆)alkenyl and (C₂-C₆)alkynyl; and R¹ is selected from the group consisting of hydrogen, a nitro group, halogen, -O-(C₁-C₆)alkyl, -S-(C₁-C₆)alkyl and a cyano group. Preferably, X is S. Preferably, R¹ is hydrogen. As appreciated by a person skilled in the art, in case R¹ is hydrogen the benzene ring is unsubstituted. Halogen is fluorine, chlorine, bromine or iodine, preferably fluorine or chlorine.

[0052] In an also preferred embodiment of the invention, the small molecule inhibitor is **1**, wherein X is O or S; Y, R², R³ and Z are each (C₁-C₆)alkyl; and R¹ is selected from the group consisting of hydrogen, a nitro group, halogen, -O-(C₁-C₆)alkyl, -S-(C₁-C₆)alkyl and a cyano group. Preferably, X is S. Preferably, R¹ is hydrogen. Halogen is fluorine, chlorine, bromine or iodine, preferably fluorine or chlorine.

[0053] In a more preferred embodiment of the invention, the small molecule inhibitor is **1**, wherein X is O or S; Y, R², R³ and Z are each independently selected from the group consisting of hydrogen, (C₁-C₃)alkyl, (C₂-C₃)alkenyl or (C₂-C₃)alkynyl; and R¹ is selected from the group consisting of hydrogen, a nitro group, halogen, -O-(C₁-C₃)alkyl, -S-(C₁-C₃)alkyl and a cyano group. Preferably, X is S. Preferably, R¹ is hydrogen. Halogen is fluorine, chlorine, bromine or iodine, preferably fluorine or chlorine.

[0054] In an also more preferred embodiment of the invention, the small molecule inhibitor is **1**, wherein X is O or S; Y, R², R³ and Z are each (C₁-C₃)alkyl; and R¹ is selected from the group consisting of hydrogen, a nitro group, halogen, -O-(C₁-C₃)alkyl, -S-(C₁-C₃)alkyl and a cyano group. Preferably, X is S. Preferably, R¹ is hydrogen. Halogen is fluorine, chlorine, bromine or iodine, preferably fluorine or chlorine.

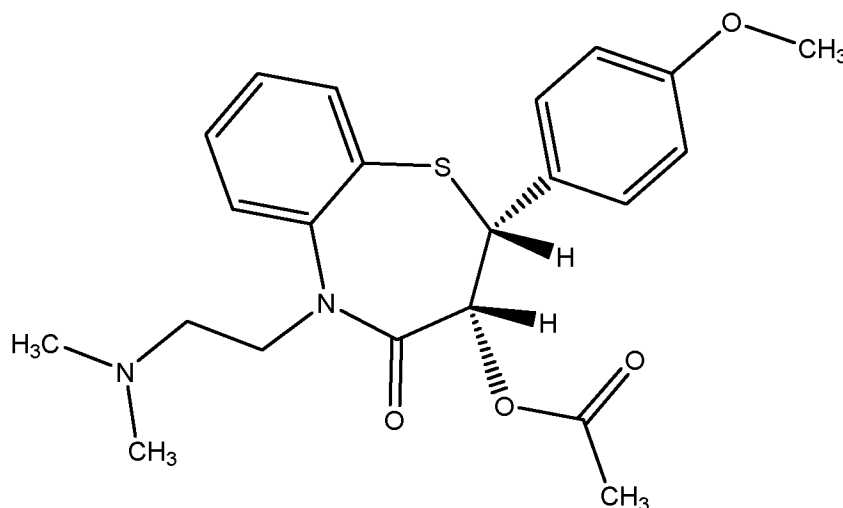
[0055] In another preferred embodiment of the invention, the small molecule inhibitor is **1**, wherein X is O or S; Y, R², R³ and Z are each independently selected from the group consisting of hydrogen and methyl; and R¹ is selected from the group consisting of hydrogen, a nitro group, halogen and a cyano group. Preferably, X is S. Preferably, R¹ is hydrogen. Halogen is fluorine, chlorine, bromine or iodine, preferably fluorine or chlorine.

[0056] In another preferred embodiment of the invention, the small molecule inhibitor is **1**, wherein X is O or S; Y, R², R³ and Z are each independently selected from the group consisting of hydrogen, (C₁-C₃)alkyl, (C₂-C₃)alkenyl and (C₂-C₃)alkynyl; and R¹ is hydrogen. Preferably, X is S. Preferably, Y, R², R³ and Z are each (C₁-C₃)alkyl.

[0057] In another preferred embodiment, the small molecule inhibitor is **1**, wherein X is S; Y, R², R³ and Z are each methyl; and R¹ is selected from the group consisting of hydrogen, a nitro group, halogen and a cyano group. Preferably, R¹ is hydrogen. Halogen is fluorine, chlorine, bromine or iodine, preferably fluorine or chlorine.

[0058] In an even more preferred embodiment, the small molecule inhibitor is **1**, wherein X is S; Y, R², R³ and Z are each methyl; and R¹ is selected from the group consisting of chlorine or fluorine.

[0059] In the most preferred embodiment, the small molecule inhibitor is **1**, wherein X is S; Y, R², R³ and Z are each methyl; and R¹ is hydrogen. This structure is also called L-cis-diltiazem ((2R,3R)-(-)-cis-3-Acetoxy-5-(2-dimethylaminoethyl)-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one) and is characterized by structure **2**.

**2**

[0060] Another group of preferred small molecule inhibitors of CNGB1 are cyclic nucleotide derivatives. A “cyclic nucleotide” (cNMP) is a single-phosphate nucleotide with a cyclic bond arrangement between the sugar and phosphate groups. Like other nucleotides, cyclic nucleotides are composed of three functional groups: a sugar, a nitrogenous base, and a single phosphate group. The 'cyclic' portion consists of two bonds between the phosphate group and the 3' and 5' hydroxyl groups of the sugar, very often a ribose. As 3',5'-cyclic adenosine monophosphate (cAMP) is an activator of CNGB1 (see Fig. 6A), it is itself not envisioned in this invention. However, mice with a knockout of CNGB1 showed an ameliorated pain behavior. Thus, derivatives of cAMP that bind to CNGB1 without activating CNGB1 are inhibitors of CNGB1 because they may block the binding site for the activator cAMP in a competitive fashion and thereby ameliorate the pain response.

[0061] All inhibitors of CNGB1 may also be available as a pharmaceutically acceptable salt.

[0062] “Neuropathic pain” as used herein is a category of pain that includes several forms of chronic pain and which results from dysfunction of nervous rather than somatic tissue. Neuropathic pain, that is pain deriving from dysfunction of the central or peripheral nervous system, may also be a consequence of damage to peripheral nerves or to regions of the central nervous system, may result from disease, or may be idiopathic.

[0063] Neuropathic pain may be caused by a lesion or a disease of the somatosensory nervous system. Preferably, neuropathic pain according to the present invention is caused by a lesion or a disease of the somatosensory nervous system. Neuropathic pain can be associated in general with allodynia (triggering of a pain response by a normally not painful stimulus),
5 hyperalgesia (increased sensitivity to pain), dysesthesia (unpleasant, abnormal sense of touch), hyperpathia (pain stimuli evoke exaggerated levels of pain), paresthesia (abnormal sensation such as tingling, tickling, pricking, numbness or burning of a person's skin with no apparent physical cause) and/or spontaneous pain (pain without apparent reason). Preferably, the neuropathic pain according to the present invention is allodynia, hyperalgesia and/or
10 spontaneous pain.

[0064] Neuropathic pain may in general occur on any part of the nervous system. However, CNG channels are highly expressed in sensory neurons. Therefore, in a preferred embodiment of the invention, a sensory neuron is affected.

[0065] For the purposes of the present invention, it may be advantageous if a subject suffering
15 from or experiencing neuropathic pain may be diagnosed whether CNGB1 is higher expressed in DRG (dorsal root ganglia) neurons in comparison to a subject not suffering from or experiencing neuropathic pain. Preferably, it is envisioned that the present invention also relates to a method of diagnosing whether in a sample obtained from a subject CNGB1 is higher expressed than in a sample from a subject not suffering from or experiencing neuropathic pain.
20 A higher expression of CNGB1 may thus be indicative of neuropathic pain. Advantageously, such a subject having a higher expression of CNGB1 may additionally be diagnosed as to whether he or she has neuropathic pain in accordance with means and methods known in the art.

[0066] In general there are many different causes for neuropathic pain and more precisely for a
25 lesion or a disease of the somatosensory nervous system. The lesion or disease of the somatosensory nervous system may be caused by a trauma, compression of a nerve, a surgery, a viral infection, pharmacotherapy, and/or retroviral treatment.

[0067] A "trauma" is an injury caused by a powerful stroke or percussion against a part of the body. This might cause injuries of the nervous system like lesions of the nervous system
30 leading to the onset of neuropathic pain. "Compression of a nerve" as used herein, refers to the compression of a nerve, wherein the compression is characterized by a pressure on the nerve. A "surgery" is a medical technique that uses operative manual and instrumental techniques on a patient to investigate or treat a pathological condition such as disease or injury, to help improve bodily function or appearance or to repair unwanted ruptured areas. By performing a surgery,
35 nerves might be harmed, also leading to the onset of neuropathic pain.

[0068] A “viral infection” as used herein relates to any infection of a subject by a virus. In a preferred embodiment of the invention, the virus is selected from the group consisting of herpes virus, human immunodeficiency virus (HIV) and papilloma virus. Those viruses are known for their ability to induce neuropathic pain.

5 **[0069]** The term “pharmacotherapy” in general means any therapy using pharmaceutical drugs, as distinguished from therapy using surgery (surgical therapy), radiation (radiation therapy), movement (physical therapy), or other modes. A pharmacotherapy targeting cancer, i.e. comprising an anti-cancer agent, is also known to induce neuropathic pain. Therefore, in a preferred embodiment of the invention, the neuropathic pain is caused by a pharmacotherapy,
10 comprising an anti-cancer agent. In a more preferred embodiment, the anti-cancer agent is selected from the group including but not limited to cisplatin, oxaliplatin, paclitaxel, docetaxel, nocodazole, thalidomide, thiouracile, vincristine, vindesine, vinblastine, and vinorelbine.

[0070] “Anti-retroviral treatment” as used herein relates to a treatment with the aim to ameliorate a retroviral infection. A preferred retroviral infection is a lentiviral infection and more
15 preferred an HIV infection. An anti-retroviral treatment comprises an anti-retroviral agent. Preferred anti-retroviral agents according to the present invention are reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors, CCR5 antagonists or fusion inhibitors.

[0071] Preferably, the lesion or disease of the somatosensory nervous system is associated with sciatica, hereditary neuropathy (including but not limited to Friedreich ataxia, familial
20 amyloid polyneuropathy, Tangier disease, Morbus Charcot-Marie-Tooth types 2B or 5, Fabry disease), metabolic disorders (including but not limited to diabetes, renal insufficiency and hypothyroidism), vitamin deficiencies (including but not limited to vitamin B12 deficiency, vitamin B6 deficiency, and vitamin E deficiency), toxic and iatrogenic neuropathies (including but not limited to alcoholism, vitamin B6 intoxication, hexacarbon intoxication, amiodarone,
25 chloramphenicol, disulfiram, isoniazide, gold, lithium, metronidazole, misonidazole, nitrofurantoin), infectious neuropathies (including but not limited to leprosy and Lyme disease), auto-immune neuropathies (including but not limited to Guillain-Barre syndrome, chronic inflammatory de-myelinating polyneuropathy, monoclonal gammopathy of undetermined significance, and polyneuropathy), trigeminal neuralgia, entrapment syndromes (including but
30 not limited to Carpel tunnel), post-traumatic neuralgia, phantom limb pain, multiple sclerosis pain, complex regional pain syndromes (including but not limited to reflex sympathetic dystrophy and causalgia), neoplasia, vasculitic/angiopathic neuropathy, and/or idiopathic neuropathy.

[0072] The “subject”, which may be treated by the inhibitors, in particular CNBG1 inhibitors, or combinations of inhibitors preferably is a vertebrate. In the context of the present invention the
35 term “subject” includes an individual in need of a treatment of neuropathic pain. Preferably, the subject is a patient suffering from neuropathic pain. Preferably, the subject is a vertebrate, more

preferably a mammal. Mammals include, but are not limited to, farm animals, sport animals, pets, primates, mice and rats. Preferably, a mammal is a human, dog, cat, cow, pig, mouse, rat etc., particularly preferred, it is a human. In some embodiments, the subject is a human subject, which optionally is more than 1 year old and less than 14 years old; between the ages of 50 and 5 65, or older than 65 years of age. In other embodiments the subject is a human subject, which is selected from the group consisting of subjects who are at least 50 years old, subjects with less than 14 years of age, subjects between 6 months and 18 years of age.

[0073] The inhibitor of CNGB1 for use according to the present invention may not only be administered to a subject alone but also in a combination with one or more additional 10 neuropathic pain reducing agents to improve the treatment. Typical anti-neuropathic agents are comprised in the following groups of substances: calcium channel anticonvulsants (gabapentine, pregabalin), tricyclic antidepressants (amitriptyline, clomipramine, imipramine), serotonin noradrenalin-reuptake inhibitors (duloxetine, venlafaxine), sodium channel anticonvulsants (carbamazepine), opioids (tramadol, morphine, oxycodone), topic local 15 anesthetics (lidocaine plasters) and/or topic capsaicin. Preferred members of the groups are mentioned in brackets.

[0074] In the use of the invention, an inhibitor of CNGB1 may be administered orally, intravenously, intrapleurally, transdermally, transmucosally, subcutaneously, intramuscularly, topically or via inhalation. Preferably, the inhibitor of CNGB1 is administered orally or 20 intravenously.

[0075] The inhibitor or inhibitors are preferably administered in a therapeutically effective amount. The "therapeutically effective amount" for an CNGB1 inhibitor or each active compound/inhibitor can vary with factors including but not limited to the activity of the compound used, stability of the active compound in the patient's body, the severity of the conditions to be 25 alleviated, the total weight of the patient treated, the route of administration, the ease of absorption, distribution, and excretion of the compound by the body, the age and sensitivity of the patient to be treated, adverse events, and the like, as will be apparent to a skilled artisan. The amount of administration can be adjusted as the various factors change over time.

[0076] The inhibitor of CNGB1 and more or more additional anti-neuropathic pain agents for the 30 use of present invention can be administered in any suitable unit dosage form. Suitable oral formulations can be in the form of tablets, capsules, suspension, syrup, chewing gum, wafer, elixir, and the like. Pharmaceutically acceptable carriers such as binders, excipients, lubricants, and sweetening or flavoring agents can be included in the oral pharmaceutical compositions. If desired, conventional agents for modifying tastes, colors, and shapes of the special forms can 35 also be included.

[0077] For injectable formulations, the inhibitor of CNGB1 and more or more additional anti-neuropathic pain agents can be in lyophilized powder in a mixture with suitable excipients in a suitable vial or tube. Before use in the clinic, the drugs may be reconstituted by dissolving the lyophilized powder in a suitable solvent system for form a composition suitable for intravenous or intramuscular injection.

[0078] In one embodiment, the inhibitor of CNGB1 and more or more additional anti-neuropathic pain agents can be in an orally administrable form (e.g., tablet or capsule or syrup etc.) with a therapeutically effective amount (e.g., from 0.1 mg to 2000 mg, 0.1 mg to 1000 mg, 0.1 mg to 500 mg, 0.1 mg to 500 mg, 0.1 mg to 200 mg, 30 mg to 300 mg, 0.1 mg to 75 mg, 0.1 mg to 30 mg).

[0079] The present invention also relates to methods of prophylaxis and/or treatment of neuropathic pain, comprising administration an inhibitor of CNGB1 to a subject.

[0080] The present invention further relates to the use of an inhibitor of CNGB1 for the preparation of a medicament for the prophylaxis and/or treatment of neuropathic pain.

[0081] The embodiments and disclosure in the context of a CNGB1 inhibitor for use in a method for the prophylaxis and/or treatment of neuropathic pain apply also to methods of treatment or second medical uses, mutatis mutandis.

[0082] The amount of an inhibitor of CNGB1 is preferably a therapeutically or prophylactically efficient amount.

[0083] It is noted that as used herein, the singular forms "a", "an", and "the", include plural references unless the context clearly indicates otherwise. Thus, for example, reference to "a reagent" includes one or more of such different reagents and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

[0084] Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

[0085] The term "and/or" wherever used herein includes the meaning of "and", "or" and "all or any other combination of the elements connected by said term".

[0086] The term "less than" or in turn "more than" does not include the concrete number.

[0087] For example, less than 20 means less than the number indicated. Similarly, more than or greater than means more than or greater than the indicated number, i.e. more than 80 % means more than or greater than the indicated number of 80 %.

5 [0088] Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. When used herein the term “comprising” can be substituted with the term “containing” or “including” or sometimes when used herein with the term “having”. When used herein “consisting of” excludes any element,
10 step, or ingredient not specified.

[0089] The term “including” means “including but not limited to”. “Including” and “including but not limited to” are used interchangeably.

[0090] It should be understood that this invention is not limited to the particular methodology, protocols, material, reagents, and substances, etc., described herein and as such can vary. The
15 terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[0091] All publications cited throughout the text of this specification (including all patents, patent application, scientific publications, instructions, etc.), whether supra or infra, are hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission
20 that the invention is not entitled to antedate such disclosure by virtue of prior invention. To the extent the material incorporated by reference contradicts or is inconsistent with this specification, the specification will supersede any such material.

[0092] The content of all documents and patent documents cited herein is incorporated by reference in their entirety.

25 [0093] A better understanding of the present invention and of its advantages will be had from the following examples, offered for illustrative purposes only. The examples are not intended to limit the scope of the present invention in any way.

EXAMPLES OF THE INVENTION

[0094] The following examples illustrate the invention. These examples should not be construed
30 as to limit the scope of the invention. The examples are included for purposes of illustration and the present invention is limited only by the claims.

Example 1: Material and methods

Animals

[0095] Experiments were performed in mice deficient for CNGB1 (CNGB1^{-/-}) (Huttl et al., 2005) backcrossed for > 10 generations on a hybrid 129SvJ and C57BL/6N background. CNGB1^{-/-} mice are viable and fertile but exhibit impaired olfaction, impaired vision, and increased postnatal mortality, which is particularly high under heterozygous breeding conditions (Michalakis S, JBC 2006). Therefore, age- and sex-matched CNGB1^{-/-} and wild type (WT) mice derived from homozygous breedings were used. Behavioral experiments with L-cis-diltiazem administration and some tissue expression studies were performed in male C57BL/6N mice obtained from Charles River (Sulzfeld, Germany). Animals were housed on a 12 h light/dark cycle with access to food and water ad libitum. All behavioral studies were carried out by observers blinded for genotype and treatment of the animals. All experiments were approved by the local Ethics Committee for Animal Research and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Behavioral tests

[0096] Rotarod test. A rotarod treadmill for mice (Ugo Basile, Varese, Italy) was used to assess motor coordination, running at a constant rotating speed of 12 rpm. After several training sessions fall-off latencies from 3-5 tests were averaged. The cut-off time was set at 120 s.

[0097] Dynamic plantar test. Mechanical sensitivity of the hindpaw was analyzed using a Dynamic Plantar Aesthesiometer (Ugo Basile). The device pushes a thin steel rod against the plantar surface of the paw from beneath with a constantly increasing force from 0 to 5 g in 10 s (ramp 0.5 g/s) and then holds 5 g for an additional 10 s (Heine et al., 2011; Kallenborn-Gerhardt et al., 2012). The latency time at which the animal withdraws the paw is recorded automatically. Paw withdrawal latency was calculated as the mean of 4-6 measurements with at least 20 s in between.

[0098] Neuropathic pain. To investigate neuropathic pain behavior, the spared nerve injury (SNI) model (Decosterd and Woolf, 2000) and a modified version of the chronic constriction injury (CCI) model were used (Bennett and Xie, 1988). For the SNI model, two branches of the sciatic nerve were ligated and cut distally leaving the sural nerve intact. For CCI, the common sciatic nerve was exposed unilaterally at the mid-thigh level by blunt dissection. Proximal to the trifurcation of the sciatic nerve, ~5 mm of nerve was freed of adhering tissue, and three silk ligatures (6-0) that constricted the nerve by ~30–50% were tied around it with ~1 mm spacing (Tegeder et al., 2006; Schmidtke et al., 2008a; Kallenborn-Gerhardt et al., 2012). Mechanical sensitivity of the affected paw was assessed using the dynamic plantar test.

[0099] Inflammatory pain. Twenty microliters of Complete Freund's Adjuvant (CFA; containing 1 mg/ml heat-killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monooleate; Sigma-Aldrich) were injected into the plantar subcutaneous space of a hindpaw

(Ferreira et al., 2001). Mechanical sensitivity of the affected paw was assessed using the dynamic plantar test.

[00100] Intraplantar drug injection. Intraplantar injection of L-cis-diltiazem (Sigma-Aldrich) was performed 14 days after SNI surgery. L-cis-diltiazem (40 µg in a volume of 20 µl) was dissolved in saline and injected subcutaneously into the plantar surface of the SNI-affected hindpaw. Paw-withdrawal latencies were measured using the dynamic plantar test every 20-60 min over a period of 6 h.

[00101] Intrathecal drug-induced pain. Intrathecal (i.t.) delivery of drugs was performed by direct lumbar puncture in awake, conscious mice as described previously (Kallenborn-Gerhardt et al., 2013; Lu and Schmidtke, 2013). The cyclic nucleotides, 8-Bromoadenosine-3',5'-cyclic monophosphorothioate, Sp-isomer (Sp-8-Br-cAMPS) and para-Chlorophenylthioguanosine-3',5'-cyclic monophosphate (8-pCPT-CGMP; both from Biolog, Bremen, Germany), were dissolved in sterile 0.9% NaCl and were i.t. administered in a volume of 5 µl. Mechanical sensitivity of both hindpaws was assessed using the dynamic plantar test.

Real-time RT-PCR

[00102] Total RNA of lumbar DRGs (L4-L5) was extracted under RNase-free conditions using a RNAqueous Micro Kit (Ambion/Life Technologies, Darmstadt, Germany). Genomic DNA contaminations were reduced by treating samples with DNase for 15 min. Reverse transcription was performed using a Verso Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed with an ABI Prism 7500 Sequence Detection System (Applied Biosystems/Life Technologies, Darmstadt, Germany) using TaqMan Gene Expression Assays for murine CNGA1 (Mm00833234_m1), CNGA2 (Mm00432614_m1), CNGA3 (Mm00802288_m1), CNGA4 (Mm01278645_m1), CNGB1 (Mm01249077_m1), CNGB1a (Mm01249065_m1), CNGB3 (Mm00489232_m1), TRPV1 (Mm01246302_m1), TRPV2 (Mm00449223_m1), TRPV4 (Mm00499025_m1), TRPA1 (Mm01227437_m1), TRPM8 (Mm01299593_m1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm99999915_g1), all purchased from Applied Biosystems/Life Technologies. Reactions were performed in duplicate or triplicate by incubating for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, including water controls to ensure specificity. Relative gene expression levels were calculated using the comparative 2-ΔΔCt method and normalized to GAPDH.

Immunohistochemistry

[00103] Immunofluorescent staining of dorsal root ganglia (L4-L5) was performed as described previously (Heine et al., 2011; Kallenborn-Gerhardt et al., 2012). Briefly, cryostat sections were permeabilized, blocked in 10% normal goat or donkey serum and 3% bovine serum albumin (BSA) in PBS, and incubated with primary antibodies overnight at 4 °C. The

following antibodies were used: mouse anti-neurofilament 200 (NF200; 1:1000; Sigma Aldrich, # N0142), mouse-anti-peripherin (1:200; Chemicon, # MAB1527), and rat anti-substance P (SubP; 1:200; BD # 556312). After rinsing in PBS, slides were incubated with secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 555 (Invitrogen/Life Technologies), Cy3 or Cy5 (Sigma-Aldrich) for 2 h at room temperature. For staining with Griffonia simplicifolia isolectin B4 (IB4), sections were incubated with Alexa Fluor 488-conjugated IB4 (Invitrogen/Life Technologies; 10 µg/ml in 0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.1 mM MnCl₂, and 0.2% Triton in PBS) for 2 h at room temperature. Finally, lipofuscin-like autofluorescence was reduced by incubating in 0.06% Sudan black B in 70% ethanol for 5 min (Schnell et al., 1999; Schmidtke et al., 2008), and slides were rinsed in PBS and coverslipped in Fluoromount G (Southern Biotech, Birmingham, USA). Images were taken using a Nikon Eclipse Ni-U microscope (Nikon, Japan) and a monochrome DS-Qi2 camera (Nikon). Final adjustment of contrast and intensity was done using Adobe Photoshop software.

In situ hybridization

[00104] Mice were perfused with 4% formaldehyde (PFA) in PBS for 5 min. DRGs were dissected and post-fixed in the same fixative for 10 minutes. Tissue was then incubated in 20% sucrose in PBS overnight, embedded in mounting media (Fluoromount G, Southern Biotech, Birmingham, US), and cryostat sections were cut at a thickness of 16 µm. In situ hybridization (ISH) was performed using an Affymetrix QuantiGene ViewRNA Tissue Assay in which target mRNA signals appear as puncta in microscopy (Solorzano et al., 2015; Osteen et al., 2016). Experiments were performed according to the instructions of the manufacture and included extensive washing steps in Wash Buffer. Briefly, sections were fixed in 4% PFA overnight, dehydrated for 10 min in a 50%, 70% and 100% ethanol series, respectively, dried for 30 min at 60 °C in a Thermobrite slide processing system (Abbott Molecular, Illinois), digested with the Protease QF Mix for 20 min, fixed again in 4% PFA for 5 min, and hybridized with hybridization probes for mouse CNGB1 (accession NM_145601, type 1 probe set, catalog # VB1-17745, Affymetrix) over night at 40 °C (Thermobrite). Then sections were consecutively incubated with PreAmplifier Mix QT, Amplifier Mix QT, an alkaline phosphatase labeled probe against the Amplifier, AP Enhancer Solution, and Fast Red Substrate (Cy3 fluorescence). Finally, sections were mounted with Vectorshield HardSet Antifade Mounting Medium with DAPI or further processed for subsequent immunohistochemistry. In immunohistochemical experiments after in situ hybridization, sections were rinsed in PBS, blocked for 60 min in 10% normal serum and 3% BSA in PBST, incubated with primary antibodies overnight, and further processed as described above.

Cell counting

[00105] For quantification of CNGB1-positive DRG neuron populations, 17 sections derived from 4 mice with at least 150 DRG neurons per animal were analyzed. The percentage

of CNGB1-positive cells versus total neuronal profiles (visualized by green basal fluorescence of DRG neurons) and the percentage of double-labeled neurons (marker and CNGB1) versus total number of CNGB1-positive cells were calculated. For quantification of DRG neuron subpopulations in CNGB1^{-/-} and WT mice, at least 3 sections from 3 mice each were analyzed and the percentage of marker-positive cells was calculated. Only DRG neurons showing CNGB1 or marker staining clearly above background level and containing nuclei were included.

Statistics

[00106] SPSS or GraphPad Prism 5 software was used for statistical analysis. Data for mRNA expression were normalized to baseline (naive animals) and analyzed by one-way ANOVA with Bonferroni post hoc test. For paired and multiple comparisons the two-way repeated-measures ANOVA followed by a Bonferroni post hoc test was used. Rotarod fall-off latencies were analyzed with the Mann-Whitney U-test and are presented as median and interquartile range. Other data are expressed as the mean $\pm$ standard error of the mean (SEM). For all tests, a probability value $P < 0.05$ was considered as statistically significant.

Example 2: Altered CNG channel expression in dorsal root ganglia after peripheral nerve injury

[00107] To assess a potential contribution of CNG channels to neuropathic pain processing, the expression levels of the 6 known CNG channel subunits (CNGA1-4, CNGB1, and CNGB3) in DRGs of naive mice and after spared nerve injury (SNI), a standard model of neuropathic pain (Decosterd and Woolf, 2000) were analyzed. In naive mice, quantitative RT-PCR experiments detected, albeit at low expression levels, CNGA2, CNGA3, CNGA4 and CNGB1, but not CNGA1 and CNGB3. The relative mRNA expression was 0.44 ± 0.07 (CNGA2), 0.69 ± 0.06 (CNGA3), 0.58 ± 0.09 (CNGA4), and 1.00 ± 0.16 (CNGB1). Notably, expression of CNGA2, CNGA4 and CNGB1 was significantly upregulated at different time points after SNI, whereas expression of CNGA3 was decreased (Fig. 1). In accordance to the results obtained from naive mice, CNGA1 and CNGB3 were not detected after SNI.

[00108] The downregulation of CNGA3 mRNA is in line with a previous study that revealed a limited function of CNGA3 in SNI-induced neuropathic pain (Heine et al., 2011). On the other hand, the upregulation of CNGA2, CNGA4 and CNGB1 mRNA points to a contribution of CNG channels containing these subunits to neuropathic pain processing. Interestingly, CNGA2, CNGA4 and CNGB1b (a splice variant of CNGB1 that lacks the N-terminal domain present in the longer splice variant CNGB1a splice variant) assemble to a functional channel in olfactory sensory neurons (Zheng et al., 2002). As the CNGB1 gene expression assay (exon boundary 29-30) of the screening experiments cannot differentiate between CNGB1a and CNGB1b, RT-PCR experiments were performed additionally using a CNGB1a-specific gene expression assay (exon boundary 1-2). In these experiments CNGB1a in DRGs of naive mice

or after SNI (data not shown) was not detected. Hence, the data suggest that an 'olfactory sensory neuron-type' CNG channel consisting of CNGA2, CNGA4 and CNGB1b subunits in DRGs might be involved in neuropathic pain processing after peripheral nerve injury.

Example 3: CNGB1 distribution in dorsal root ganglia

5 **[00109]** Considering that i) CNGB1 showed higher relative mRNA expression in DRGs as compared to other CNG subunits, ii) the SNI-induced upregulation of CNGB1 was more pronounced as compared to CNGA2 and CNGA4, and iii) CNGB1 knockout mice are readily available (Huttl et al., 2005), CNGB1 was focused in the remainder of the study. Using fluorescent in situ hybridization the cellular distribution of CNGB1 mRNA in DRGs was
10 investigated. CNGB1 mRNA was expressed in $45.6 \pm 4.7\%$ of total neuronal profiles (Fig. 2). To estimate the localization of CNGB1 in DRG neuron subpopulations, in situ hybridization of CNGB1 mRNA were combined with immunostaining for established neuronal markers. It was observed that $52.6 \pm 2.8\%$ of CNGB1-positive neurons were positive for NF200, a marker of large-diameter, myelinated DRG neurons (Fig. 2A and B). Furthermore, $40.0 \pm 7.3\%$ of
15 CNGB1-positive neurons expressed peripherin, which labels the small-diameter, unmyelinated C-fiber DRG cell population (Fig. 2C and D). These data suggest that CNGB1 is expressed in both unmyelinated and myelinated DRG neurons.

Example 4: Ameliorated neuropathic pain behavior in CNGB1^{-/-} mice

[00110] Next, the functional contribution of CNG channels to neuropathic pain processing
20 in vivo was assessed using CNGB1^{-/-} mice. The expression of other CNG subunits in DRGs of CNGB1^{-/-} mice did not significantly differ from that in WT mice (Fig. 3A), suggesting that there is no compensatory regulation due to the lack of CNGB1. The percentage of DRG neuron subpopulations positive for established markers (NF200, isolectin B4 and substance P) were comparable in CNGB1^{-/-} and WT mice (Fig. 3B), indicating that there are no developmental
25 defects affecting DRG neuron subpopulations in CNGB1^{-/-} mice. Furthermore, the motor coordination was not impaired in CNGB1^{-/-} mice, as analyzed in the rotarod test (median fall-off latencies: CNGB1^{-/-} mice, 107 s [interquartile range 83.75–119.5 s]; WT mice, 96.42 s [interquartile range 65–118.75 s]; $p = 0.59$; $n = 10$).

[00111] The neuropathic pain behavior of CNGB1^{-/-} and WT mice was then investigated
30 in the SNI model. Under baseline conditions, mechanical sensitivity of the hindpaws were similar in both genotypes, as measured using a dynamic plantar aesthesiometer (paw withdrawal latencies: CNGB1^{-/-} mice, 8.6 ± 0.2 s; WT mice, 9.0 ± 0.2 s; $p = 0.18$; $n = 19-21$). After SNI, mechanical hypersensitivity of the affected hindpaw developed in mice of both genotypes (Fig. 4A). Interestingly however, the hypersensitivity was significantly ameliorated in
35 CNGB1^{-/-} mice as compared to WT mice at all tested time points (3-21 days after SNI; Fig. 4A).

These data suggest that CNGB1 contributes to the induction and maintenance of neuropathic pain processing after SNI.

[00112] The behavior of CNGB1^{-/-} mice was also tested in a second model of peripheral nerve injury, the chronic constriction injury (CCI) model. Similar to SNI, considerably reduced hindpaw hypersensitivity in CNGB1^{-/-} mice as compared to WT mice did occur after CCI (Fig. 4B), confirming that the observed phenotype is consistent across different models. Together, these data suggest that CNGB1-containing CNG channels contribute to the processing of neuropathic pain after peripheral nerve injury.

Example 5: Ameliorated neuropathic pain behavior after CNGB1 inhibition

[00113] Whether pharmacological inhibition of CNGB1 can ameliorate neuropathic pain once it has already established was investigated next. For that purpose, C57BL/6N mice were subjected to SNI surgery to induce mechanical hypersensitivity. Fourteen days after SNI, when mechanical hypersensitivity was fully developed, mice were treated with L-cis-diltiazem, a potent inhibitor of CNGB1-positive channels (Chen et al., 1993). Notably, intraplantar injection of L-cis-diltiazem (40 µg) ameliorated the neuropathic pain behavior as compared to vehicle treated control animals (Fig. 4C). Significant differences between groups occurred during 60 to 120 min after drug administration. Importantly, motor coordination, as assessed with the rotarod test, was not impaired at 60, 120 and 180 min after intraplantar injection of 40 µg L-cis-diltiazem (all mice reached the 120 s cut-off time; n = 6). These data further confirm that CNGB1-positive CNG channels contribute to neuropathic pain processing, and suggest that inhibition of CNGB1 could present new strategy for treatment of neuropathic pain.

Example 6: Normal inflammatory pain behavior in CNGB1^{-/-} mice

[00114] To address the question if CNGB1 also plays a role in inflammatory pain processing, the behavior of CNGB1^{-/-} and WT mice was investigated after injection of Complete Freund's Adjuvant (CFA) into a hindpaw. As shown in Fig. 5, the CFA injection resulted in mechanical hypersensitivity in both groups, which persisted over several days. However, the hypersensitivity was indistinguishable between groups, suggesting that CNGB1 is dispensable for the processing of persistent inflammatory pain. These data also exclude the possibility that a general pain-processing defect in CNGB1^{-/-} mice could account for the observed phenotype in the neuropathic pain models.

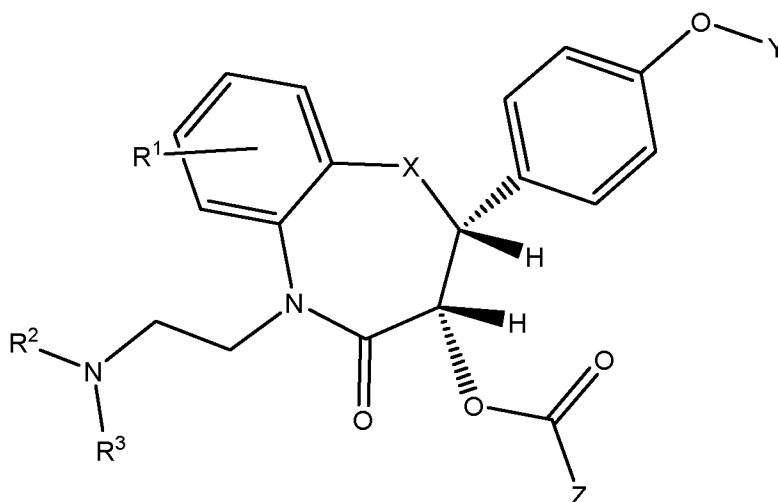
Example 7: cAMP-induced, but not cGMP-induced pain behavior is modulated in CNGB1^{-/-} mice

[00115] Next, the role of CNGB1 in cAMP- and cGMP-mediated pain processing in vivo was assessed. For that purpose, the extent of mechanical hindpaw hypersensitivity of CNGB1^{-/-} and WT mice following intrathecal (i.t.) administration of membrane-permeable CNG channel-

activating analogs of cAMP and cGMP was determined. As shown in Fig. 6A, a cAMP analog (Sp-8-Br-cAMPs; 20 nmol i.t.) evoked transient mechanical hindpaw hypersensitivity in both groups. However, the hypersensitivity was significantly less pronounced in CNGB1^{-/-} mice as compared to WT. In contrast, administration of a cGMP analog (8-pCPT-cGMP; 10 nmol i.t.) evoked mechanical allodynia in both genotypes to a similar extent (Fig. 6B). In control experiments, i.t. delivery of vehicle (saline) had no effect on mechanical hypersensitivity in both CNGB1^{-/-} and WT mice (Fig. 6C). These data suggest that cAMP but not cGMP-mediated mechanical hindpaw hypersensitivity depends on the presence of CNGB1.

Items

- 10 **[00116]** The invention is also characterized by the following items:
- [00117]** 1. An inhibitor of CNGB1 for use in a method for the prophylaxis and/or treatment of neuropathic pain in a subject.
- [00118]** 2. The inhibitor for the use of item 1, wherein the inhibitor is selected from the group consisting of peptides, proteins, antibodies, nucleic acids and small molecules.
- 15 **[00119]** 3. The inhibitor for the use of item 1 or 2, wherein the small molecule is a benzodiazepine derivative.
- [00120]** 4. The inhibitor for the use of item 3, wherein the benzodiazepine derivative is characterized by structure 1



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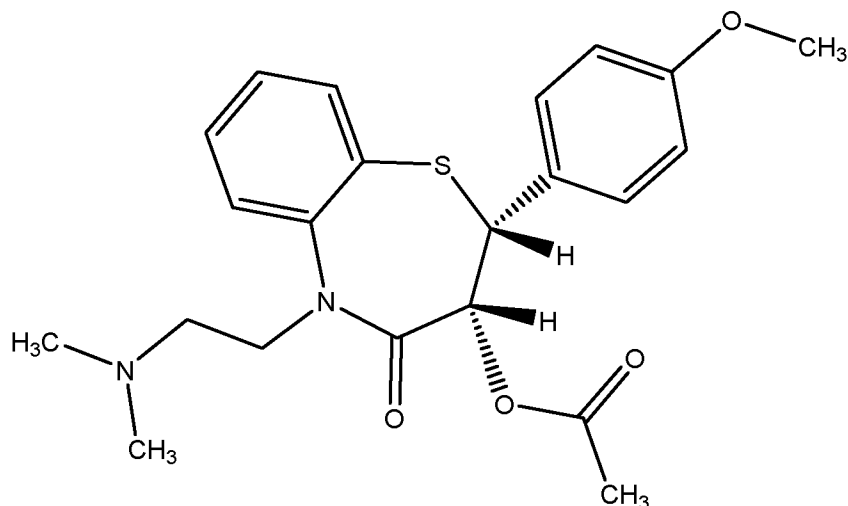
wherein

X is O or S;

Y, R², R³, Z are each independently selected from the group consisting of hydrogen, (C₁-C₆)alkyl, (C₂-C₆)alkenyl and (C₂-C₆)alkynyl; and

R¹ is selected from the group consisting of hydrogen, a nitro group, halogen, -O-(C₁-C₆)alkyl, -S-(C₁-C₆)alkyl and a cyano group.

[00121] 5. The inhibitor for the use of item 3 or 4, wherein the small molecule is L-cis-diltiazem characterized by structure **2**:

**2**

[00122] 6. The inhibitor for the use of item 2, wherein the small molecule is a cyclic nucleotide derivative.

[00123] 7. The inhibitor for the use of anyone of the preceding items, wherein neuropathic pain is caused by a lesion or disease of the somatosensory nervous system.

[00124] 8. The inhibitor for the use of anyone of the preceding items, wherein a sensory neuron is affected.

[00125] 9. The inhibitor for the use of item 7, wherein the lesion or disease of the somatosensory nervous system is caused by a trauma, compression of a nerve, a surgery, a viral infection, pharmacotherapy, and/or antiretroviral treatment.

[00126] 10. The inhibitor for use of item 9, wherein the viral infection is mediated by a virus selected from the group consisting of herpes virus, human immunodeficiency virus (HIV), and papilloma virus.

[00127] 11. The inhibitor for the use of item 9, wherein the pharmacotherapy comprises an anti-cancer agent, and wherein the anti-cancer agent preferably is selected from the group consisting of but not limited to cisplatin, oxaliplatin, paclitaxel, docetaxel, nocodazole, thalidomide, thiouracile, vincristine, vindesine, vinblastine, and vinorelbine.

[00128] 12. The inhibitor for the use of item 9, wherein the antiretroviral treatment comprises an anti-retroviral agent, and wherein the anti-retroviral agent preferably is selected

from the group consisting of reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors, CCR5 antagonists or fusion inhibitors.

[00129] 13. The inhibitor for the use of item 7, wherein the lesion or disease of the somatosensory nervous system is associated with sciatica, hereditary neuropathy (including but not limited to Friedreich ataxia, familial amyloid polyneuropathy, Tangier disease, Morbus Charcot-Marie-Tooth types 2B or 5, Fabry disease), metabolic disorders (including but not limited to diabetes, renal insufficiency and hypothyroidism), vitamin deficiencies (including but not limited to vitamin B12 deficiency, vitamin B6 deficiency, and vitamin E deficiency), toxic and iatrogenic neuropathies (including but not limited to alcoholism, vitamin B6 intoxication, hexacarbon intoxication, amiodarone, chloramphenicol, disulfiram, isoniazide, gold, lithium, metronidazole, misonidazole, nitrofurantoin), infectious neuropathies (including but not limited to leprosy and Lyme disease), auto-immune neuropathies (including but not limited to Guillain-Barre syndrome, chronic inflammatory de-myelinating polyneuropathy, monoclonal gammopathy of undetermined significance, and polyneuropathy), trigeminal neuralgia, entrapment syndromes (including but not limited to Carpel tunnel), post-traumatic neuralgia, phantom limb pain, multiple sclerosis pain, complex regional pain syndromes (including but not limited to reflex sympathetic dystrophy and causalgia), neoplasia, vasculitic/angiopathic neuropathy, and/or idiopathic neuropathy.

[00130] 14. The inhibitor for the use of anyone of the preceding items, wherein the neuropathic pain is allodynia, hyperalgesia and/or spontaneous pain.

[00131] 15. The inhibitor for the use of anyone of the preceding items, wherein the subject is human.

[00132] 16. The inhibitor for the use of anyone of the preceding items, wherein the inhibitor is used in combination with one or more additional neuropathic pain reducing agent(s).

[00133] 17. The inhibitor for the use of anyone of the preceding items, wherein the inhibitor is administered to the subject orally, topically, transdermally, transmucosally, subcutaneously, intramuscularly and/or intravenously.

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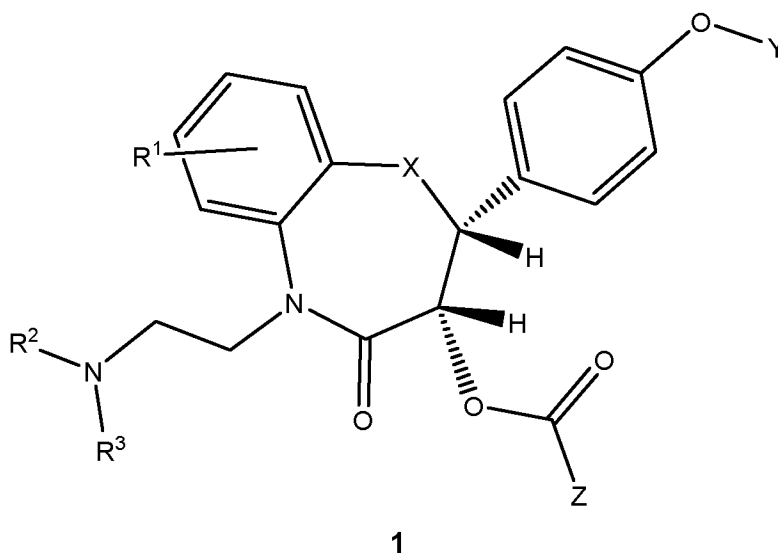
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CLAIMS

1. An inhibitor of CNGB1 for use in a method for the prophylaxis and/or treatment of neuropathic pain in a subject.
2. The inhibitor for the use of claim 1, wherein the inhibitor is selected from the group consisting of peptides, proteins, antibodies, nucleic acids and small molecules.
3. The inhibitor for the use of claim 1 or 2, wherein the small molecule is a benzodiazepine derivative.
4. The inhibitor for the use of claim 3, wherein the benzodiazepine derivative is characterized by structure 1



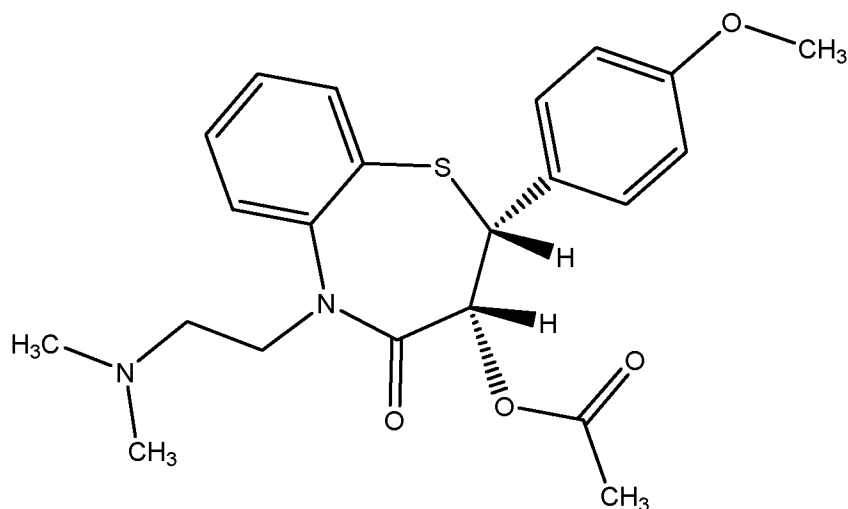
wherein

X is O or S;

Y, R², R³, Z are each independently selected from the group consisting of hydrogen, (C₁-C₆)alkyl, (C₂-C₆)alkenyl and (C₂-C₆)alkynyl; and

R¹ is selected from the group consisting of hydrogen, a nitro group, halogen, -O-(C₁-C₆)alkyl, -S-(C₁-C₆)alkyl and a cyano group.

5. The inhibitor for the use of claim 3 or 4, wherein the small molecule is L-cis-diltiazem characterized by structure 2:



2

6. The inhibitor for the use of claim 2, wherein the small molecule is a cyclic nucleotide derivative.
7. The inhibitor for the use of anyone of the preceding claims, wherein neuropathic pain is caused by a lesion or disease of the somatosensory nervous system.
8. The inhibitor for the use of claim 7, wherein the lesion or disease of the somatosensory nervous system is caused by a trauma, compression of a nerve, a surgery, a viral infection, pharmacotherapy, and/or antiretroviral treatment.
9. The inhibitor for use of claim 8, wherein the viral infection is mediated by a virus selected from the group consisting of herpes virus, human immunodeficiency virus (HIV), and papilloma virus.
10. The inhibitor for the use of claim 8, wherein the pharmacotherapy comprises an anti-cancer agent, and wherein the anti-cancer agent preferably is selected from the group consisting of but not limited to cisplatin, oxaliplatin, paclitaxel, docetaxel, nocodazole, thalidomide, thiouracile, vincristine, vindesine, vinblastine, and vinorelbine.
11. The inhibitor for the use of claim 8, wherein the antiretroviral treatment comprises an anti-retroviral agent, and wherein the anti-retroviral agent preferably is selected from the group consisting of reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors, CCR5 antagonists or fusion inhibitors.
12. The inhibitor for the use of claim 7, wherein the lesion or disease of the somatosensory nervous system is associated with sciatica, hereditary neuropathy (including but not limited to Friedreich ataxia, familial amyloid polyneuropathy, Tangier disease, Morbus Charcot-Marie-Tooth types 2B or 5, Fabry disease), metabolic disorders (including but not limited to diabetes, renal insufficiency and hypothyroidism), vitamin deficiencies

(including but not limited to vitamin B12 deficiency, vitamin B6 deficiency, and vitamin E deficiency), toxic and iatrogenic neuropathies (including but not limited to alcoholism, vitamin B6 intoxication, hexacarbon intoxication, amiodarone, chloramphenicol, disulfiram, isoniazide, gold, lithium, metronidazole, misonidazole, nitrofurantoin), infectious neuropathies (including but not limited to leprosy and Lyme disease), auto-immune neuropathies (including but not limited to Guillain-Barre syndrome, chronic inflammatory de-myelinating polyneuropathy, monoclonal gammopathy of undetermined significance, and polyneuropathy), trigeminal neuralgia, entrapment syndromes (including but not limited to Carpel tunnel), post-traumatic neuralgia, phantom limb pain, multiple sclerosis pain, complex regional pain syndromes (including but not limited to reflex sympathetic dystrophy and causalgia), neoplasia, vasculitic/angiopathic neuropathy, and/or idiopathic neuropathy.

13. The inhibitor for the use of anyone of the preceding claims, wherein the neuropathic pain is allodynia, hyperalgesia and/or spontaneous pain.
14. The inhibitor for the use of anyone of the preceding claims, wherein the subject is human.
15. The inhibitor for the use of anyone of the preceding claims, wherein the inhibitor is used in combination with one or more additional neuropathic pain reducing agent(s).

Figure 1

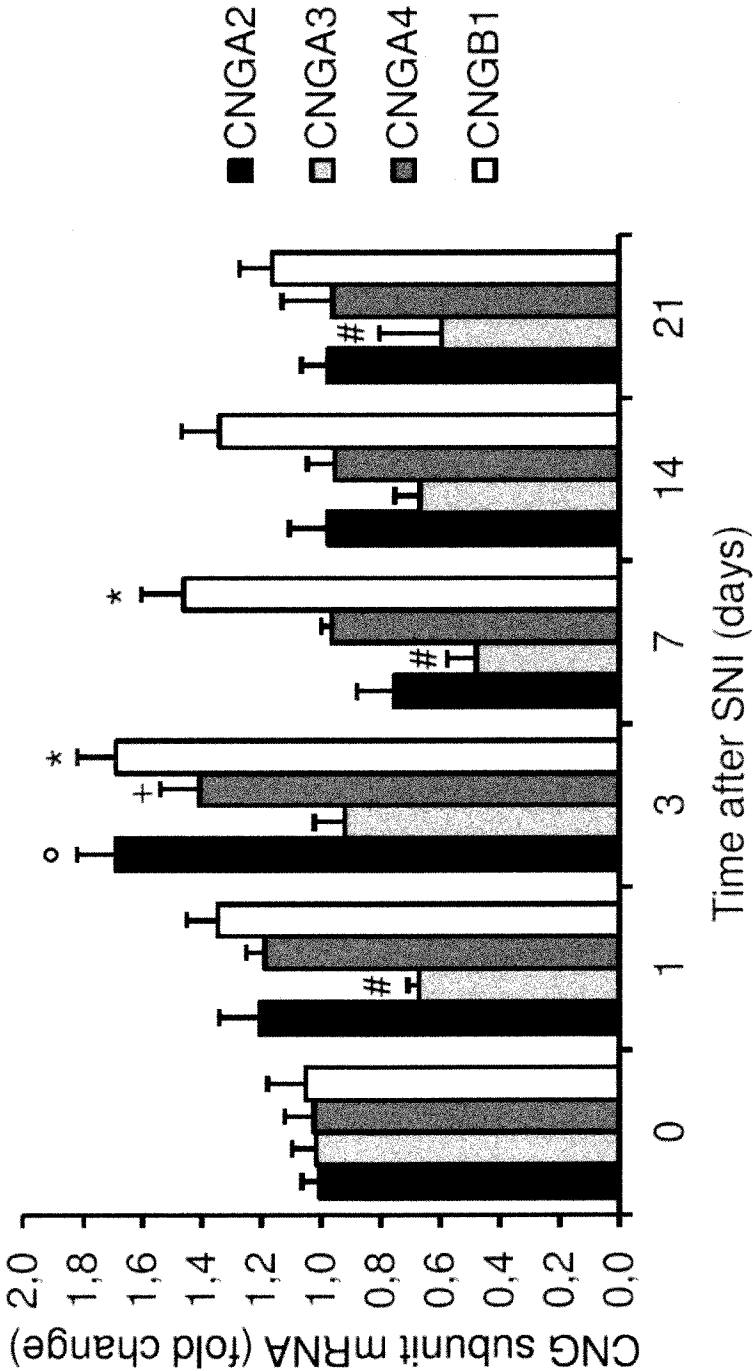


Figure 2

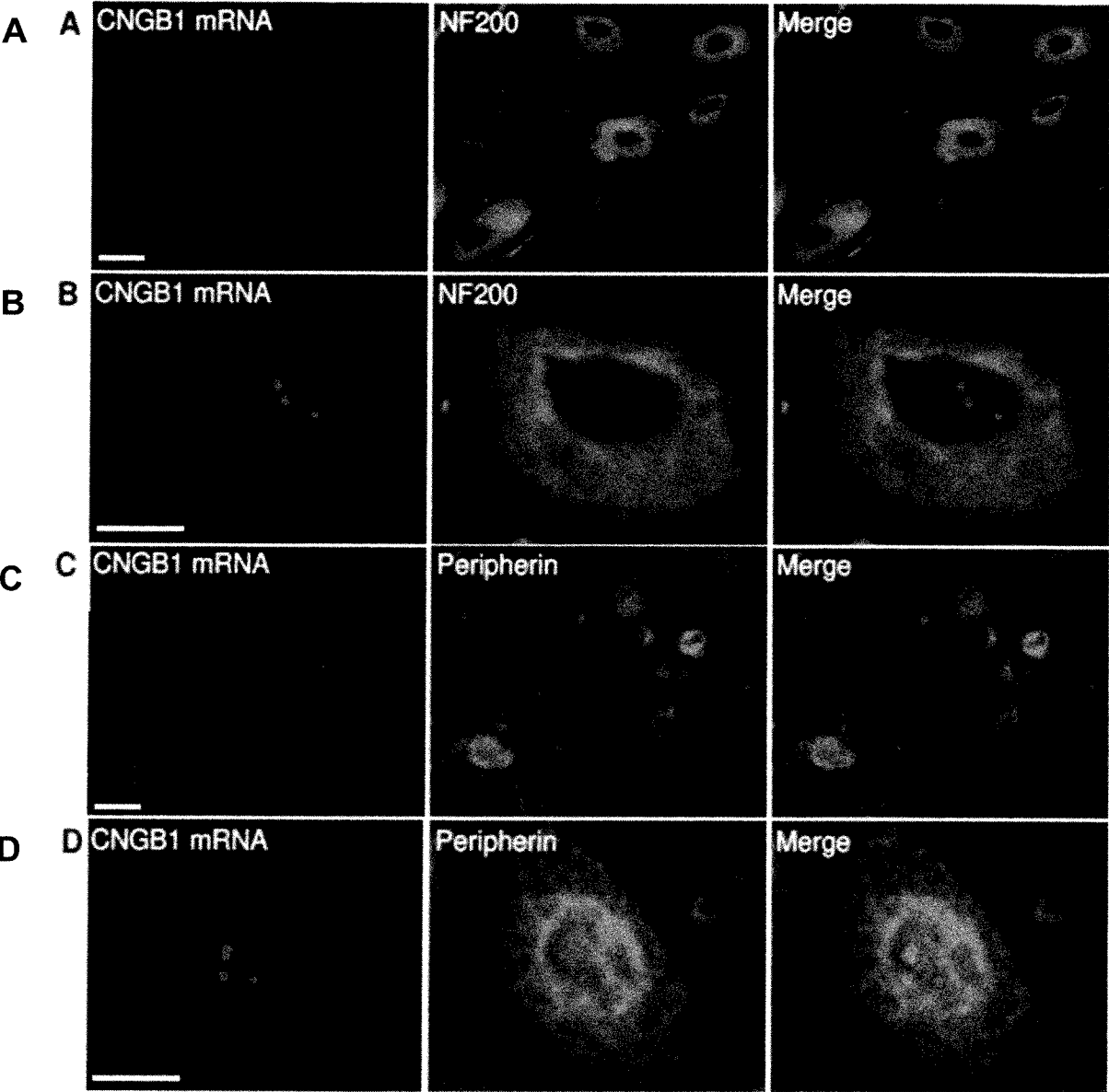


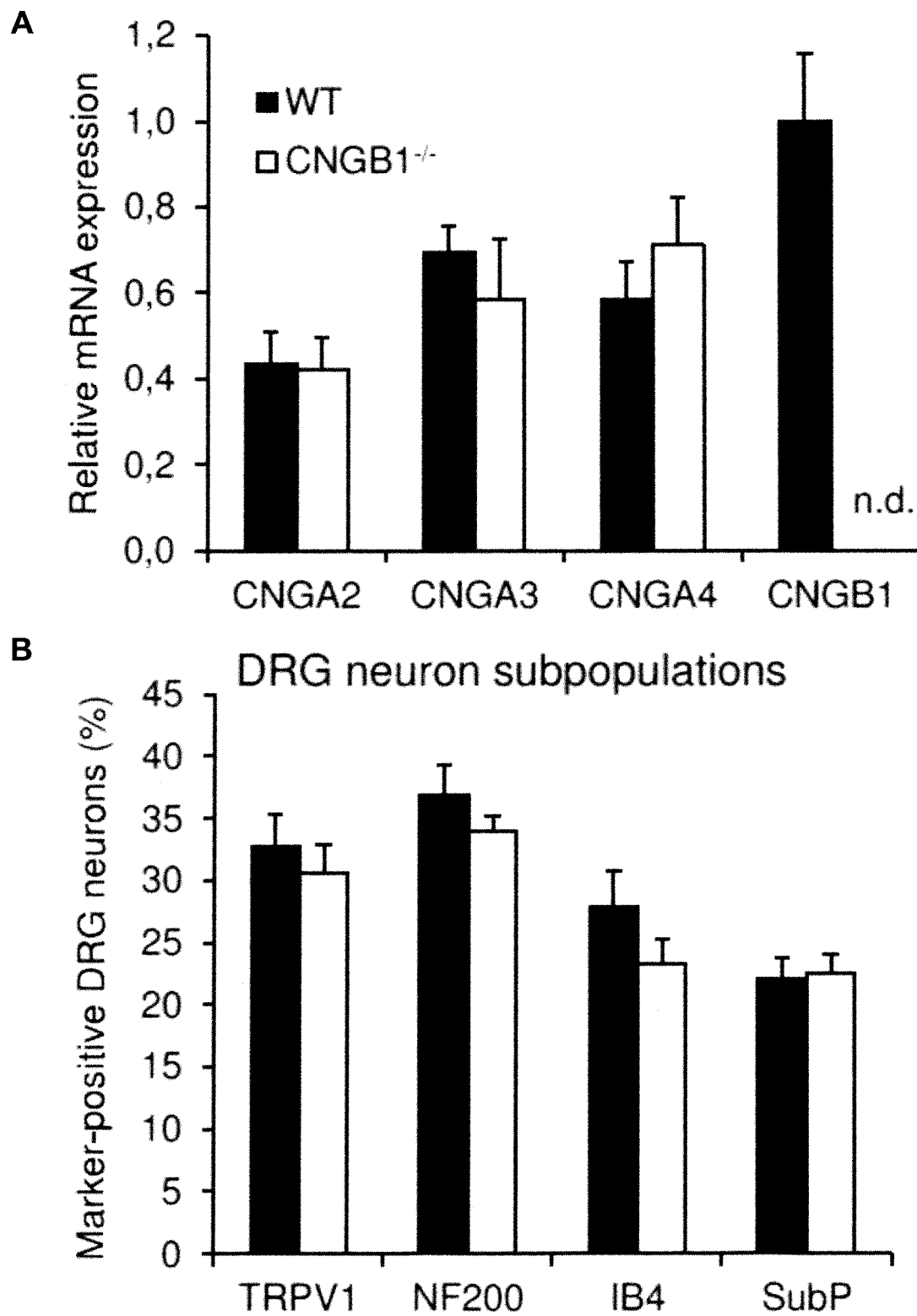
Figure 3

Figure 4

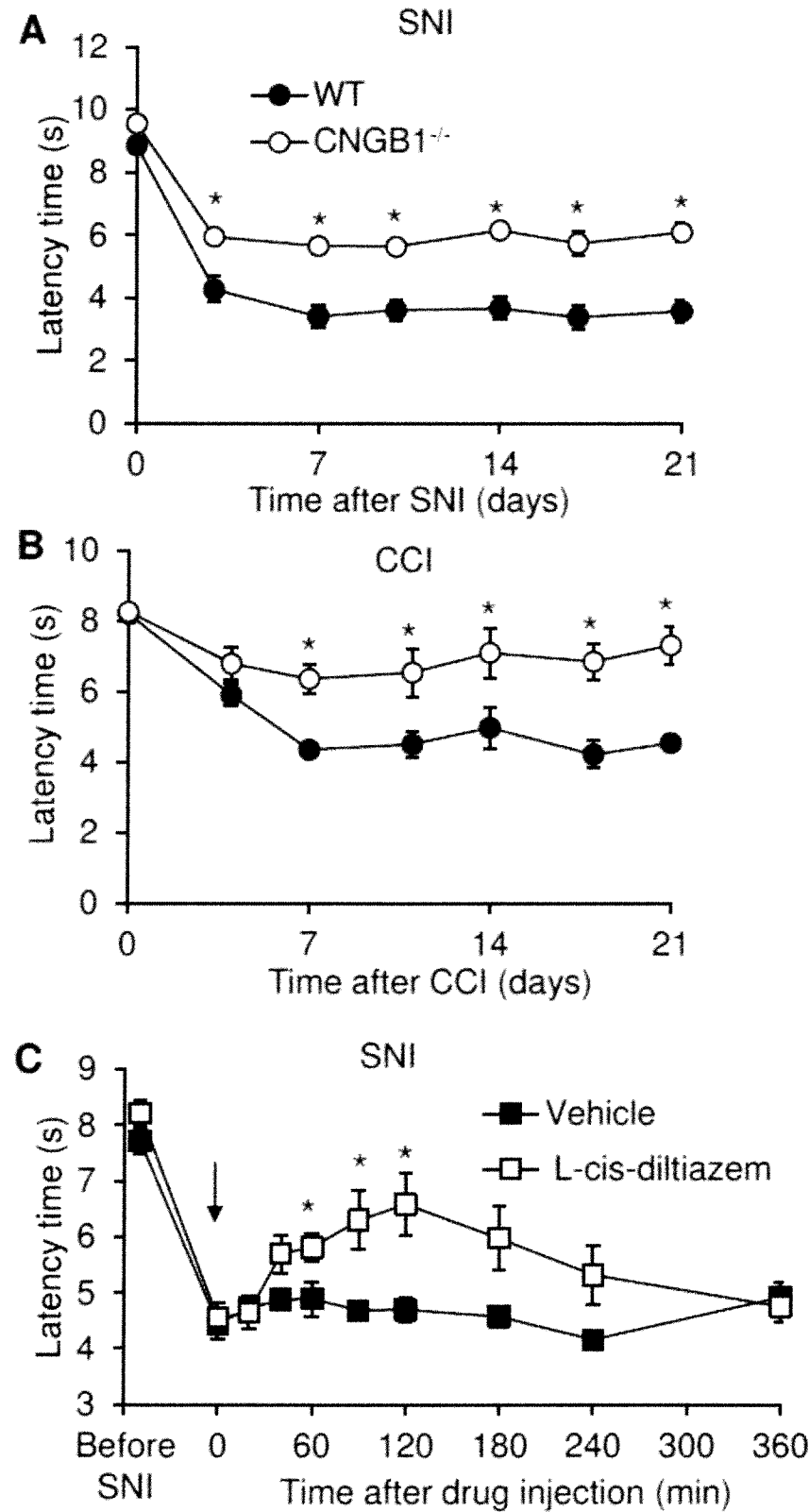


Figure 5

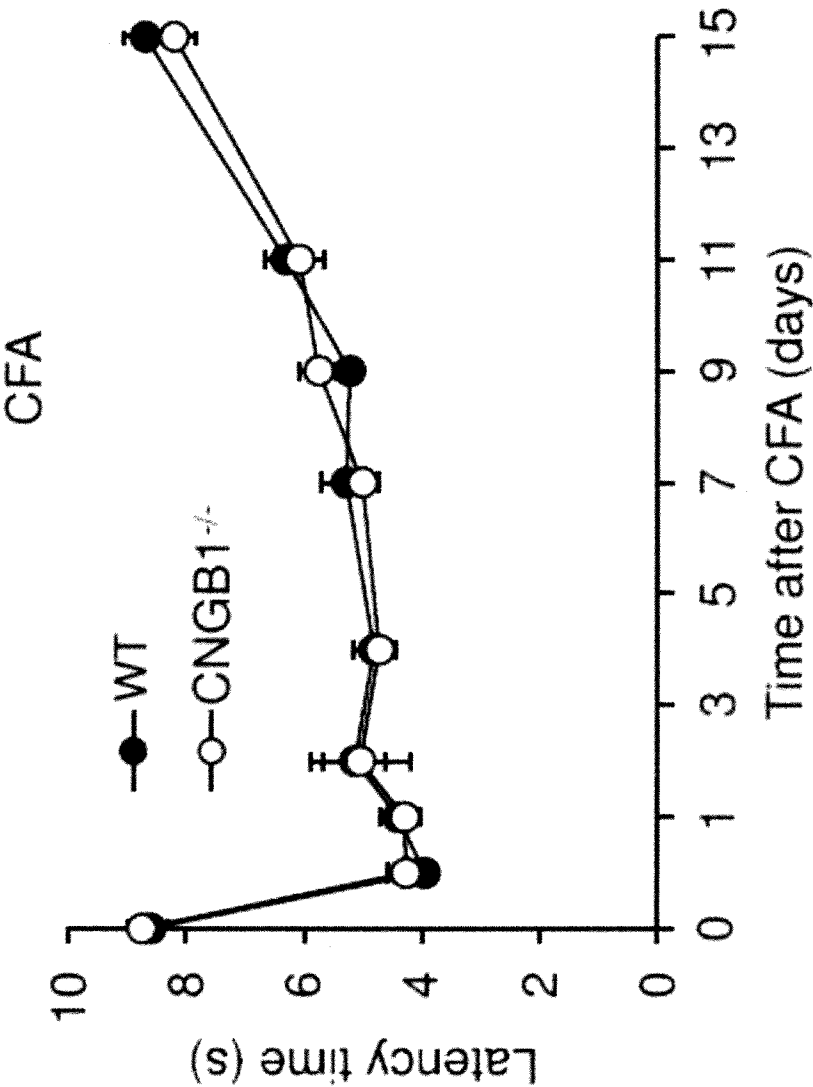


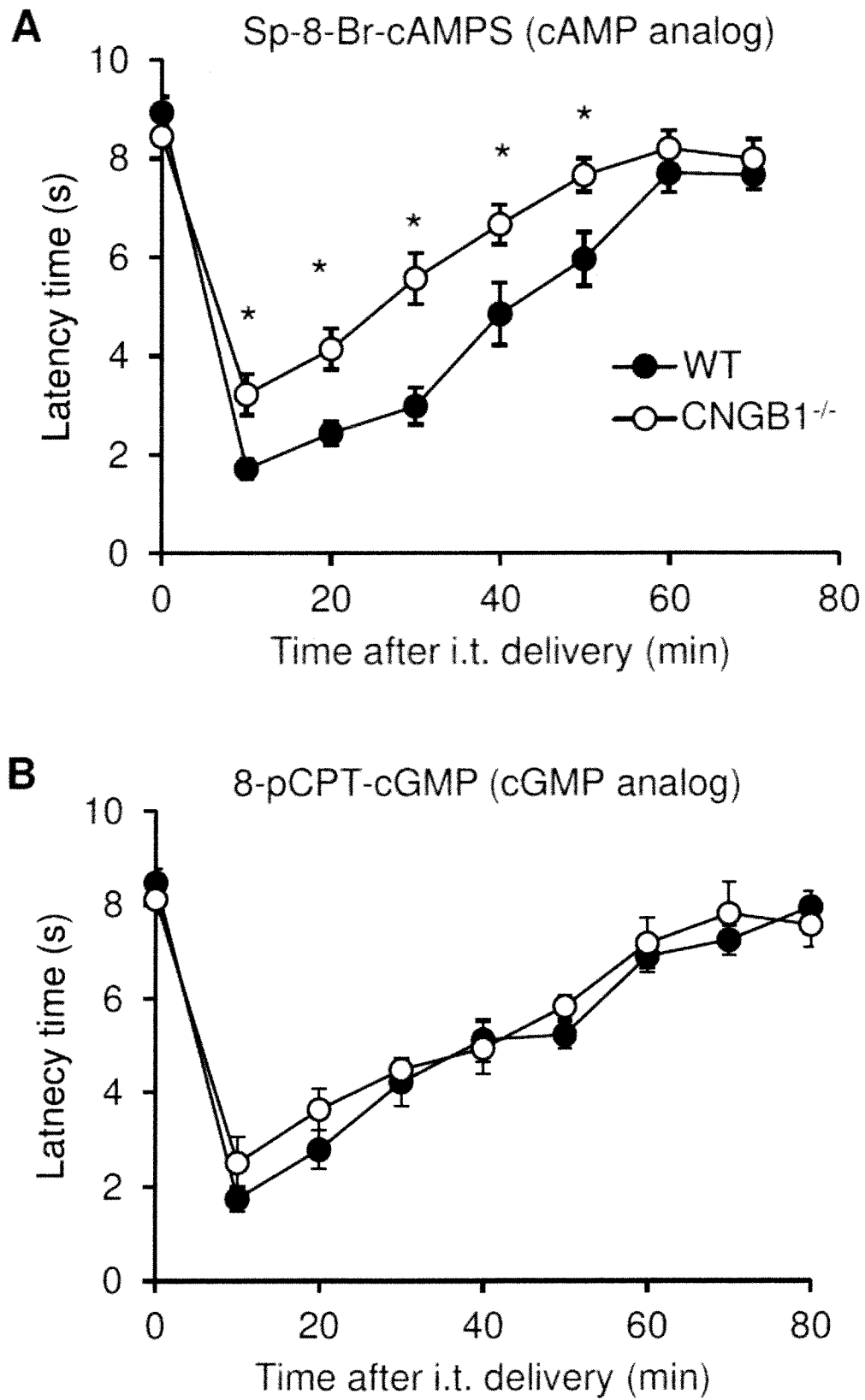
Figure 6

Figure 6 (continued)



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/065915

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/554 A61K45/06 A61P25/04
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, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2 266 558 A2 (ANALGESIC NEUROPHARMACEUTICALS LLC [US]) 29 December 2010 (2010-12-29)	1,2,7,8, 12-15
Y	Fig. 3; p. 9	1-3,6-15
X	US 2005/250767 A1 (WEINER DAVID M [US] ET AL) 10 November 2005 (2005-11-10)	1-3, 13-15
Y	[0002], [0055], [0068]	1-3,6-15
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Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

7 August 2018

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International application No

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