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A NOVEL AGENT ABLE TO MODIFY PERIPHERAL AFFERENT FUNCTION

Technical Field

This invention relates to a novel agent which is able to modify peripheral afferent function. The agent may inhibit neurotransmitter release from discrete populations of neurons, and thereby reduce, or preferably prevent, the transmission of afferent pain signals from peripheral to central pain fibres. The agent may be used in or as a pharmaceutical for the treatment of pain, particularly chronic pain.

Background

The sense of touch has traditionally been regarded as one of the five classical senses, but in reality it is highly complex, transducing a number of different sensations. These sensations are detected in the periphery by a variety of specialised nerve endings and associated structures. Some of these are specific for mechanical stimuli of various sorts such as touch, pressure, vibration, and the deformation of hairs or whiskers. Another class of nerves are able to detect temperatures, with different fibres being activated by heat and cold. A further population of nerve endings are not normally excited by mild stimuli, but by strong stimuli only. Sensory nerves of this category often respond to more than one stimulus, and are known as high-threshold polymodal fibres. They may be used to sense potentially damaging situations or objects. The polymodal fibres also transduce chemical signals such as the "burning" sensation evoked by acid. Thus, the sense of touch can transmit a very detailed description of objects and serve to both inform and warn of events.

The transduction of sensory signals from the periphery to sensation itself is achieved by a multi-neuronal pathway and the information processing centres of the brain. The first nerve cells of the pathway involved in the transmission of sensory stimuli are called primary sensory afferents. The cell bodies for the primary sensory afferents from the head and some of the internal organs reside in various of the ganglia associated with the cranial nerves, particularly the trigeminal nuclei and the nucleus of the solitary tract. The cell bodies for the primary sensory afferents for the remainder of the body lie in the dorsal root ganglia of the spinal column. The primary sensory afferents and their processes have been classified histologically; the cell bodies fall into two classes: A-type are large (60-120 μm in diameter), while B-type are smaller (14-30 μm) and more numerous. Similarly the processes fall into two categories: C-fibres lack the myelin sheath that A-fibres possess. A-fibres can be further sub-divided into A β -fibres, that are large diameter with well developed myelin, and A δ -fibres, that are thinner with less well developed myelin. It is generally believed that A β -fibres arise from A-type cell bodies and that A δ - and C-fibres arise from B-type cell bodies. These classifications can be further extended and subdivided by studying the selective expression of a range of molecular markers.

Functional analyses indicate that under normal circumstances A β -fibres transmit the senses of touch and moderate temperature discrimination, whereas the C-fibres are mainly equivalent to the polymodal high-threshold fibres mentioned above. The role of A δ -fibres is less clear as they seem to have a variety of responsive modes, with both high and low thresholds.

After the activation of the primary sensory afferents, the next step in the transduction of sensory signals is the activation of the projection neurons, which carry the signal to higher parts of the central nervous system such as the thalamic nuclei. The cell bodies of these neurons (other than those related to the cranial nerves) are located in the dorsal horn of the spinal cord. This is also where the synapses between the primary afferents and the projection neurons are located. The dorsal horn is organised into a series of laminae that are stacked, with lamina I being most dorsal followed by lamina II etc. The different classes of primary afferents make synapses in different laminae. For cutaneous primary afferents, C-fibres make synapses in laminae I and II, A δ -fibres in laminae I, II, and V, and A β -fibres in laminae III, IV, and V. Deeper laminae (V-VII, X) are thought to be involved in the sensory pathways arriving from deeper tissues such as muscles and the viscera.

The predominant neurotransmitter at the synapses between primary afferents and projection neurons is glutamate, although importantly the C-fibres contain several neuropeptides such as substance P and calcitonin-gene related peptide (CGRP). A-fibres may also express neuropeptides such as neuropeptide Y under some circumstances.

The efficiency of transmission of these synapses can be altered via descending pathways and by local interneurons in the spinal cord. These modulatory neurons release a number of mediators that are either inhibitory (e.g. opioid peptides, glycine) or excitatory (e.g. nitric oxide, cholecystokinin), to provide a mechanism for enhancing or reducing awareness of sensations.

A category of sensation that requires such physiological modulation is pain. Pain is a sensation that can warn of injury or illness, and as such is essential in everyday life. There are times, however, when there is a need to be able to ignore it, and physiologically this is a function of, for example, the opioid peptides. Unfortunately, despite these physiological mechanisms, pain can continue to be experienced during illnesses or after injuries long after its utility has passed. In these circumstances pain becomes a symptom of disease that would be better alleviated.

Clinically, pain can be divided into three categories: (1) Acute pain, usually arising from injury or surgery that is expected to disappear when that injury is healed. (2) Chronic pain arising from malignant disease; the majority of people with metastatic cancer have moderate to severe pain and this is resolved either by successful treatment of the disease or by the death of the patient. (3) Chronic pain not caused by malignant disease; this is a heterogeneous complaint, caused by a variety of illnesses, including arthritis and peripheral neuropathies, that are usually not life-threatening but which may last for decades with increasing levels of pain.

The physiology of pain that results from tissue damage is better understood than that which is caused by central nervous system defects. Under normal circumstances the sensations that lead to pain are first transduced by the A δ - and C-fibres that carry high threshold signals. Thus the synapses in laminae I and II are involved in the transmission of the pain signals, using glutamate and the peptides released by C-fibres to produce activation of the appropriate projection neurons. There is, however, evidence that in some chronic pain states other A-fibres (including A β -fibres) can carry pain signals, and thus act as primary nociceptive afferents, for example in the hyperalgesia and allodynia associated with neuropathic pain. These changes have been associated with the expression of peptides such as neuropeptide Y in A fibres. During various chronic pain conditions the synapses of the various sensory afferents with projection neurons may be modified in several ways: there may be changes in morphology leading to an increase in the number of synapses, the levels and ratios of the different peptides may change, and the sensitivity of the projection neuron may change.

Given the enormity of the clinical problem presented by pain, considerable effort has been expended in finding methods for its alleviation. The most commonly used pharmaceuticals for the alleviation of pain fall into two categories: (1) Non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and ibuprofen; (2) Opioids, including morphine.

NSAIDs have their main analgesic action at the periphery by inhibiting the production of prostaglandins by damaged tissues. Prostaglandins have been shown to be peripheral mediators of pain and inflammation and a reduction in their concentration provides relief to patients. This is especially the case in mild arthritic disease, where inflammation is a major cause of pain. It has been suggested that prostaglandins are involved in the mediation of pain in the spinal cord and the brain; this may explain why NSAIDs have analgesic effects in some pain states that do not involve inflammation or peripheral tissue damage. As prostaglandins, however, are only one of several mediators of pain, NSAIDs alone are only effective in reducing some types of mild pain to acceptable levels. They are regarded as having a ceiling of activity above which increasing doses do not give increasing pain relief. Furthermore they have side effects that limit their usefulness in chronic complaints. The use of NSAIDs is associated with irritation of the gastro-intestinal tract and prolonged use may lead to the development of extensive ulceration of the gut. This is particularly true in elderly patients who form the largest cohort of patients with, for example, arthritis.

Opioids act at the level of the spinal cord to inhibit the efficiency of neurotransmission between the primary nociceptive fibres (principally C-fibres) and the projection neurons. They achieve this by causing a prolonged hyperpolarization of both elements of these synapses. The use of opioids is effective in alleviating most types of acute pain and chronic malignant pain. There are, however, a number of chronic malignant pain conditions which are partly or completely refractory to opioid analgesia, particularly those which involve nerve compression, e.g. by tumour formation. Unfortunately opioids also have unwanted systemic side-effects including: (1) depression of the respiratory system at the level of the respiratory centres in the

brain stem; (2) the induction of constipation by a variety of effects on the smooth musculature of the gastro-intestinal tract; and (3) psychoactive effects including sedation and the induction of euphoria. These side effects occur at doses similar to those that produce analgesia and therefore limit the doses that can be given to patients.

Delivery of opioids at the spinal level can reduce the side-effect profile, but requires either frequently repeated spinal injections or fitting of an catheter, both of which carry increased risk to the patient. Fitting of a catheter requires that the patient is essentially confined to bed thus further restricting their quality of life.

The use of opioids for the treatment of some other types of chronic pain is generally ineffective or undesirable. Examples include the pain associated with rheumatoid arthritis and neuromas that develop after nerve injury. The undesirable nature of opioid treatment in these patients is related not only to side-effects already mentioned and the probable duration of the disease but also to the fourth major side-effect of the opioids: dependence. Opioids such as morphine and heroin are well-known drugs of abuse that lead to physical dependence, this last side-effect involves the development of tolerance: the dose of a drug required to produce the same analgesic affect increases with time. This may lead to a condition in which the doses required to alleviate the pain are life-threatening due to the first three side-effects.

Although NSAIDS and opioids have utility in the treatment of pain there is general agreement that they are often not appropriate for the adequate treatment of pain, particularly chronic and severe pains.

Other treatments are also used, particularly for the treatment of chronic severe pain including surgical lesions of the pain pathways at several levels from peripheral nerves through dorsal root section and cordotomy to pituitary destruction. These are, however, mostly severe operations that are all associated with significant risk to the patient.

It can be seen, therefore, that there remains a significant need for the development of new classes of pharmaceuticals for the treatment of pain of many types. The desired properties of such new therapies can be briefly expressed as follows: (1) the ability to provide significant relief of pain including severe pain; (2) the lack of systemic side effects that significantly impair the patients quality of life; (3) long-lasting actions that do not require frequent injections or long-term catheterisation of patients; (4) provision of agents which do not lead to tolerance and associated dependence.

Statement of Invention

The present invention relates to an agent which can reduce and preferably prevent the transmission of pain signals from the periphery to the central nervous system, thereby alleviating the sensation of pain. Specifically, the invention can provide an agent which can reduce and preferably prevent the transmission of pain signals from nociceptive afferents to projection neurons. More specifically, the invention can

provide an agent which can inhibit the exocytosis of at least one neurotransmitter or neuromodulator substance from at least one category of nociceptive afferents.

In a first aspect of the invention, there is provided an agent which can be administered systemically, and can specifically target defined populations of nociceptive afferents to inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nerves.

In a second aspect of the invention, there is provided an agent which can be locally administered at the periphery, and which is able to inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nociceptive afferents transmitting the pain signal from the periphery.

In a third aspect of the invention, an agent is provided which can be administered into the spinal cord, and which can inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nociceptive afferents terminating in that region of the spinal cord.

In a fourth aspect of the invention, there is provided an agent which can specifically target defined populations of afferent neurons, so that the effect of the agent is limited to that cell type.

In a fifth aspect of the invention, there is provided a method of treatment of pain which comprises administering an effective dose of the agent according to the invention.

In a sixth aspect of the invention, the agent can be expressed recombinantly as a fusion protein which includes the required components of the agent.

Definitions

Without wishing to be limited by the definitions set down, it is intended in this description that the following terms have the following meanings:

Light chain means the smaller of the two polypeptide chains which form clostridial neurotoxins; it has a molecular mass of approximately 50 kDa and is commonly referred to as L-chain or simply L.

Heavy chain means the larger of the two polypeptide chains which form clostridial neurotoxins; it has a molecular mass of approximately 100 kDa and is commonly referred to as H-chain or simply H.

H_C fragment means a fragment derived from the H-chain of a clostridial neurotoxin approximately equivalent to the carboxy-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain. It contains the natural toxins binding domain for motor neurons.

H_N fragment means a fragment derived from the H-chain of a clostridial neurotoxin approximately equivalent to the amino-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain. It contains a domain involved in the translocation the L-chain across endosomal membranes.

LH_N means a fragment derived from a clostridial neurotoxin that contains the L-chain, or a functional fragment thereof coupled to the H_N fragment. It is commonly derived from the intact neurotoxin by proteolysis.

Targeting Moiety (TM) means any chemical structure of an agent which functionally interacts with a binding site causing a physical association between the agent and the surface of a primary sensory afferent.

Binding site (BS) means a structure on the surface of a cell with which exogenous molecules are able to interact in such a way as to bring about a physical association with the cell.

Primary sensory afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system.

Primary nociceptive afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system, where that information can result in a sensation of pain.

Detailed Description of the Invention

It can be seen that an agent for reducing or preventing the transmission of pain signals from peripheral, nociceptive afferent neurons to projection neurons, has many potential applications in the reduction of the sensation of pain, particularly of severe, chronic pain.

According to the invention, there is provided an agent which can inhibit the release of at least one neurotransmitter or neuromodulator or both from the synaptic terminals of nociceptive afferents.

The agent has a number of discrete functions:

- 1) It binds to a surface structure (the Binding Site [BS]) which is characteristic of, and has a degree of specificity for, nociceptive afferent neurons.
- 2) It enters the neuron. The entry of molecules into a cell can occur by a process of endocytosis. Only certain cell surface BSs undergo endocytosis, and preferably the BS to which the agent binds is one of these. In one aspect of this invention, the BS is

present on the peripheral, sensory fibres of the nociceptive afferent neuron and, following internalization, undergoes retrograde transport to the cell body and central processes of the neuron, in such a manner that the agent is also delivered to these regions of the neuron. In another aspect of this invention, the BS to which the agent binds is present on the central processes or cell body of the nociceptive afferent neuron.

3) The agent enters the cytosol.

4) The agent modifies components of the exocytotic machinery present in the synaptic terminals of the central processes of those neurons, such that the release of at least one neurotransmitter or neuromodulator from the synaptic terminal is reduced or preferably prevented.

Surprisingly, an agent of the present invention can be produced by modifying a clostridial neurotoxin or fragment thereof.

The clostridial neurotoxins are proteins with molecular masses of the order of 150 kDa. They are produced by various species of the genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*. There are at present eight different classes of the neurotoxins known: tetanus toxin, and botulinum neurotoxin in its serotypes A, B, C1, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesized by the bacterium as a single polypeptide that is modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H), which has a molecular mass of approximately 100 kDa, and the light chain (L), which has a molecular mass of approximately 50 kDa. The clostridial neurotoxins bind to an acceptor site on the cell membrane of the motor neuron at the neuromuscular junction and are internalised by an endocytotic mechanism. The internalized clostridial neurotoxins possess a highly specific zinc-dependent endopeptidase activity which hydrolyses a specific peptide bond in one of three proteins, synaptobrevin, syntaxin or SNAP-25, which are crucial components of the neurosecretory machinery. This activity of the clostridial toxins results in a prolonged muscular paralysis. The zinc-dependent endopeptidase activity of clostridial neurotoxins is found to reside in the L-chain. The clostridial neurotoxins are highly selective for motoneurons due to the specific nature of the acceptor site on those neurons. The specific neuromuscular junction binding activity of clostridial neurotoxins is known to reside in the carboxy-terminal portion of the heavy chain component of the dichain neurotoxin molecule, a region known as H_C.

Surprisingly, by covalently linking a clostridial neurotoxin, or a hybrid of two clostridial neurotoxins, in which the H_C region of the H-chain has been removed or modified, to a new molecule or moiety, the Targeting Moiety (TM), that binds to a BS on the surface of sensory neurons, a novel agent capable of inhibiting the release of at least one neurotransmitter or neuromodulator from nociceptive afferents is produced. A further surprising aspect of the present invention is that if the L-chain of a clostridial neurotoxin, or a fragment of the L-chain containing the endopeptidase activity, is covalently linked to a TM which can also effect internalisation of the L-chain, or fragment thereof, into the cytoplasm of a sensory neuron, this also produces a novel agent capable of inhibiting

the release of at least one neurotransmitter or neuromodulator. The covalent linkages used to couple the component parts of the agent may include appropriate spacer regions.

The TM provides specificity for the BS on the nociceptive afferent neuron. The TM component of the agent can comprise one of many cell binding molecules, including, but not limited to, antibodies, monoclonal antibodies, antibody fragments (Fab, F(ab)₂, Fv, ScFv, etc.), lectins and ligands to the receptors for hormones, cytokines, growth factors or neuropeptides. A list of possible TMs is given in Table 1, this list is illustrative and is not intended to be limiting to the scope of TMs which could fulfil the requirements of this invention. In one embodiment of the invention the TM binds to a BS which undergoes retrograde transport.

It is known in the art that the H_C portion of the neurotoxin molecule can be removed from the other portion of the heavy chain, known as H_N, such that the H_N fragment remains disulphide linked to the light chain (L-chain) of the neurotoxin molecule to provide a fragment known as LH_N. Thus, in one embodiment of the present invention the LH_N fragment of a clostridial neurotoxin is covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the H_C domain of a clostridial neurotoxin is mutated or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction. This modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the heavy chain of a clostridial neurotoxin, in which the H_C domain is mutated or modified, e.g. by chemical modification, to reduce or preferably incapacitate its ability to bind the neurotoxin to receptors at the neuromuscular junction is combined with the L-chain of a different clostridial neurotoxin. The hybrid, modified clostridial neurotoxin is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention, the H_N portion of a clostridial neurotoxin is combined with the L-chain of a different clostridial neurotoxin. The hybrid LH_N is then covalently linked, using linkages which may include one or more spacer regions, to a TM.

In another embodiment of the invention the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is linked, using linkages which may include one or more spacer regions, to a TM which can also effect the internalization of the light chain, or fragment thereof containing endopeptidase activity, into the cytoplasm of the cell.

In another embodiment of the invention the agent is expressed recombinantly as a fusion protein which includes an appropriate fragment of a Targeting Moiety in addition to any desired spacer domains. The recombinantly expressed agent may be derived wholly from the gene encoding one serotype of neurotoxin or be a chimera derived from the genes encoding two different serotypes.

In another embodiment of the invention the required LH_N, which may be a hybrid of an L and H_N from different clostridial toxin types, is expressed recombinantly as a fusion protein with the TM, and may also include one or more spacer regions.

In another embodiment of the invention the light chain of a clostridial neurotoxin, or a fragment of the light chain containing the endopeptidase activity, is expressed recombinantly as a fusion protein with a TM which can also affect the internalization of the light chain, or fragment thereof containing the endopeptidase activity, into the cytoplasm of the cell. The expressed fusion protein may also include one or more spacer regions.

Exploitation in Industry

The agent described in this invention can be used in vivo, either directly or as a pharmaceutically acceptable salt, for treatment of pain.

For example, an agent according to the invention can be used systemically for the treatment of severe chronic pain. A specific example of this is the use in treatment of clinical pain associated with rheumatoid arthritis affecting multiple joints.

In another example, an agent according to the invention can be locally applied for the treatment of pain. A specific example of this is treatment by local injection into a joint affected by inflammatory pain.

In further example an agent according to the invention can be administered by spinal injection (epidural or intrathecal) at the level of the spinal segment involved in the innervation of an affected organ for the treatment of pain. This is, for example, applicable in the treatment of deep tissue pain, such as chronic malignant pain.

The present invention will now be described by reference to the following examples:

Example 1. Synthesis of a conjugate of NGF and the LH_N fragment of BoNT/A.

Lyophilised murine 2.5 S NGF was dissolved by the addition of water and dialysed into MES buffer (0.1 M MES, 0.1 M sodium chloride, pH 5.0). To this solution (at a concentration of about 0.3 mg/ml) was added PDPH (100 mg/ml in DMF) to a final concentration of 1.25 mg/ml. After mixing, solid EDAC was added to produce a final concentration of about 1.25 mg/ml. The reaction was allowed to proceed for at least 30 min at room temperature. Excess PDPH was then removed by repeated concentration and dilution using Microcon 10 microconcentrators (Amicon) and MES buffer.

Meanwhile an equal weight of BoNT/A LH_N (produced essentially by the method of Shone C.C., Hambleton, P., and Melling, J. 1987, *Eur. J. Biochem.* 167, 175-180),

dissolved in triethanolamine buffer (0.02 M triethanolamine/HCl, 0.1 M sodium chloride, pH 7.8) at a concentration of about 1 mg/ml, was reacted with Traut's reagent (100 mM stock solution in 1 M triethanolamine/HCl, pH 7.8) at a final concentration of 2 mM. After one hour the LHN was desalted into PBSE (phosphate buffered saline with 1 mM EDTA, degassed before use) using a PD-10 column (Pharmacia). The protein peak from the column eluate was concentrated using a Microcon 50 (Amicon) to a concentration of about 2 mg/ml.

The derivatized NGF was subjected to a final concentration step resulting in a reduction in volume to less than 10 % of the starting volume and then mixed with the derivatized LHN overnight at room temperature. The products of the reaction were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE).

The conjugate resulting from the above reaction was partially purified by size exclusion chromatography over Superose 6 (Pharmacia). The elution profile was followed by measuring the optical density at 280 nm and SDS-PAGE analysis of the fractions. This allowed the separation of conjugate from free NGF and by-products of the reaction. The fractions containing conjugate were stored at 4 C until required.

Example 2. Activities of NGF-LHN conjugate in PC-12 cells.

PC12 cells are a cell-line of neuroectodermal derivation that are commonly used as a model system for the study of nerve function. As a model system for testing the function of an NGF-LHN conjugate they have two necessary features: firstly they are well known to possess cell-surface receptors for NGF that have been shown to be involved in a differentiation process in response to low concentrations of NGF. Secondly they have been shown to contain the exocytotic machinery for neurotransmitter release including, importantly in this example, SNAP-25.

PC12 cells were plated out into a 24-well plate that had been coated with MATRIGEL basement membrane matrix (Collaborative Biomedical Products) at a density of approximately 5×10^5 cells per well. After 24 h in culture (RPMI 1640 with 10% horse serum and 5% foetal calf serum, 37 C, 5% CO₂) the medium was replaced with fresh medium containing added conjugate (prepared as described in example 1) or LHN or NGF alone or in combination. After a further period in culture the medium was removed and the cells washed once with PBS. Cells were then lysed by the addition of 0.5 ml sodium hydroxide (0.2 M) for 30 min. After this time the solutions were neutralised by the addition of 0.5 ml hydrochloric acid (0.2 M) followed by 0.1 ml of Tris/HCl (1 M, pH 8.0). To extract the membrane proteins from these mixtures Triton-X-114 (10%, v/v) was added and incubated on ice for 30 min, the insoluble material was removed by centrifugation and the supernatants were then warmed to 37 C for 30 min. The resulting two phases were separated by centrifugation and the upper phase discarded. The proteins in the lower phase were precipitated with chloroform/methanol for analysis by Western blotting.

These samples were separated by SDS-PAGE and transferred to nitrocellulose. Cleavage of SNAP-25, a crucial component of the neurosecretory process and the substrate for the zinc-dependent endopeptidase activity of BoNT/A, was then detected by immunoblotting with an antibody that recognises the newly revealed carboxy-terminus of the cleaved SNAP-25. No immunoreactivity was observed in samples from control cells whereas a band corresponding to a molecular mass of 29 kDa was observed weakly in samples incubated with LHN and NGF and strongly in samples incubated with NGF-LHN conjugate.

Example 3. The production of a chimaeric LHN whereof the L chain is derived from BoNT/B and the HN fragment from BoNT/A.

The HN fragment of BoNT/A is produced according to the method described by Shone C.C., Hambleton, P., and Melling, J. (1987, *Eur. J. Biochem.* 167, 175-180) and the L-chain of BoNT/B according to the method of Sathyamoorthy, V. and DasGupta, B.R. (1985, *J. Biol. Chem.* 260, 10461-10466). The free cysteine on the HN fragment of BoNT/A is then derivatised by the addition of a ten-fold molar excess of dipyrindyl disulphide followed by incubation at 4 C overnight. The excess dipyrindyl disulphide and the thiopyridone by-product are then removed by desalting the protein over a PD10 column (Pharmacia) into PBS.

The derivatised HN is then concentrated to a protein concentration in excess of 1 mg/ml before being mixed with an equimolar portion of L-chain from BoNT/B (>1 mg/ml in PBS). After overnight incubation at room temperature the mixture is separated by size exclusion chromatography over Superose 6 (Pharmacia) and the fractions analysed by SDS-PAGE. The chimaeric LHN is then available for derivatisation to produce a targeted conjugate as described in Example 1.

Table 1 - Targeting Moieties (TM)

Growth Factors:

1. Nerve growth factor (NGF);
2. Leukaemia inhibitory factor (LIF);
3. Basic fibroblast growth factor (bFGF);
4. Brain-derived neurotrophic factor (BDNF);
5. Neurotrophin-3 (NT-3);
6. Hydra head activator peptide (HHAP);
7. Transforming growth factor β 1 (TGF- β 1);

Table 1 contd.

8. Transforming growth factor $\beta 2$ (TGF- $\beta 2$);
9. Transforming growth factor α (TGF- α);
10. Epidermal growth factor (EGF);
11. Ciliary neuron trophic factor (CNTF).

Cytokines:

1. Tumour necrosis factor α (TNF- α);
2. Interleukin-1 α (IL-1 α);
3. Interleukin-1 β (IL-1 β);
4. Interleukin-8 (IL-8).

Peptides:

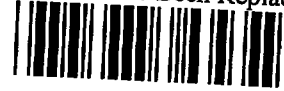
1. β -Endorphin;
2. Methionine - enkephalin;
3. D-Ala²-D-Leu⁵-enkephalin;
4. Bradykinin.

Antibodies:

1. Antibodies against the lactoseries carbohydrate epitopes found on the surface of dorsal root ganglion neurons (e.g. monoclonal antibodies 1B2 and LA4);
2. Antibodies against any of the receptors for the ligands given above.



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Claims

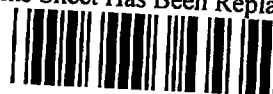
1. An agent specific for peripheral sensory afferents for inhibiting the transmission of signals between a primary sensory afferent and a projection neuron by controlling the release of at least one neurotransmitter or neuromodulator from the primary sensory afferent.
2. An agent according to Claim 1 which comprises a molecule capable of introducing a protease activity into the cytosol of a primary sensory afferent.
3. An agent according to Claim 2 in which the protease activity is the Zn^{2+} -dependent protease activity of the light chain (L-chain) of a clostridial neurotoxin or a fragment thereof retaining the Zn^{2+} -dependent protease activity.
4. An agent according to any preceding Claim which comprises a Targeting Moiety (TM) coupled to a clostridial neurotoxin in which the heavy chain (H-chain) is removed or modified, the TM being capable of functionally interacting with a binding site causing a physical association between the agent and the surface of a primary sensory afferent.
5. An agent according to any preceding Claim which comprises a Targeting Moiety (TM) coupled to a clostridial neurotoxin in which the HC part of the H-chain is removed or modified.
6. An agent according to Claim 5 in which the modified H-chain is the H_N -fragment of a clostridial neurotoxin.
7. An agent according to any of Claims 3-6 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin.
8. An agent according to any of Claims 3-7 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type A.
9. An agent according to any of Claims 3-7 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type B.
10. An agent according to any of Claims 3-9 in which the H-chain is modified by chemical derivatisation to reduce or remove its native binding affinity for motor neurons.



11. An agent according to any of Claims 3-9 in which the H-chain is modified by mutation to reduce or remove its native binding affinity for motor neurons.
12. An agent according to any of Claims 3-9 in which the H-chain is modified by proteolysis.
13. An agent according to Claim 12 which is formed by the coupling of a TM to the LHN fragment of botulinum neurotoxin type A.
14. An agent according to any of Claims 3-12 in which the H-chain is obtained from a different clostridial neurotoxin than that from which the L-chain is obtained.
15. An agent according to Claim 14 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from botulinum neurotoxin type B.
16. An agent according to Claim 15 which is composed of a TM linked to the HN fragment of botulinum neurotoxin type A and the L-chain of botulinum neurotoxin type B.
17. An agent according to any of Claims 3-16 in which the L-chain or L-chain fragment is linked to the H-chain by a direct covalent linkage.
18. An agent according to any of Claims 3-13 in which the L-chain or L-chain fragment is linked to the H-chain by a covalent linkage which includes one or more spacer regions.
19. An agent according to any of Claims 3-18 in which the TM is capable of delivering the L-chain or L-chain fragment into the cytosol of a primary sensory afferent unaided.
20. An agent according to any of Claims 3-19 in which the ability to deliver the L-chain or L-chain fragment into the cytosol of a primary sensory afferent is entirely contained within the TM.
21. An agent according to any of Claims 4-20 in which the TM comprises a ligand to a cell-surface binding site on a primary sensory afferent.
22. An agent according to any of Claims 4-21 in which the TM binds to a binding site which is characteristic of a particular defined population of primary sensory afferents.
23. An agent according to any of Claims 4-22 in which the TM binds to a binding site which is characteristic of a particular defined population of primary nociceptive afferents.
24. An agent according to any of Claims 4-23 in which the TM binds to a binding site which undergoes retrograde transport within a primary sensory afferent.



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25. An agent according to any of Claims 4-24 in which the TM binds to a binding site which undergoes retrograde transport within a primary nociceptive afferent.
26. An agent according to any of Claims 4-25 in which the TM comprises a ligand to a cell-surface receptor on a primary sensory afferent.
27. An agent according to any of Claims 4-26 in which the TM comprises a ligand to a growth factor receptor on a primary sensory afferent.
28. An agent according to any of Claims 4-27 in which the TM comprises a ligand to a neuropeptide receptor on a primary sensory afferent.
29. An agent according to any of Claims 4-27 in which the TM comprises a ligand to a cytokine receptor on a primary sensory afferent.
30. An agent according to any of Claims 4-27 in which the TM comprises a ligand to a hormone receptor on a primary sensory afferent.
31. An agent according to any of Claim 4-30 in which the TM comprises a monoclonal antibody or is derived from a monoclonal antibody to a surface antigen on a primary sensory afferent.
32. An agent according to Claim 27 in which the TM comprises a ligand to a nerve growth factor receptor.
33. An agent according to Claim 32 in which the TM comprises nerve growth factor.
34. An agent according to Claim 33 which comprises nerve growth factor linked to the LHN fragment of botulinum neurotoxin type A.
35. An agent according to any preceding Claim in which the TM is linked to the clostridial neurotoxin-derived component by a direct covalent linkage.
36. An agent according to any preceding Claim in which the TM is linked to the clostridial neurotoxin-derived component by a covalent linkage which includes one or more spacer regions.
37. An agent according to any preceding Claim which prevents the release of a neurotransmitter or neuromodulator from a primary sensory afferent.
38. An agent according to any preceding Claim which inhibits the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent.
39. A method for obtaining an agent according to any preceding Claim which comprises the covalent attachment of a TM to a modified clostridial neurotoxin or a fragment of a clostridial neurotoxin.



40. A method for obtaining an agent according to any of Claims 1-38 which comprises the covalent attachment of a TM to a modified clostridial neurotoxin or a fragment of a clostridial neurotoxin with the inclusion of one or more spacer regions.
41. A method for obtaining an agent according to any of Claims 1-38 which comprises constructing a genetic construct which codes for the agent, incorporating said agent into a host organism and expressing the construct to produce the agent.
42. A method of controlling the release of a neurotransmitter or neuromodulator from a primary sensory afferent by applying the agent of any one of Claims 1-38.
43. A method of controlling the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent by applying the agent of any one of Claims 1-38.
44. A method of controlling the transmission of sensory information from a primary sensory afferent to a projection neuron by applying the agent of any one of Claims 1-38.
45. A method of controlling the transmission of sensory information from a primary nociceptive afferent to a projection neuron by applying the agent of any one of Claims 1-38.
46. A method of controlling the sensation of pain by applying the agent of any one of Claims 1-38.
47. Use of the agent according to any one of Claims 1-38 or a pharmaceutically acceptable salt thereof as a medicament for the alleviation of pain.
48. Use of the agent according to any one of Claims 1-38 or a pharmaceutically acceptable salt thereof as a medicament for the prevention of pain.
49. Use of the agent according to any one of Claims 1-38 in the manufacture of a medicament for the alleviation of pain.
50. Use of the agent according to any one of Claims 1-38 in the manufacture of a medicament for the prevention of pain.
51. A method of alleviating pain which comprises administering an effective dose of the agent according to any one of Claims 1-38.
52. A method of preventing pain which comprises administering an effective dose of the agent according to any one of Claims 1-38.



53. An agent specific for peripheral sensory afferents which can inhibit the release of at least one neurotransmitter or neuromodulator or both from the synaptic terminals of nociceptive afferents, the agent having the following discrete functions:

1) It binds to a surface structure (the Binding Site [BS]) which is characteristic of, and has a degree of specificity for, nociceptive afferent neurons;

2) It enters the neuron;

3) It enters the cytosol; and

4) It modifies components of the exocytotic machinery present in the synaptic terminals of the central processes of the neurons, such that the release of at least one neurotransmitter or neuromodulator from the synaptic terminal is reduced or prevented.