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(54) Title: CHEMOKINE RECEPTORS

(57) Abstract: The invention provides the use of an agonist of a CX3C receptor in the preparation of a medicament for the prevention or treatment of pain and to improve nerve regeneration after nerve in ury or damage. The pain may, for example, be neuropathic pain, inflammatory j pain or pain associated with cancer (other than neuropathic pain), surgery, visceral damage, headache or trauma. The agonist may be fractalkine or a derivative thereof.
CHEMOKINE RECEPTORS

FIELD OF INVENTION

This invention relates to the field of pain (especially but not exclusively neuropathic or inflammatory pain) and nerve injury. In particular, the invention relates to the use of ligands of CX3C receptors, in particular but not exclusively the CX3CRI receptor, in the treatment or prevention of pain and/or promotion of nerve regeneration or repair. The invention also relates to drug discovery methods for determining candidate drugs for use in the treatment or prevention of pain and/or promotion of nerve regeneration or repair, and to pharmaceutical compositions for the prevention or treatment of pain and/or promotion of nerve regeneration or repair.

BACKGROUND

Injury to a peripheral nerve induces changes within the cell bodies of sensory neurons located in the dorsal root ganglion (DRG) that promote survival and axonal regeneration. Under favourable conditions, for instance following a crush injury, most nerve fibres successfully regenerate. However, in many clinically relevant circumstances, traumatic or disease-induced nerve injury has a poor outcome with only a limited return of function and often with considerable delay. In such cases, neuropathic or chronic pain states may develop.

Pain is normally associated with injury or damage and results in guarding and immobilisation of the affected area. Nociception (the neuronal signalling underlying the sensation of pain) therefore results in protection and the promotion of rapid healing, albeit triggering an unpleasant sensory and emotional experience. In many pathological situations, nociceptive inputs can result in functional changes that are actively detrimental to the organism.

Chronic inflammation or nerve injury results in the alteration of many of the properties of primary afferent neurons and their central connections in the spinal cord, leading to allodynia (the perception of pain from a normally innocuous stimulus), hyperalgesia (an
exaggerated response to any given pain stimulus) and an expansion of the receptive field (i.e. the area that is "painful" when a stimulus is applied). The majority of chronic pain conditions arise as a result of damage to either central or peripheral nervous tissue.

Neuropathic pain can be defined as pain deriving from damage to, or inflammation of, the central or peripheral nervous systems. Examples of pain syndromes and causes of pain of this class include painful diabetic sensory neuropathy, alcoholic neuropathy, trigeminal neuralgia, cancer-related pain due to tumour invasion of a nerve, post-herpetic neuralgia, temporomandibular disorder, myofascial pain, back pain (sciatica), peripheral nerve or spinal cord trauma or transection (including surgery), limb amputation and stump pain, arteriovenous malformations, Vitamin B12 deficiency, pain caused by the side-effects of anti-cancer and anti-AIDS therapies, post-stroke pain, complex regional pain syndrome, fibromyalgia-associated neuropathic pain, reflex sympathetic dystrophy, phantom limb syndrome, multiple sclerosis-associated pain, HIV-associated neuropathic pain, carpal tunnel-associated neuropathic pain, pain associated with inflammation or infection of a tooth (toothache), or visceral pain.

Similarly, pain ("inflammatory pain") may also be induced by inflammatory conditions such as connective tissue diseases which include, without limitation, rheumatoid arthritis, Wallenberg's syndrome, systemic lupus erythematosus, multiple sclerosis and polyarteritis nodosa. In addition, inflammatory pain may be caused by various chemical burns and various local and systemic infections.

Neuropathic pain may occur in all body regions. Burn injury also often leads to neuropathic hyperalgesia in the affected body area. In humans, neuropathic pains tend to be chronic. There is general agreement amongst clinicians that neuropathic pain is usually resistant, non-responsive, or only partially responsive to treatment with opioid analgesics. Consequently, alternate therapies for the management of neuropathic pain are widely sought. These include the use of antidepressants and anti-epileptics. Both classes of drugs are effective in only 25-35% of cases. Further, the beneficial effects of these drugs are short lived, rarely persist after a few weeks or months and are often at the expense of severe side-effects. Side effects of these classes of drugs include sedation, confusion,
abdominal pain, diarrhoea, vomiting, renal toxicity and liver toxicity. Similarly, current therapies for inflammatory pain include drugs in the classes of non-steroidal anti-inflammatory drugs and cyclo-oxygenase-II (cox-2) inhibitors. Both these classes of drugs have major side-effects including gastrointestinal upset, nausea and vomiting, gastrointestinal bleeding and gastritis.

The patho-physiological mechanisms that underlie neuropathic pain and its relationship to disordered peripheral nerve regeneration are poorly understood and remain important clinical and scientific issues. Many research groups have attempted to further elucidate the mechanisms that underlie the adaptive response of the peripheral nervous system to injury, by studying factors and/or receptors whose levels and expression patterns are known to change in primary sensory neurons after injury, for example, neurotrophins, the TGFβ superfamily, and various neuropeptides and their receptors.

In recent years, a number of compounds have been identified that modulate neuropathic and/or inflammatory pain behaviour. For example:

WO-A-03/007936 relates to the use of carbamate compounds for the prevention and treatment of neuropathic pain;


WO-A-03/032910 relates to the use of carbinols in the treatment of neuropathic pain; and

EP-A-1243262 relates to the use of a known class of chemical compounds in the treatment of inflammatory pain, for example, rheumatoid arthritis pain. The compounds are also said to show antinociceptive effects.

Chemokines and their receptors

Chemokines are a family of pro-inflammatory mediators that promote recruitment and activation of multiple lineages of leukocytes (e.g. lymphocytes, macrophages). They can
be released by many kinds of tissue cells after activation. Continuous release of chemokines at sites of inflammation can mediate the ongoing migration and recruitment of effector cells to sites of chronic inflammation. The chemokines are related in primary structure and share four conserved cysteines, which form disulfide bonds. Based upon this conserved cysteine motif, the chemokine family can be divided into distinct families, including the C-C chemokines, the C-X-C chemokines, and the C-XXX-C chemokines (or CX3C chemokines), in which the first two conserved cysteines are adjacent or separated by one or three intervening residues, respectively (see e.g., Bagnoli and Dahinden (1994), Immunology Today 15 127-133; Bazan, J. F. et al. (1997) Nature, 385 640-644). The CX3C chemokines include fractalkine, which is also referred to as neurotactin (Pan, Y. et al. (1997) Nature 387 611-617), CX3CL1, and CXXXCL1; ABCD-3 (Schaniel C., et al. (1999) Eur. J. Immunol. 29 2934-2947) and SCYD1 (Nomiymama H. et al. (1998) Cytogenet. Cell Genet. 81 10-11).


The chemokine receptors are members of a superfamily of G protein-coupled receptors (GPCR) which share structural features that reflect a common mechanism of action of signal transduction (Gerard, C. and Gerard, N. P. (1994) Annu. Rev. Immunol. 12 775-808; Gerard, C. and Gerard, N. P. (1994) Curr. Opin. Immunol. 6 140-145). Conserved features include seven hydrophobic domains spanning the plasma membrane, which are connected by hydrophilic extracellular and intracellular loops. The majority of the primary sequence homology occurs in the hydrophobic transmembrane regions with the hydrophilic regions being more diverse. The human CX3C chemokine receptor 1 (CX3CR1, also referred to as CXXXCR1 and V28 (Raport, C. J. et al. (1995) Gene 163 295-299; WO94/12635; Godiska et al. U.S. Patent No. 5,759,804)) can bind fractalkine and is expressed by a variety of different cells and tissues including peripheral blood leukocytes (PBL), spleen and brain (Raport, C. J., et al. (1995) Gene 163 295-299). An alignment of human and mouse forms of the CX3CR1 chemokine receptor is shown in the accompanying Figure 1.

The fractalkine-CX3CR1 interaction is unusual in that fractalkine is the only ligand known to bind and activate the CX3CR1 receptor and in that fractalkine does not bind any other known chemokine receptor. Thus, the normal promiscuity that typifies the chemokine superfamily is absent, suggesting that the roles played by fractalkine and CX3CR1 are non-
redundant and may be of critical importance to the nervous system. Further, activation of CX3CR1 by fractalkine increases the phosphorylation (and hence activation) of both Akt (protein kinase B) and extracellular regulated kinases (Meucci, O., et al. (2000) Proc. Natl. Acad. Sci. U.S.A. 97 8075-8080; Maciejewski-Lenoir, et al. (1999) J. Immunol. 163 1628-1635). These signalling cascades have been shown to play trophic and/or neuroprotective roles in various subsets of neurons in both the peripheral and central nervous systems.

Cultured rat dorsal root ganglion (DRG) neurons have been demonstrated to express a wide variety of chemokine receptors, including CX3CR1. Addition of fractalkine to these cultured neurons increased intracellular calcium levels in approximately 9% of neurons implying that a small sub-population of DRG neurons express the CX3CR1 receptor. Further, fractalkine also increased electrical excitability of a small subset of these cultured DRG neurons. The authors went on to demonstrate that a number of chemokines (including RANTES, SDF-1α and MDC, but not fractalkine) induced allodynia after injection into the rat paw. The authors concluded “these results provide evidence that chemokines may produce painful effects via direct actions on chemokine receptors expressed by nociceptive neurons. Chemokine receptor antagonists (and specifically CX3CR1 antagonists) may be important therapeutic interventions in the pain that is associated with HIV-1 infection and inflammation” (S. B. Oh et al. (2001) Journal of Neuroscience 21 5027-5035).

Another group have also demonstrated that, after facial motor nerve axotomy, dramatic changes in the levels of CX3CR1 and fractalkine in the facial nucleus were evident. These included increases in the number and perineuronal location of CX3CR1-expressing microglia, decreased levels of motor neuron-expressed fractalkine mRNA, and an alteration in the forms of fractalkine protein expressed. These data describe mechanisms of cellular communication between neurons and microglia, involving fractalkine and CX3CR1, which occur in both normal and pathological states of the central nervous system. No data was presented on the levels of fractalkine or CX3CR1 in the DRG, or responses to nerve injury in the DRG (J. K. Harrison et al. (1998) Proc. Natl. Acad. Sci. U.S.A. 95 10896-1090).

WO-A-01/60406 relates to the use of an antagonist of CX3CR1 in a method of treating a
subject having inflammatory arthritis.

WO-A-02/076990 relates to the use of a compound, which is an antagonist of CX3CR1, in the treatment or prevention of diseases such as neurodegenerative disorders or a demyelinating disease, and in the treatment of pain.


The present applicants have unexpectedly found that expression of the chemokine receptor CX3CR1 in the DRG is markedly increased after nerve injury. Infusion of fractalkine (the natural ligand for CX3CR1), markedly and unexpectedly attenuates neuropathic and inflammatory pain in the mouse. Further, CX3CR1 knock-out mice have increased neuropathic pain (allodynia) after nerve injury. These findings contrast with the conclusion drawn by Oh, S.B. et al. mentioned above which indicated the use of chemokine receptor antagonists in the treatment of pain. The applicants have also shown that fractalkine speeds up neurite outgrowth from cultured DRG neurons, indicating that it is also a pro-regenerative and growth-promoting molecule.

SUMMARY OF THE INVENTION

According to one aspect of the invention there is provided the use of an agonist of a CX3C receptor in the preparation of a medicament for the prevention or treatment of pain.

Advantageously, the use of an agonist of a CX3C receptor allows pain to be prevented or treated, as the result of the ability of an agonist of a CX3C receptor to prevent or reduce a pain response as the result of, for example, tissue injury or nerve injury, damage or disease.

The pain may be neuropathic pain, preferably centrally mediated neuropathic pain. The pain may be chronic, allodynia (the perception of pain from a normally innocuous stimulus), hyperalgesia (an exaggerated response to any given pain stimulus) and an expansion of the receptive field (i.e. the area that is “painful” when a stimulus is applied), phantom pain or inflammatory pain.
In a further alternative, the pain may be one of: painful diabetic peripheral neuropathy, post-herpetic neuralgia, trigeminal neuralgia, post-stroke pain, multiple sclerosis-associated pain, neuropathy-associated pain such as in idiopathic or post-traumatic neuropathy and mononeuritis, HIV-associated neuropathic pain, cancer-associated neuropathic pain, carpal tunnel-associated neuropathic pain, spinal cord injury-associated pain, complex regional pain syndrome, fibromyalgia-associated neuropathic pain, lumbar and cervical pain, reflex sympathetic dystrophy, phantom limb syndrome or peripheral nerve or spinal cord trauma, entrapment neuropathy, nerve transection including surgery, Lissauer tract section, limb amputation and stump pain, neuroma/tumour compression, arteriovenous malformation, Vitamin B12 deficiency, diabetic neuropathy, alcoholic neuropathy, pain caused by the side effects of anti-cancer and anti-AIDS therapies, pain associated with inflammation or infection of a tooth (toothache), visceral pain, pain caused by chemical burns, pain caused by local or systemic infection, or pain caused by connective tissue disease. The connective tissue disease may be one of: rheumatoid arthritis, Wallenberg’s syndrome, systemic lupus erythematosus, multiple sclerosis, or polyarteritis nodosa.

In a yet further alternative, the pain may be associated with cancer, surgery, visceral damage, headache or trauma.

A CX3C receptor is preferably one that is activated by fractalkine. Most preferably, the receptor is a human CX3C receptor, but agonists of CX3C receptors from other species may be useful in the treatment or prevention of pain in humans or animals.

The agonist may be fractalkine or a derivative thereof. Suitable derivatives may include polypeptides comprising a portion of the fractalkine amino acid sequence. For example, a derivative of fractalkine may comprise a portion of the chemokine portion of native fractalkine. Other agonists, especially novel or known small chemical entities, are also contemplated for use in accordance with the invention. Other agonists which may be used are selected from compounds that activate a CX3C receptor. Preferably, agonists for use in accordance with the invention bind to the CX3C receptor with a binding affinity of less than or equal to 100µM, more preferably less than or equal to 1µM.
According to another aspect of the invention there is provided a method for preventing or treating pain comprising administering an effective amount of an agonist of a CX3C receptor to an individual in need of such prevention or treatment. An effective amount is sufficient to prevent or treat pain in the individual.

The pain may be neuropathic pain, preferably centrally mediated neuropathic pain. The pain may be chronic, allodynia (the perception of pain from a normally innocuous stimulus), hyperalgesia (an exaggerated response to any given pain stimulus) and an expansion of the receptive field (i.e. the area that is “painful” when a stimulus is applied), phantom pain or inflammatory pain.

In a further alternative, the pain may be one of: painful diabetic peripheral neuropathy, post-herpetic neuralgia, trigeminal neuralgia, post-stroke pain, multiple sclerosis-associated pain, neuropathy-associated pain such as in idiopathic or post-traumatic neuropathy and mononeuritis, HIV-associated neuropathic pain, cancer-associated neuropathic pain, carpal tunnel-associated neuropathic pain, spinal cord injury-associated pain, complex regional pain syndrome, fibromyalgia-associated neuropathic pain, lumbar and cervical pain, reflex sympathetic dystrophy, phantom limb syndrome or peripheral nerve or spinal cord trauma, entrapment neuropathy, nerve transection including surgery, Lissauer tract section, limb amputation and stump pain, neuroma/tumour compression, arteriovenous malformation, Vitamin B12 deficiency, diabetic neuropathy, alcoholic neuropathy, pain caused by the side effects of anti-cancer and anti-AIDS therapies, pain associated with inflammation or infection of a tooth (toothache), visceral pain, pain caused by chemical burns, pain caused by local or systemic infection, or pain caused by connective tissue disease. The connective tissue disease may be one of: rheumatoid arthritis, Wallenberg’s syndrome, systemic lupus erythematosus, multiple sclerosis, or polyarteritis nodosa.

In a yet further alternative, the pain may be associated with cancer, surgery, visceral damage, headache or trauma.

According to another aspect of the invention there is provided a method of selecting
candidate drugs for use in the prevention or treatment of pain, the method comprising:

a) determining whether a compound is an agonist of a CX3C receptor, and

b) selecting compounds which are agonists of the CX3C receptor as candidate drugs, or for further development.

Preferably the CX3C receptor is a CX3CR1 receptor, most preferably the human CX3CR1 receptor.

Receptors from other species may be used in methods of selecting candidate drugs in accordance with the invention. The use of chimeric receptor constructs is also contemplated.

Selected candidate drugs preferably bind to the CX3C receptor with a binding affinity of less than or equal to 100μM, more preferably less than or equal to 1μM.

In a typical method in accordance with this aspect of the invention, a large selection of test compounds are screened in a high throughput screening (HTS) assay. Such HTS systems are well known.

According to another aspect of the invention there is provided a pharmaceutical composition for use in the treatment or prevention of pain, the composition comprising a pain treating or preventing amount of at least one agonist of a CX3C receptor, preferably the CX3CR1 receptor, and a suitable excipient.

The agonist may be fractalkine or a derivative thereof. Suitable derivatives may include polypeptides comprising a portion of the fractalkine amino acid sequence. For example, a derivative of fractalkine may comprise a portion of the chemokine portion of native fractalkine. Other agonists, especially novel or known small chemical entities, are also contemplated for use in pharmaceutical compositions in accordance with the invention.

Pharmaceutical compositions of this invention may comprise a CX3C receptor agonist compound and pharmaceutically acceptable salts thereof, with any pharmaceutically
acceptable carrier, adjuvant or vehicle. Pharmaceutically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions of this invention include, for example, ion exchangers, alumina, aluminium stearate, lecithin, serum proteins such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polycrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

Pharmaceutical compositions of this invention may be administered orally, parenterally, by injection, by needle-free device, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. Oral administration or administration by injection or needle-free device is preferred. The term "parenteral" as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intra-articular, intrasynovial, intrasternal, intrathecal, intralesional and intracranial injection or infusion techniques.

Where the pharmaceutical composition is administered by injection or needle-free device, it may be in the form of a sterile injectable preparation or a form suitable for delivery by needle-free device, which may be an aqueous or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as, for example, Tween 80) and suspending agents. The sterile injectable preparation or form suitable for delivery by needle-free device may also be a solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions.
These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant such as Ph. Helv or a similar alcohol.

Pharmaceutical compositions of this invention may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, and aqueous suspensions and solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, can also typically be added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are administered orally, the active ingredient is combined with emulsifying and suspending agents. If desired, sweetening and/or flavouring and/or colouring agents may be added.

Pharmaceutical compositions of this invention may also be administered in the form of suppositories for rectal administration. These compositions can be prepared by mixing a compound of this invention with a suitable non-irritating excipient which is solid at room temperature but liquid at rectal temperature and therefore will melt in the rectum to release the active components. Such materials include, but are not limited to, cocoa butter, beeswax and polyethylene glycols.

Topical administration of the pharmaceutical compositions of this invention is especially useful when the desired treatment involves areas or organs readily accessible by topical application. For application topically to the skin, the pharmaceutical composition should be formulated with a suitable ointment containing the active components suspended or dissolved in a carrier. Carriers for topical administration of the compounds of this invention include, but are not limited to, mineral oil, liquid petroleum, white petroleum, propylene glycol, polyoxyethylene-polyoxypropylene compounds, emulsifying wax and water. Alternatively, the pharmaceutical composition can be formulated with a suitable lotion or cream containing the active compound suspended or dissolved in a carrier. Suitable carriers include, for example, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water. Pharmaceutical compositions of this invention may also be topically applied to the lower
intestinal tract by rectal suppository formulation or in a suitable enema formulation. Topical-transdermal patches are also included in this invention.

The pharmaceutical compositions of this invention may be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

According to a further aspect of the invention there is provided the use of an agonist of a CX3C receptor in the preparation of a medicament for the promotion of nerve regeneration or repair.

Advantageously, the use of an agonist of a CX3C receptor allows the promotion of nerve regeneration and/or repair, as the result of the ability of an agonist of a CX3C receptor to promote nerve regeneration and/or repair following, for example, nerve injury, disease or damage.

The agonist may be fractalkine or a derivative thereof.

Preferably the agonist activates the CX3CR1 receptor, most preferably the human CX3CR1 receptor.

According to another aspect of the invention there is provided a method of promoting nerve regeneration or repair comprising administering an effective amount of an agonist of a CX3C receptor to an individual in need of such treatment.

According to another aspect of the invention there is provided a method of selecting candidate drugs for use in the promotion of nerve regeneration or repair, the method comprising:

a) determining whether a compound is an agonist of a CX3C receptor, and

b) selecting compounds which are agonists of the CX3C receptor as candidate
drugs, or for further development.

The receptor may be CX3CR1, preferably the human receptor.

Selected candidate drugs may bind to the CX3C receptor with a binding affinity of less than or equal to 100\(\mu\)M, preferably less than or equal to 1\(\mu\)M.

The invention also provides a pharmaceutical composition for use in the promotion of nerve regeneration or repair, the composition comprising an effective amount of at least one agonist of a CX3C receptor, and a suitable excipient.

**DEFINITIONS**

The following definitions are provided by way of explanation:

“CX3C receptor”: a mammalian receptor which is activated by fractalkine, including without limitation the human, rat or mouse receptors described as CX3CR1, CXXXCR1 and V28 and polypeptides which act as receptors with amino acid sequences having at least 80% homology with such receptors. Activation may be determined, for example, by an increase in intracellular calcium levels. The receptor may also be chimeric in form (i.e. including sequences from different species) or truncated (i.e. shorter than a native sequence) or extended (i.e. including additional sequence beyond that of a native sequence).

BRIEF DESCRIPTION OF THE.Drawings

The use of ligands of CX3C receptor in the prevention or treatment of pain and neurite outgrowth and drug design methods in accordance with the invention will now be described with reference to the further accompanying drawings Figures 1 to 9 in which:

Figures 1A and 1B show the alignment of the human and mouse CX3CR1 DNA sequences;

Figure 2 is a graph illustrating the results of an analysis of CX3CR1 levels in the DRG of mice before and after axotomy (transection of the sciatic nerve);

Figure 3 is a picture demonstrating expression of CX3CR1 and fractalkine by immunohistochemical staining on sections of intact adult mouse DRG;

Figure 4A is a graph illustrating the results of experiments on thermal nociception by injecting fractalkine into the sciatic nerve in mice;

Figure 4B is a graph illustrating the results of experiments on mechanical nociception by injecting fractalkine into the sciatic nerve in mice;

Figure 5 is a graph illustrating the results of experiments on the effect of the injection of fractalkine on mechanical allodynia in mice over the first week in a spared nerve injury model of neuropathic pain;

Figure 6 is a graph illustrating the results of experiments on mechanical allodynia in control, wild-type mice compared to CX3CR1 knock-out mice, over the two weeks in a spared nerve injury model;

Figure 7 is a graph illustrating the results of experiments on the effect of the intra-sciatic injection of fractalkine on inflammatory pain behaviour in mice after an intra-dermal injection of formalin;

Figure 8 is a graph illustrating the effects of fractalkine on neurite length in cultured sensory neurons from wild-type mice; and
Figure 9 is a schematic outlining a method of selecting candidate drugs for use in the prevention and treatment of pain or for use in the promotion of nerve regeneration or repair.

MODES OF CARRYING OUT THE INVENTION

1. Identifying G-Protein Coupled Receptors

In order to identify nerve injury-responsive G-Protein Coupled Receptors (GPCRs) in the DRG, two sets of degenerate primer pairs were designed, based upon the conserved transmembrane domains of over 100 known GPCRs that fall into the broad class of neuropeptide receptors were designed:-

Forward  5' - gca ccg (ct)5a t(gct)a g(ct)g t5g ata g(ag)t aca - 3'
Reverse  5' - GCA GGA AG(GC) C(AG)T AGA (GT) AG

The following PCR conditions were then used on adult intact mouse 129OlaHsd DRG:-

\[
\begin{align*}
94 \degree C & \quad 2 \text{ mins} \\
94 \degree C & \quad 1 \text{ min} \\
65 \degree C & \quad 20 \text{ sec} \\
55 \degree C & \quad 5 \text{ sec} \\
45 \degree C & \quad 15 \text{ sec} \\
72 \degree C & \quad 1 \text{ min} \\
72 \degree C & \quad 5 \text{ mins}
\end{align*}
\]

PCR amplicons were sub-cloned and then sequenced. The CX3CR1 receptor was identified in the mouse DRG and was identical to EMBL accession number AF074912. PCR
amplicons were sub-cloned and then sequenced. The CX3CR1 receptor was identified in the mouse DRG and was identical to EMBL accession number AF074912 (Combadiere et al (1998) Biochem. Biophys. Res. Commun. 253 728-732).

2. Expression of CX3CR1 in the DRG

Semi-quantitative RT-PCR was then performed (using an ABI Taqman® 7900, Applied Biosystems, Cheshire, UK) to analyse expression of CX3CR1 in DRG from intact wild-type mice and at two time points after axotomy (N=4).

Forward primer  
5'-TCC GCA ACT CGG AAG TCA AC-3'

Reverse primer  
5'-GAA GTA GCA AAA GCT CAT GAT AAG CA-3'

PCR conditions:

\[
\begin{align*}
95 ^\circ\text{C} & \quad 10 \text{ mins} \\
95 ^\circ\text{C} & \quad 15 \text{ secs} \\
60 ^\circ\text{C} & \quad 1 \text{ min}
\end{align*}
\]

50 cycles

As shown in Figure 2, these data demonstrate that there is a highly significant 6-fold rise in the levels of the CX3CR1 receptor at 1 and 3 weeks after axotomy (P<0.001).

Following on from this, these mRNA studies were extended to expression of CX3CR1 and fractalkine at the protein levels using immunocytochemistry, as previously described (Holmes et al Proc. Natl. Acad. Sci. U.S.A. 100 6180-6185 (2003)). In brief, mice were intracardially perfused with 4% paraformaldehyde/PBS and the spinal columns removed and post-fixed for 4 hours at room temperature. The L4 and L5 DRGs were dissected and equilibrated in 20% sucrose overnight at 4°C, embedded in Optimal Cutting Temperature (OCT) mounting medium (Tissue Tek Ltd., Eastbourne, UK), frozen on dry ice and then cryostat sectioned (16μM sections for DRG). Sections were blocked and permeabilised in
10% normal donkey serum/PBS 0.2% Triton-X-100 (PBST) for 1 hour at room temperature. Sections were then incubated in rabbit polyclonal antibody to CX3CR1 (Santa Cruz Biotechnology, Inc., California, USA; at final dilution of 1:20) or fractalkine (R&D BioSystems, Abingdon, UK; at final dilution of 1:100) in PBST overnight at room temperature, washed 3 x 10 minutes in PBS and incubated in donkey anti-rabbit cy3 (Jackson Laboratory, Westgrove, PA, USA) at 1:800 for 3 hours at room temperature. After washing, sections were mounted in Vectashield™ (Vector Laboratories Inc., Burlington, CA, USA). Images were taken using a Leica fluorescent microscope (Leica Microsystems, Milton Keynes, UK) with RT Color Spot camera and Spot Advance image capture system software (Diagnostic Instruments, Sterling Heights, MI, USA). Results (Fig. 3) show that a subset (approx. 30%) of DRG neurons express CX3CR1 whilst the vast majority express fractalkine.

3. Effect of CX3CR1 ligands on thermal or mechanosensory nociception in mice

Having demonstrated that the levels of CX3CR1 are increased in the DRG after nerve injury, the effects of direct injection of 2µl of mouse fractalkine (R&D Systems (Abingdon, UK; cat no. 472-FF) (100mg/Kg body weight), into the sciatic nerve of wild-type mice of the 129OlaHsd strain, were studied. No effects of the chemokine were noted on either thermal or mechanosensory nociception (as illustrated in Fig. 4A and 4B respectively), consistent with the low levels of CX3CR1 expression in the intact DRG. Specifically, Fig. 4A shows that there is no significant difference between the mean latency response in mice injected with saline or fractalkine in a Hargreaves test. Fig. 4B shows that there is no significant difference between the 50% withdrawal threshold in mice injected with saline or fractalkine in a von Frey Hairs test.

4. Effect of CX3CR1 ligands on chronic neuropathic and inflammatory pain in mice

The effects of the same dose of fractalkine on chronic neuropathic pain were then explored in the same strain of mice, using the recently described SNI model (Holmes et al (2003) Proc. Natl. Acad. Sci. U.S.A. 100 6180-6185). The spared nerve injury model (SNI), is performed by ligation of two branches of the sciatic nerve after it trifurcates, leaving one
branch intact. This procedure generates a partial denervation of the sciatic nerve which induces allodynia and an expansion of the receptive pain field. The pain behaviours in these models closely resemble a number of human neuropathic pain conditions, such as those associated with diabetes mellitus, alcoholism and trauma (Decosterd and Woolf (2000) Pain 87 149-158; Woolf and Doubell (1994) Curr.Opin.Neubiol. 4 525-534). Injection of fractalkine (same conditions as in Example 3) at the same time as the SNI injury model markedly attenuated chronic pain behaviour (Fig. 5) for 7 days.

To further demonstrate that activation of CX3CR1 by fractalkine reduces pain, CX3CR1 knock-out mice were tested (Combadiere et al (2003) Circulation 107 1009-1016) compared to age- and strain-matched wild-type controls, using the same SNI model of neuropathic pain described above. The knock-out mice developed more pain after nerve injury over 14 days than the wild-type animals, once again demonstrating that activation of CX3CR1 inhibits neuropathic pain (Fig. 6).

The effect of intra-sciatic fractalkine (400ng given 2 days prior to formalin administration) on inflammatory pain was tested, using the standard intra-dermal injection of 30μl of 1% formalin as previously described (Kerr et al (2001) Pain 93 267-277). The administration of fractalkine significantly reduced nociceptive behaviour in response to the formalin injection (Fig. 7, P<0.05).

The results from the above Examples indicate that ligands for the CX3CR1 receptor, in particular agonists for that receptor, will be effective at preventing or reducing pain, particularly but not exclusively neuropathic and/or inflammatory pain, in humans.

5. Effect of CX3CR1 ligands on nerve regeneration in mice

The up-regulation of CX3CR1 after nerve injury may also contribute to an appropriate regenerative response after nerve damage. To test this, nerve outgrowth (neurite axonal length) from cultured DRG (sensory) neurons in vitro was measured in the presence or absence of fractalkine as previously described (Holmes et al (2000) Proc. Natl. Acad. Sci. U.S.A. 97 11563-11568). Fractalkine at two doses stimulates neurite outgrowth over an 8 hour period (Fig. 8).
6. Screening Assays to determine CX3C receptor ligands

Compounds which may be suitable for use in preventing or reducing pain in methods in accordance with the invention, or for use in the promotion of nerve regeneration or repair in accordance with the invention, may be isolated by screening libraries of compounds for compounds which are ligands for a CX3C receptor. Once ligands have been identified, cells transfected with and stably expressing the cDNA encoding the human CX3C receptor are incubated with such CX3C receptor ligands, under conditions permitting binding of compounds to CX3C. Activation of the CX3C receptor, indicating that the compound is a CX3C receptor agonist, is detected by a variety of measures including radioligand binding, an increase in intracellular calcium levels or change in intracellular pH. Such methods are known in the art, for example Warrior et al (2003) J. Biomolec. Screening 8 324-331; Yan et al (2002) J. Biomolec. Screening 7 451-459; Rao et al (2002) Analyt. Biochem. 307 117-130; Rudiger et al (2001) J. Biomolec. Screening 6 29-37; and Chen et al (1998) Mol. Pharm. 53 177-181. Preferably, compounds which bind to the CX3C receptor with high affinity are selected for further development as drug candidates or as the basis for the design of further compounds (see Figure 9).

The receptor used in the screening assay may be the human, rat or mouse form of the receptor but is preferably the human form of the receptor. The receptor may be entire or may include only a portion of the full length native receptor sequence. Preferably the receptor has the full human receptor amino acid sequence.
CLAIMS

1. A method of selecting candidate drugs for use in the prevention or treatment of pain, the method comprising:

   a) determining whether a compound is an agonist of a CX3C receptor, and

   b) selecting compounds which are agonists of the CX3C receptor as candidate drugs.

2. A method of selecting candidate drugs according to claim 1 in which the receptor is CX3CR1.

3. A method according to claim 1 or 2 in which the receptor is a human receptor.

4. A method according to any one of claims 1 to 3 in which a selected candidate drug binds to the CX3C receptor or to CX3CR1 with a binding affinity of less than or equal to 100μM.

5. A method according to claim 4 in which a selected candidate drug binds to the CX3C receptor or to CX3CR1 with a binding affinity of less than or equal to 1μM.

6. The use of an agonist of a CX3C receptor in the preparation of a medicament for the prevention or treatment of pain.

7. The use according to claim 6 in which the pain is neuropathic pain.

8. The use according to claim 7 wherein the pain is centrally mediated neuropathic pain.

9. The use according to claim 6 wherein the pain is chronic, allodynia, hyperalgesic pain, phantom pain or inflammatory pain.

10. The use according to any of claims 6 to 9 wherein the pain is one of: painful diabetic peripheral neuropathy, post-herpetic neuralgia, trigeminal neuralgia, post-
stroke pain, multiple sclerosis-associated pain, neuropathy-associated pain such as in idiopathic or post-traumatic neuropathy and mononeuritis, HIV-associated neuropathic pain, cancer-associated neuropathic pain, carpal tunnel-associated neuropathic pain, spinal cord injury-associated pain, complex regional pain syndrome, fibromyalgia-associated neuropathic pain, lumbar and cervical pain, reflex sympathetic dystrophy, phantom limb syndrome or peripheral nerve or spinal cord trauma, entrapment neuropathy, nerve transection including surgery, Lissauer tract section, limb amputation and stump pain, neuroma/tumour compression, arteriovenous malformation, Vitamin B12 deficiency, diabetic neuropathy, alcoholic neuropathy, pain caused by the side effects of anti-cancer and anti-AIDS therapies, HIV-associated neuropathic pain, pain associated with inflammation or infection of a tooth (toothache), visceral pain, pain caused by chemical burns, pain caused by local or systemic infection, or pain caused by connective tissue disease.

11. The use according to claim 10 wherein the connective tissue disease is one of: rheumatoid arthritis, Wallenberg's syndrome, systemic lupus erythematosus, multiple sclerosis or polyarteritis nodosa.

12. The use according to any of claims 6 to 11 in which the pain is that associated with cancer, surgery, visceral damage, headache or trauma.

13. The use according to any of claims 6 to 12 in which the agonist is fractalkine or a derivative thereof.

14. The use according to any of claims 6 to 13 in which the agonist activates the CX3CR1 receptor.

15. The use according to claim 14 in which the agonist activates the human CX3CR1 receptor.

16. A method of preventing or treating pain comprising administering an effective amount of an agonist of a CX3C receptor to an individual in need of such prevention or treatment.
17. A method according to claim 16 wherein said pain is neuropathic pain.

18. A method according to claim 17 wherein the neuropathic pain is centrally mediated neuropathic pain.

19. A method according to claim 16 wherein the pain is chronic, allodynia, hyperalgesic pain, phantom pain or inflammatory pain.

20. A method according to any of claims 16 to 19 wherein the pain is one of: painful diabetic peripheral neuropathy, post-herpetic neuralgia, trigeminal neuralgia, post-stroke pain, multiple sclerosis-associated pain, neuropathy-associated pain such as in idiopathic or post-traumatic neuropathy and mononeuritis, HIV-associated neuropathic pain, cancer-associated neuropathic pain, carpal tunnel-associated neuropathic pain, spinal cord injury-associated pain, complex regional pain syndrome, fibromyalgia-associated neuropathic pain, lumbar and cervical pain, reflex sympathetic dystrophy, complex regional pain syndrome, phantom limb syndrome or peripheral nerve or spinal cord trauma, entrapment neuropathy, nerve transection including surgery, Lissauer tract section, limb amputation and stump pain, neuroma/tumour compression, arteriovenous malformation, vitamin B12 deficiency, diabetic neuropathy, alcoholic neuropathy, pain caused by the side effects of anti-cancer and anti-AIDS therapies, HIV-associated neuropathic pain, pain associated with inflammation or infection of a tooth (toothache), visceral pain, pain caused by chemical burns, pain caused by local or systemic infection or pain caused by connective tissue disease.

21. A method according to claim 20 wherein the connective tissue disease is one of: rheumatoid arthritis, Wallenberg’s syndrome, systemic lupus erythematosus, multiple sclerosis or polyarteritis nodosa.

22. A method according to any of claims 16 to 21 in which the pain is that associated with cancer, surgery, visceral damage, headache or trauma.

23. A method according to any one of claims 16 to 22 in which the agonist is
fractalkine or a derivative thereof.

24. A method according to any one of claims 16 to 23 in which the agonist activates the human CX3CR1 receptor.

25. A pharmaceutical composition for use in the treatment or prevention of pain, the composition comprising a pain treating or preventing amount of at least one agonist of a CX3C receptor, and a suitable excipient.

26. A pharmaceutical composition according to claim 25 in which the agonist is fractalkine.

27. A pharmaceutical composition according to claim 25 or 26 in which the receptor is CX3CR1.

28. A pharmaceutical composition according to claim 27 in which the receptor is the human CX3CR1 receptor.

29. The use of an agonist of a CX3C receptor in the preparation of a medicament for the promotion of nerve regeneration or repair.

30. The use according to claim 29 in which the agonist is fractalkine or a derivative thereof.

31. The use according to claim 29 or 30 in which the agonist activates the CX3CR1 receptor.

32. The use according to claim 31 in which the agonist activates the human CX3CR1 receptor.

33. A method of promoting nerve regeneration or repair comprising administering an effective amount of an agonist of a CX3C receptor to an individual in need of such treatment.

34. A method of selecting candidate drugs for use in the promotion of nerve
regeneration or repair, the method comprising:

a) determining whether a compound is an agonist of a CX3C receptor, and

b) selecting compounds which are agonists of the CX3C receptor as candidate drugs.

35. A method of selecting candidate drugs according to claim 34 in which the receptor is CX3CR1.

36. A method according to claim 34 or 35 in which the receptor is a human receptor.

37. A method according to any one of claims 34 to 35 in which a selected candidate drug binds to the CX3C receptor or to CX3CR1 with a binding affinity of less than or equal to 100μM.

38. A method according to claim 37 in which a selected candidate drug binds to the CX3C receptor or to CX3CR1 with a binding affinity of less than or equal to 1μM.

39. A pharmaceutical composition for use in the promotion of nerve regeneration or repair, the composition comprising an effective amount of at least one agonist of a CX3C receptor, and a suitable excipient.

40. A pharmaceutical composition according to claim 39 in which the agonist is fractalkine.

41. A pharmaceutical composition according to claim 39 or 40 in which the receptor is CX3CR1.

42. A pharmaceutical composition according to claim 41 in which the receptor is the human CX3CR1 receptor.
Fig 2

CX3CR1 mRNA (Arbitrary Units)

Control

0 100 200 300 400 500 600 700

1 week post-axonotomy

3 weeks post-axonotomy

***

***
**Partial Diagonals**

**Fig 4**

**A**

**Thermal Nociception (Hargreaves)**

- **Mean Latency Response (secs)**
  - Saline Injection
  - Fractalkine Injection

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean Latency Response (secs)</th>
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</thead>
<tbody>
<tr>
<td>Control (Saline)</td>
<td>20</td>
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<tr>
<td>Fractalkine (100mg/ul) Injection</td>
<td>20</td>
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</tbody>
</table>

**B**

**Mechanical Nociception (VonFrey Hairs)**

- **50% Withdrawal Threshold (g)**
  - Saline Injection
  - Fractalkine Injection

<table>
<thead>
<tr>
<th>Condition</th>
<th>50% Withdrawal Threshold (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Saline)</td>
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</tr>
<tr>
<td>Fractalkine (100mg/ul) Injection</td>
<td>2</td>
</tr>
</tbody>
</table>
Fig 5

Sham Injection
Fractalkine

50% Withdrawal Threshold

Days Post Sciatic Nerve Injury
Fig 6

CX3CR1 KO

WT

DAYS POST SCIATIC NERVE INJURY

50% WITHDRAWAL THRESHOLD

Day 1
Day 2
Day 7
Day 9
Day 12
Day 14

Baseline

1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fig 9

Screen compound library for putative CX3CR agonists

Binding affinity studies & CX3CR activation studies

Compound selection

Compound development (including clinical trials)