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(54) Title: COMPOSITION FOR REDUCING NERVOUS SYSTEM INJURY AND METHOD OF MAKING AND USE THEREOF

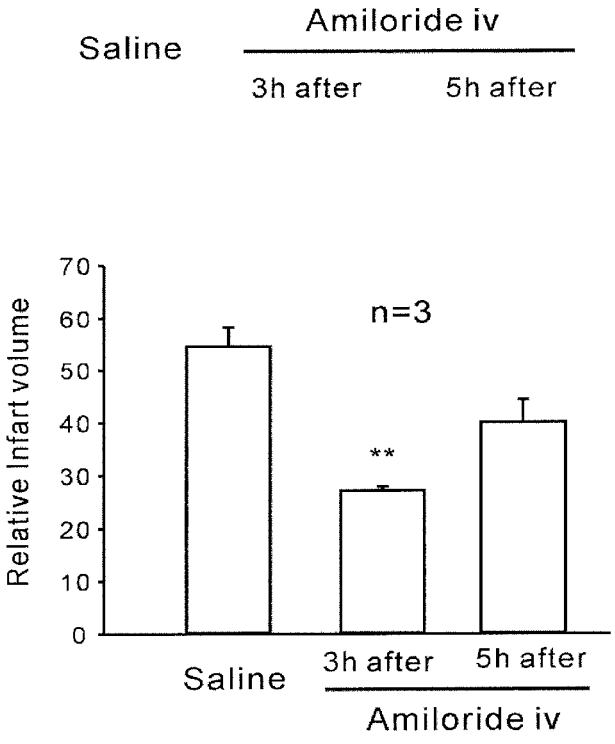

FIG. 20

(57) Abstract: This application discloses a composition comprising an amiloride and/or an amiloride analog which can be used for reducing nerve injury or nervous system injury in a subject. The formulation of such composition is also disclosed. The application further directs to methods for treating nerve injury or nervous system injury by administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof.

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TITLE

COMPOSITION FOR REDUCING NERVOUS SYSTEM INJURY AND METHOD OF MAKING AND USE THEREOF

FIELD

[0001] This application relates to the field of neurology. In particular, this application directs to compositions comprising an amiloride and/or an amiloride analog which can be used for reducing nerve injury or nervous system injury in a subject..

BACKGROUND

[0002] Nerve injuries may be caused by many conditions, such as degenerative nerve diseases, stroke, ischemia, chemical and mechanical injury to the nervous system. Many types of nerve injury result in changes in the ion flux into neurons which, in turn, lead to neuron cell death. Accordingly, various ion channels may be candidates for mediating this altered ion flux, thus reducing the extent of nerve injuries.

SUMMARY

[0003] One aspect of the present application relates to a method for reducing nerve injury in a subject. The method comprises administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising an active ingredient selected from the group consisting of amiloride and amiloride analogs. In some embodiments, the pharmaceutical composition is administered intravenously, intrathecally or intracerebroventricularlly.

[0004] In some embodiments, the active ingredient comprises amiloride or a pharmaceutically acceptable salt thereof.

[0005] In other embodiments, the active ingredient comprises an amiloride analog or a pharmaceutically acceptable salt thereof. In a related embodiment, the amiloride analog is

selected from the group consisting of benzamil, phenmil, 5-(N-ethyl-N-isobutyl)-amiloride (EIPA), bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride. In another related embodiment, the amiloride analog is benzamil. In another related embodiment, the amiloride analog is a methylated analog of benzamil. In another related embodiment, the amiloride analog comprises a ring formed on a guanidine group. In another related embodiment, the amiloride analog comprises an acylguanidino group. In another related embodiment, the amiloride analog comprises a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

[0006] In some embodiments, the amiloride, amiloride analog or a pharmaceutically acceptable salt thereof is given in a dose range of 0.1 mg - 10 mg /kg body weight.

[0007] In some embodiments, the pharmaceutical composition is administered within one hour of the onset of an ischemic event, within five hours of the onset of an ischemic event, or between one hour and five hours of the onset of an ischemic event.

[0008] In some embodiments, the nerve injury is brain injury.

[0009] Another aspect of the present application relates to a method for treating brain injury in a subject. The method comprises administering to said subject a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog, or a pharmaceutically acceptable salt thereof. In some embodiments, the pharmaceutical composition is administered intravenously, intrathecally or intracerebroventricularly.

[0010] In some embodiments, the amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride. In a related embodiment, the amiloride analog is benzamil. In other embodiments, the amiloride analog is selected from the group consisting of methylated

analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

[0011] Another aspect of the present application relates to a method for reducing nervous system injury caused by a change of ion flux into neurons. The method comprises administering to a subject in need of such treatment a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof.

[0012] In some embodiments, the pharmaceutical composition is administered intravenously, intrathecally, intracerebroventricularly or intramuscularly.

[0013] In other embodiments, the amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride. In a related embodiment, the amiloride analog is benzamil. In other embodiments, the amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

[0014] Another aspect of the present application relates to a method for reducing nervous system injury. The method comprises administering to a subject in need of such treatment a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof.

[0015] In some embodiments, the pharmaceutical composition is administered intravenously, intrathecally, intracerebroventricularly or intramuscularly.

[0016] In other embodiments, the amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride. In a related embodiment, the amiloride analog is benzamil. In other embodiments, the amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

[0017] Another aspect of the present application relates to a pharmaceutical composition for reducing nervous system injury. The pharmaceutical composition comprises an effective amount of amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof; and a pharmaceutically acceptable carrier, wherein the pharmaceutical composition is formulated for intravenous, intrathecal or intracerebroventricular injection.

[0018] In some embodiments, the pharmaceutical composition comprises an amiloride analog or a pharmaceutically acceptable salt thereof, wherein the amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

[0019] In other embodiments, the pharmaceutical composition comprises an amiloride analog or a pharmaceutically acceptable salt thereof, wherein the amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an

acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

[0020] Another aspect of the present application relates to a pharmaceutical composition for reducing nervous system injury. The pharmaceutical composition comprises an effective amount of an amiloride analog or a pharmaceutically acceptable salt thereof; and a pharmaceutically acceptable carrier.

[0021] In some embodiments, the pharmaceutical composition is formulated for intravenous, intrathecal, intracerebroventricular or Intramuscular injection.

[0022] In one embodiment, the amiloride analog is a methylated analog of benzamil. In another embodiment, the amiloride analog comprises a ring formed on a guanidine group. In another embodiment, the amiloride analog comprises an acylguanidino group. In yet another embodiment, the amiloride analog comprises a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] FIG. 1 is a view of a flowchart illustrating an exemplary method of reducing neuroinjury in an ischemic subject.

[0024] FIG. 2 is a view of a flowchart illustrating an exemplary method of identifying drugs for treating ischemia-related nerve injury.

[0025] FIG. 3 is a series of graphs presenting exemplary data related to the electrophysiology and pharmacology of acid sensing ion channel (ASIC) proteins in cultured mouse cortical neurons.

[0026] FIG. 4 is an additional series of graphs presenting exemplary data related to the electrophysiology and pharmacology of ASIC proteins in cultured mouse cortical neurons.

[0027] FIG. 5 is a set of graphs and traces presenting exemplary data showing that modeled ischemia may enhance activity of ASIC proteins, in accordance with aspects of the present teachings.

[0028] FIGS. 6 and 7 are a set of graphs and traces presenting exemplary data showing that ASIC proteins in cortical neurons may be Ca^{2+} permeable, and that Ca^{2+} permeability may be ASIC1a dependent.

[0029] FIG. 8 is a series of graphs presenting exemplary data showing that acid incubation may induce glutamate receptor-independent neuronal injury that is protected by ASIC blockade.

[0030] FIG. 9 is a series of graphs presenting exemplary data showing that ASIC1a may be involved in acid-induced injury *in vitro*.

[0031] FIG. 10 is a series of graphs with data showing neuroprotection in brain ischemia in vivo by ASIC1a blockade and by ASIC1 gene knockout.

[0032] FIG. 11 is a graph plotting exemplary data for the percentage of ischemic damage produced by stroke in an animal model system as a function of the time and type of treatment.

[0033] FIG. 12 is a view of the primary amino acid sequence of an exemplary cystine knot peptide, PcTx1, with various exemplary peptide features shown.

[0034] FIG. 13 is a comparative view of the cystine knot peptide of FIG. 12 aligned with various exemplary deletion derivatives of the peptide.

[0035] FIG. 14 is an exemplary graph plotting the amplitude of calcium current measured in cells as a function of the ASIC family member(s) expressed in the cells.

[0036] FIG. 15 is a graph presenting exemplary data related to the efficacy of nasally administered PcTx venom in reducing ischemic injury in an animal model system.

[0037] FIG. 16 is a composite showing representative ASIC 1a current traces in CHO cells treated with benzamil (panel A) or 5-(N-ethyl-N-isopropyl) amiloride (EIPA) (panel B), and dose-dependent blockade of ASIC 1a current expressed in CHO cells by amiloride and amiloride analogs (panel C).

[0038] FIG. 17 is a composite showing representative ASIC 2a current traces in CHO cells treated with benzamil (panel A) or amiloride (panel B), and dose-dependent blockade of ASIC 2a current expressed in CHO cells by amiloride and amiloride analogs (panel C).

[0039] FIG. 18 is a graph showing reduction of infarct volume in mice by intracerebroventricular injections of amiloride or amiloride analogs.

[0040] FIG. 19 is a composite showing reduction of infarct volume in the cortical tissue of mice by intravenous injection of saline or amiloride 60 min after MCAO.

[0041] FIG. 20 is a composite showing reduction of infarct volume in the cortical tissue of mice by intravenous injection of saline or amiloride 3 hours or 5 hours after MCAO.

[0042] FIG. 21 shows structure activity relationship (SAR) for hydrophobic amiloride analogs on various channels.

DETAILED DESCRIPTION

[0043] The present application provides methods and compositions for reducing nerve injury. The nerve injury may be caused by degenerative nervous system diseases, stroke, ischemia, trauma, chemical and mechanical injury to the nervous system. As used herein, the term "nervous system" includes both the central nervous system and the peripheral nervous system." The term "central nervous system" or "CNS" includes all cells and tissue of the brain and spinal cord of a vertebrate. The term "peripheral nervous system" refers to all cells and tissue of the portion of the nervous system outside the brain and spinal

cord, such as the motor neurons that mediate voluntary movement, the autonomic nervous system that includes the sympathetic nervous system and the parasympathetic nervous system and regulates involuntary functions, and the enteric nervous system that controls the gastrointestinal system. Thus, the term "nervous system" includes, but is not limited to, neuronal cells, glial cells, astrocytes, cells in the cerebrospinal fluid (CSF), cells in the interstitial spaces, cells in the protective coverings of the spinal cord, epidural cells (*i.e.*, cells outside of the dura mater), cells in non-neural tissues adjacent to or in contact with or innervated by neural tissue, cells in the epineurium, perineurium, endoneurium, funiculi, fasciculi, and the like.

[0044] In some embodiments, the nerve injury is a nervous system injury. In other embodiments, the nerve injury is brain injury. In some embodiments, the nerve injury is a nervous system injury caused by changes in the ion flux into neurons. For example, stroke/brain ischemia is a leading cause of morbidity and mortality. Over-activation of the postsynaptic glutamate receptors and subsequent Ca^{2+} toxicity plays a critical role in ischemic brain injury. The present application demonstrates that activation of Ca^{2+}-permeable acid-sensing ion channels (ASICs) is involved in acidosis-induced, glutamate receptor-independent, ischemic brain injury and provides a new direction for neuroprotection by targeting ASIC family members (ASICs). The present application further provides novel inhibitors of ASICs that have increased potency to homomeric ASICs channel and increased aqueous solubility. In some embodiments, the present application provides pharmaceutical compositions and methods for reducing nerve injury by inhibiting ASIC1a channel.

[0045] One aspect of the present application relates to a method for reducing nerve injury in a subject. The method comprises administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising an active ingredient selected from the group consisting of amiloride and amiloride analogs. In some embodiments, the

pharmaceutical composition is administered intravenously, intrathecally or intracerebroventricularlly.

[0046] In some embodiments, the active ingredient comprises amiloride or a pharmaceutically acceptable salt thereof. In other embodiments, the active ingredient comprises an amiloride analog or a pharmaceutically acceptable salt thereof. In a related embodiment, the amiloride analog is selected from the group consisting of benzamil, phenmil, 5-(N-ethyl-N-isobutyl)-amiloride (EIPA), bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride. In another related embodiment, the amiloride analog is benzamil. In another related embodiment, the amiloride analog is a methylated analog of benzamil. In another related embodiment, the amiloride analog comprises a ring formed on a guanidine group. In another related embodiment, the amiloride analog comprises an acylguanidino group. In another related embodiment, the amiloride analog comprises a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

[0047] In some embodiments, the amiloride, amiloride analog or a pharmaceutically acceptable salt thereof is given in a dose range of 0.1 mg - 10 mg /kg body weight. In other embodiments, the pharmaceutical composition is administered within one hour of the onset of an ischemic event, within five hours of the onset of an ischemic event, or between one hour and five hours of the onset of an ischemic event.

[0048] Another aspect of the present application relates to a method for treating brain injury in a subject. The method comprises administering to said subject a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog, or a pharmaceutically acceptable salt thereof. In some embodiments, the pharmaceutical composition is administered intravenously, intrathecally or intracerebroventricularly.

[0049] In some embodiments, the amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride. In a related embodiment, the amiloride analog is benzamil. In other embodiments, the amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

[0050] Another aspect of the present application relates to a method for reducing nervous system injury caused by a change of ion flux into neurons. The method comprises administering to a subject in need of such treatment a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof.

[0051] Another aspect of the present application provides a composition for treating ischemia or reducing injury resulting from ischemia. The method comprises the step of administering intravenously or intrathecally to a subject in need of such treatment a therapeutically effective amount of an active ingredient selected from the group consisting of amiloride, amiloride analogs, and salts thereof. The methods of the present application may provide one or more advantages over other methods of ischemia treatment. These advantages may include (1) less ischemia-induced injury, (2) fewer side effects of treatment (*e.g.,* due to selection of a more specific therapeutic target), and/or (3) a longer time window for effective treatment, among others.

[0052] FIG. 1 shows a flowchart 20 with exemplary steps 22, 24 that may be performed in a method of reducing nerve injury in an ischemic subject. The steps may be

performed any suitable number of times and in any suitable combination. In the method, an ischemic subject (or subjects) may be selected for treatment, indicated at 22. An ASIC-selective inhibitor then may be administered to the ischemic subject(s), indicated at 24. Administration of the inhibitor to the ischemic subject may be in a therapeutically effect amount, to reduce ischemia-induced injury to the subject, for example, reducing the amount of brain damage resulting from a stroke.

[0053] A potential explanation for the efficacy of the ischemia treatment of FIG. 1 may be offered by the data of the present teachings (*e.g.*, *see* Example 1). In particular, the damaging effects of ischemia may not be equal to acidosis, that is, acidification of tissue/cells via ischemia may not be sufficient to produce ischemia-induced injury. Instead, ischemia-induced injury may be caused, in many cases, by calcium flux into cells mediated by a member(s) of the ASIC family, particularly ASIC1a. Accordingly, selective inhibition of the channel activity of ASIC1a may reduce this harmful calcium flux, thereby reducing ischemia-induced injury.

[0054] FIG. 2 shows a flowchart 30 with exemplary steps 32, 34 that may be performed in a method of identifying drugs for treatment of ischemia. The steps may be performed any suitable number of times and in any suitable combination. In the method, one or more ASIC-selective inhibitors may be obtained, indicated at 32. The inhibitors then may be tested on an ischemic subject for an effect on ischemia-induced injury, indicated at 34.

NERVE INJURIES

[0055] The present application is directed to pharmaceutical compositions and methods for reducing nerve injuries in a subject. As used herein, the term "nerve injury" means an acute or chronic injury to or adverse condition of a nervous system tissue or cell resulting from physical transaction or trauma, contusion or compression or surgical lesion, vascular pharmacologic insults including hemorrhagic or ischemic damage, or from

neurodegenerative or any other neurological disease, or any other factor causing the injury to or adverse condition of the nervous system tissue or cell. In some embodiments, the nerve injury is caused by cognitive disorders, psychotic disorders, neurotransmitter-mediated disorders or neuronal disorders. Nerve injury includes injuries to the nervous system (*i.e.*, nervous system injuries) and brain injury.

[0056] As used herein, the term "cognitive disorders" refers to and intends diseases and conditions that are believed to involve or be associated with or do involve or are associated with progressive loss of structure and/or function of neurons, including death of neurons, and where a central feature of the disorder may be the impairment of cognition (*e.g.*, memory, attention, perception and/or thinking). These disorders include pathogen-induced cognitive dysfunction, *e.g.* HIV associated cognitive dysfunction or Lyme disease associated cognitive dysfunction. In some embodiments, the cognitive disorders are degenerative cognitive disorders. Examples of degenerative cognitive disorders include Alzheimer's Disease, Huntington's Disease, Parkinson's Disease, amyotrophic lateral sclerosis (ALS), autism, mild cognitive impairment (MCI), stroke, traumatic brain injury (TBI), age-associated memory impairment (AAMI) and epilepsy.

[0057] As used herein, the term "psychotic disorders" refers to and intends mental diseases or conditions that are believed to cause or do cause abnormal thinking and perceptions. Psychotic disorders are characterized by a loss of reality which may be accompanied by delusions, hallucinations (perceptions in a conscious and awake state in the absence of external stimuli which have qualities of real perception, in that they are vivid, substantial, and located in external objective space), personality changes and/or disorganized thinking. Other common symptoms include unusual or bizarre behavior, as well as difficulty with social interaction and impairment in carrying out the activities of daily living.

Exemplary psychotic disorders are schizophrenia, bipolar disorders, psychosis, anxiety, depression and chronic pain.

[0058] As used herein, the term "neurotransmitter-mediated disorders" refers to and intends diseases or conditions that are believed to involve or be associated with or do involve or are associated with abnormal levels of neurotransmitters such as histamine, glutamate, serotonin, dopamine, norepinephrine or impaired function of aminergic G protein-coupled receptors. Exemplary neurotransmitter-mediated disorders include spinal cord injury, diabetic neuropathy, allergic diseases and diseases involving geroprotective activity such as age-associated hair loss (alopecia), age-associated weight loss and age-associated vision disturbances (cataracts). Abnormal neurotransmitter levels are associated with a wide variety of diseases and conditions including, but not limited, to Alzheimer's disease, Parkinson's Disease, autism, Guillain-Barre syndrome, mild cognitive impairment, schizophrenia, anxiety, multiple sclerosis, stroke, traumatic brain injury, spinal cord injury, diabetic neuropathy, fibromyalgia, bipolar disorders, psychosis, depression and a variety of allergic diseases.

[0059] As used herein, the term "neuronal disorders" refers to and intends diseases or conditions that are believed to involve, or be associated with, or do involve or are associated with neuronal cell death and/or impaired neuronal function or decreased neuronal function. Exemplary neuronal indications include neurodegenerative diseases and disorders such as Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease, canine cognitive dysfunction syndrome (CCDS), Lewy body disease, Menkes disease, Wilson disease, Creutzfeldt-Jakob disease, Fahr disease, an acute or chronic disorder involving cerebral circulation, such as ischemic or hemorrhagic stroke or other cerebral hemorrhagic insult, age-associated memory impairment (AAMI), mild cognitive impairment (MCI), injury-related mild cognitive impairment (MCI), post-concussion syndrome, post-

traumatic stress disorder, adjuvant chemotherapy, traumatic brain injury (TBI), neuronal death mediated ocular disorder, macular degeneration, age-related macular degeneration, autism, including autism spectrum disorder, Asperger syndrome, and Rett syndrome, an avulsion injury, a spinal cord injury, myasthenia gravis, Guillain-Barre syndrome, multiple sclerosis, diabetic neuropathy, fibromyalgia, neuropathy associated with spinal cord injury, schizophrenia, bipolar disorder, psychosis, anxiety or depression, and chronic pain..

[0060] In some embodiments, the nerve injuries or nervous system injuries are caused by a change in the ion flux into neurons or a nervous system tissue. As used herein, the term "nervous system tissue" refers to animal tissue comprising nerve cells, the neuropil, glia, neural inflammatory cells, and endothelial cells in contact with " nervous system tissue". "Nerve cells" may be any type of nerve cell known to those of skill in the art including, but not limited to neurons. As used herein, the term "neuron" represents a cell of ectodermal embryonic origin derived from any part of the nervous system of an animal. Neurons express well-characterized neuron-specific markers, including neurofilament proteins, NeuN (Neuronal Nuclei marker), MAP2, and class III tubulin. Included as neurons are, for example, hippocampal, cortical, midbrain dopaminergic, spinal motor, sensory, enteric, sympathetic, parasympathetic, septal cholinergic, central nervous system and cerebellar neurons. "Glial cells" useful in the present invention include, but are not limited to astrocytes, Schwan cells, and oligodendrocytes. "Neural inflammatory cells" useful in the present invention include, but are not limited to cells of myeloid origin including macrophages and microglia.

[0061] In some embodiments, the pharmaceutical compositions and methods of the present application relate to reducing nerve injuries caused by ischemia or an ischemia-related condition. Ischemia, as used herein, is a reduced blood flow to an organ(s) and/or tissue(s). The reduced blood flow may be caused by many mechanisms, including but are not

limited to, a partial or complete blockage (an obstruction), a narrowing (a constriction), and/or a leak/rupture , of one or more blood vessels that supply blood to the organ(s) and/or tissue(s). Ischemia may be created by thrombosis, an embolism, atherosclerosis, hypertension, hemorrhage, an aneurysm, surgery, trauma, medication, and the like. The reduced blood flow thus may be chronic, transient, acute or sporadic.

[0062] Any organ or tissue may experience a reduced blood flow and required treatment for ischemia. Exemplary organs and/or tissues include, but are not limited to, brain, arteries, heart, intestines and eye (*e.g.*, the optic nerve). Ischemia-induced injuries (*i.e.*, disease and/or damage produced by various types of ischemia) include, but are not limited to, ischemic myelopathy, ischemic optic neuropathy, ischemic colitis, coronary heart disease, and/or cardiac heart disease (*e.g.*, angina, heart attack, *etc.*), among others. Ischemia-induced injury thus may damage and/or kill cells and/or tissue, for example, producing necrotic (infarcted) tissue, inflammation, and/or tissue remodeling, among others, at affected sites within the body. Treatment according to aspects of the present application may reduce the incidence, extent, and/or severity of this injury.

[0063] An ischemia-related condition may be any consequence of ischemia. The consequence may be substantially concurrent with the onset ischemia (*e.g.*, a direct effect of the ischemia) and/or may occur substantially after ischemia onset and/or even after the ischemia is over (*e.g.*, an indirect, downstream effect of the ischemia, such reperfusion of tissue when ischemia ends). Exemplary ischemia-related conditions may include any combination of the symptoms (and/or conditions) listed above. Alternatively, or in addition, the symptoms may include local and/or systemic acidosis (pH decrease), hypoxia (oxygen decrease), free radical generation, and/or the like.

[0064] In some embodiments, the ischemia-related condition is stroke. Stroke, as used herein, is brain ischemia produced by a reduced blood supply to a part (or all) of the

brain. Symptoms produced by stroke may be sudden (such as loss of consciousness) or may have a gradual onset over hours or days. Furthermore, the stroke may be a major ischemic attack (a full stroke) or a more minor, transient ischemic attack, among others. Symptoms produced by stroke may include, for example, hemiparesis, hemiplegia, one-sided numbness, one-sided weakness, one-sided paralysis, temporary limb weakness, limb tingling, confusion, trouble speaking, trouble understanding speech, trouble *see*ing in one or both eyes, dim vision, loss of vision, trouble walking, dizziness, a tendency to fall, loss of coordination, sudden severe headache, noisy breathing, and/or loss of consciousness. Alternatively, or in addition, the symptoms may be detectable more readily or only via tests and/or instruments, for example, an ischemia blood test (*e.g.,* to test for altered albumin, particular protein isoforms, damaged proteins, *etc.*), an electrocardiogram, an electroencephalogram, an exercise stress test, brain CT or MRI scanning and/or the like.

[0065] Acid-base balance is important for biological systems. Normal brain function depends on the complete oxidation of glucose, with the end product of CO_2 and H_2O for its energy requirements. During ischemia, increased anaerobic glycolysis, due to the lack of oxygen supply, leads to lactic acid accumulation. Accumulation of lactic acid, along with increased H^+ release from ATP hydrolysis, causes decreases in tissue pH. Extracellular pH (pH_o) typically falls to 6.5 during ischemia, and it can fall below 6.0 during severe ischemia or under hyperglycemic conditions.

SUBJECTS OF NERVE INJURY

[0066] The method and pharmaceutical composition of the present application can be used in any subject that has a nerve injury or a history of nerve injury and/or a significant chance of developing nerve injury after treatment begins and during a time period in which the treatment is still effective. In some embodiments, the subject is an ischemic subjects. An ischemic subject, as used herein, is any person (a human subject) or animal (an animal

subject) that has ischemia, an ischemia-related condition, a history of ischemia, and/or a significant chance of developing ischemia after treatment begins and during a time period in which the treatment is still effective.

[0067] The subject may be an animal. The term "animal," as used herein, refers to any animal that is not human. Exemplary animals that may be suitable include any animal with a bloodstream, such as rodents (mice, rats, *etc.*), dogs, cats, birds, sheep, goats, non-human primates, *etc.* The animal may be treated for its own sake, *e.g.*, for veterinary purposes (such as treatment of a pet). Alternatively, the animal may provide an animal model of nerve injury, such as ischemia, to facilitate testing drug candidates for human use, such as to determine the candidates' potency, window of effectiveness, side effects, *etc.*

[0068] Ischemic subjects for treatment may be selected by any suitable criteria. Exemplary criteria may include any detectable symptoms of ischemia, a history of ischemia, an event that increases the risk of (or induces) ischemia (such as a surgical procedure, trauma, administration of a medication, *etc.*), and/or the like. A history of ischemia may involve one or more prior ischemic episodes. In some examples, a subject selected for treatment may have had an onset of ischemia that occurred at least about one, two, or three hours before treatment begins, or a plurality of ischemic episodes (such as transient ischemic attacks) that occurred less than about one day, twelve hours, or six hours prior to initiation of treatment.

ASIC INHIBITORS, AMILORIDE AND AMILORIDE ANALOGS

[0069] Inhibitors of ASIC family members, as used herein, are substances that reduce (partially, substantially, or completely block) the activity or one or more members of the ASIC family, that is, ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4, among others. In some examples, the inhibitors may reduce the channel activity of one or more members, such as the ability of the members to flux ions (*e.g.*, sodium, calcium, and/or potassium ions, among others) through cell membranes (into and/or out of cells). The substances may be

compounds (small molecules of less than about 10 kDa, peptides, nucleic acids, lipids, *etc.*), complexes of two or more compounds, and/or mixtures, among others. Furthermore, the substances may inhibit ASIC family members by any suitable mechanism including competitive, noncompetitive, uncompetitive, and/or mixed inhibition, among others.

[0070] The inhibitor may be an ASIC1a inhibitor that inhibits acid sensing ion channel 1a (ASIC1a). ASIC1a, as used herein, refers to an ASIC1a protein or channel from any species. For example, an exemplary human ASIC1a protein/channel is described in Waldmann, R., *et al.* 1997, Nature 386, pp. 173-177, which is incorporated herein by reference.

[0071] The expression "ASIC1a inhibitor" may refer to a product which, within the scope of sound pharmacological judgment, is potentially or actually pharmaceutically useful as an inhibitor of ASIC1a, and includes reference to substances which comprise a pharmaceutically active species and are described, promoted, or authorized as an ASIC1a inhibitor.

[0072] An ASIC1a inhibitor may be selective within the ASIC family. Selective inhibition of ASIC1a, as used herein, is inhibition that is substantially stronger on ASIC1a than on another ASIC family member(s) when compared (for example, in cultured cells) after exposure of each to the same (sub-maximal) concentration(s) of an inhibitor. The inhibitor may inhibit ASIC1a selectively relative to at least one other ASIC family member (ASIC1b, ASIC2a, ASIC2b, ASIC3, ASIC 4, *etc.*) and/or selectively relative to every other ASIC family member. The strength of inhibition for a selective inhibitor may be described by an inhibitor concentration at which inhibition occurs (*e.g.*, an IC_{50} (inhibitor concentration that produces 50% of maximal inhibition) or a K_i value (inhibition constant or dissociation constant)) relative to different ASIC family members. An ASIC1a-selective inhibitor may inhibit ASIC1a activity at a concentration that is at least about two-, four-, or ten-fold lower

(one-half, one-fourth, or one-tenth the concentration or lower) than for inhibition of at least one other or of every other ASIC family member. Accordingly, an ASIC1a-selective inhibitor may have an IC_{50} and/or K_i for ASIC1a inhibition that is at least about two-, four-, or ten-fold lower (one-half, one-fourth, or one-tenth or less) than for inhibition of at least one other ASIC family member and/or for inhibition of every other ASIC family member.

[0073] An ASIC1a-selective inhibitor, in addition to being selective, also may be specific for ASIC1a. ASIC1a-specific inhibition, as used herein, is inhibition that is substantially exclusive to ASIC1a relative to every other ASIC family member, such as ASIC2a and ASIC3a. An ASIC1a-specific inhibitor may inhibit ASIC1a at an inhibitor concentration that is at least about twenty-fold lower (5% of the concentration or less) than for inhibition of every other ASIC family member. Accordingly, an ASIC1a-specific inhibitor may have an IC_{50} and/or K_i for ASIC1a relative to every other member of the ASIC family that is at least about twenty-fold lower (five percent or less), such that, for example, inhibition of other ASIC family members is at least substantially (or completely) undetectable. In some embodiments, the ASIC1a-selective inhibitor has increased potency to homomeric ASIC1a channel and increased aqueous solubility comparing to the commercially available amiloride-related ASIC1a inhibitors such as amiloride benzamil, phenamil, 5-(N-ethyl-N-isobutyl) amiloride (EIPA), bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

[0074] Any suitable ASIC inhibitor or combination of inhibitors may be used. For example, a subject may be treated with an ASIC1a-selective inhibitor and a nonselective ASIC inhibitor, or with an ASIC1a-selective inhibitor and an inhibitor to a non-ASIC channel protein, such as a non-ASIC calcium channel. In some examples, a subject may be treated with an ASIC1a-selective inhibitor and an inhibitor of NMDA receptors, such as a glutamate antagonist.

[0075] The inhibitor may be or include a peptide. The peptide may have any suitable number of amino acid subunits, generally at least about ten and less than about one-thousand subunits. In some examples, the peptide may have a cystine knot motif. A cystine knot, as used herein, generally comprises an arrangement of six or more cysteines. A peptide with these cysteines may create a "knot" including (1) a ring formed by two disulfide bonds and their connecting backbone segments, and (2) a third disulfide bond that threads through the ring. In some examples, the peptide may be a conotoxin from an arachnid and/or cone snail species. For example, the peptide may be PcTx1 (psalmotoxin 1), a toxin from a tarantula (Psalmopoeus cambridgei (Pc)).

[0076] In some examples, the peptide may be structurally related to PcTx1, such that the peptide and PcTx1 differ by at least one deletion, insertion, and/or substitution of one or more amino acids. For example, the peptide may have at least about 25% or at least about 50% sequence identity, and/or at least about 25% or at least about 50% sequence similarity with PcTx1 (*see* below). Further aspects of peptides that may be suitable as inhibitors are described below in Example 3.

[0077] Methods of alignment of amino acid sequences for comparison and generation of identity and similarity scores are well known in the art. Exemplary alignment methods that may be suitable include (Best Fit) of Smith and Waterman, a homology alignment algorithm (GAP) of Needleman and Wunsch, a similarity method (Tfasta and Fasta) of Pearson and Lipman, and/or the like. Computer algorithms of these and other approaches that may be suitable include, but are not limited to: CLUSTAL, GAP, BESTFIT, BLASTP, FASTA, and TFASTA.

[0078] As used herein, "sequence identity" or "identity" in the context of two peptides relates to the percentage of residues in the corresponding peptide sequences that are the same when aligned for maximum correspondence. In some examples, peptide residue positions

that are not identical may differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (*e.g.* charge or hydrophobicity) and therefore are expected to produce a smaller (or no) effect on the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards, to give a "similarity" of the sequences, which corrects for the conservative nature of the substitutions. For example, each conservative substitution may be scored as a partial rather than a full mismatch, thereby correcting the percentage sequence identity to provide a similarity score. The scoring of conservative substitutions to obtain similarity scores is well known in the art and may be calculated by any suitable approach, for example, according to the algorithm of Meyers and Miller, Computer Applic. Biol Sci., 4: 11-17 (1988), *e.g.*, as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif., USA).

[0079] Amiloride, a guanidinium group containing pyrazine derivative, has been used for the treatment of mild hypertension with little reported side effect. Amiloride works by directly blocking the epithelial sodium channel (ENaC) thereby inhibiting sodium reabsorption in the late distal convoluted tubules, connecting tubules, and collecting ducts in the kidneys. This promotes the loss of sodium and water from the body, but without depleting potassium. As used herein, the term "amiloride" refers to both amiloride and salts of amiloride, such as amiloride hydrochloride.

[0080] Amiloride analogs, as used herein, refer to chemical compounds having biological activities similar to those of amiloride but with a slightly altered chemical structure. Examples of amiloride analogs include, but are not limited to, benzamil, phenamil, 5-(N-ethyl-N-isobutyl) amiloride (EIPA), bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride. Other examples include amiloride analogs with a hydrophobic substitutent at

the C_5-NH_2 position and/or on the guanidino group, as shown in FIG. 21, as well as methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group. In some embodiments, the amiloride analogs do not to block the human Na^+/Ca^{2+} ion exchanger. In other embodiments, , the amiloride analogs are weak inhibitor of the Na^+/Ca^{2+} ion exchanger and help maintaining low levels of intracellular Ca^{2+}. In other embodiments, the amiloride analogs are very weak inhibitor of the Na^+/Ca^{2+} ion exchanger with an IC_{50} of 1.1 mM or less. In some other embodiments, the amiloride analogs do not block the ASIC2a and/or ASIC3 channels. In one embodiment, the amiloride analogs have increased selectivity for ASIC1a over the ASIC3 channel and/or ASIC2 channel.

[0081] As used herein, the term "amiloride analog" refers to both amiloride analog and salts of amiloride analog, such as 5-(N,N-dimenthyl)amiloride hydrochloride.

[0082] In some embodiments, amiloride and/or amiloride analogs are used in conjunction with other ASIC inhibitors such as PcTx1 and derivatives thereof.

ADMINISTRATION OF INHIBITORS

[0083] Administration (or administering), as used herein, includes any route of subject exposure to an inhibitor, under any suitable conditions, and at any suitable time(s). Administration may be self-administration or administration by another, such as a health-care practitioner (*e.g.*, a doctor, a nurse, *etc.*). Administration may be by injection (*e.g.*, intravenous, intramuscular, subcutaneous, intracerebral, introcerebroventricular, epidural, and/or intrathecal, among others), ingestion (*e.g.*, using a capsule, lozenge, a fluid composition, *etc.*), inhalation (*e.g.*, an aerosol (less than about 10 microns average droplet diameter) inhaled nasally and/or orally), absorption through the skin (*e.g.*, with a skin patch)

and/or mucosally (*e.g.*, through oral, nasal, and/or pulmonary mucosa, among others), and/or the like. Mucosal administration may be achieved, for example, using a spray (such as a nasal spray), an aerosol that is inhaled), and/or the like. A spray may be a surface spray (droplets on average greater than about 50 microns in diameter) and/or a space spray (droplets on average about 10-50 microns in diameter). In some examples, ischemia may produce an alteration of the blood-brain barrier of an ischemic subject, thus increasing the efficiency with which an inhibitor that is introduced (*e.g.*, by injection and/or absorption) into the bloodstream of a subject can reach the brain. Administration may be performed once or a plurality of times, and at any suitable time relative to ischemia diagnosis, to provide treatment. Accordingly, administration may be performed before ischemia has been detected (*e.g.*, prophylactically,) after a minor ischemic episode, during chronic ischemia, after a full stroke, and/or the like. In some embodiments, amiloride or amiloride analog is administered intravenously. In other embodiments, amiloride or amiloride analog is administered intracerebrally. In other embodiments, amiloride or amiloride analog is administered intracerebroventricularly. In other embodiments, amiloride or amiloride analog is administered intramuscularly. In other embodiments, amiloride or amiloride analog is administered intrathecally.

[0084] A therapeutically effective amount (or simply "an effective amount") of an inhibitor may be administered. A therapeutically effective amount or an effective amount of an inhibitor, as used herein, is any amount of the inhibitor that, when administered to subjects, reduces, in a significant number of the subjects, the degree, incidence, and/or extent of ischemia-induced injury in the subjects. Accordingly, a therapeutically effective amount may be determined, for example, in clinical studies in which various amounts of the inhibitor are administered to test subjects (and, generally, compared to a control group of subjects).

The therapeutically effective amount of inhibitor or inhibitors may be given by a single injection or multiple injections in a volume of 0.1-50 ml per injection.

[0085] In some embodiments, the inhibitor is amiloride, an amiloride analog or a salt thereof and is given at a daily dose (as a single dose or multiple dose) in the range of 0.01-30 mg/kg body weight, 0.01-10 mg/kg body weight, 0.01-3 mg/kg body weight, 0.01-1 mg/kg body weight, 0.01-0.3 mg/kg body weight, 0.01-0.1 mg/kg body weight, 0.01-0.03 mg/kg body weight, 0.03-30 mg/kg body weight, 0.03-10 mg/kg body weight, 0.03-3 mg/kg body weight, 0.03-1 mg/kg body weight, 0.03-0.3 mg/kg body weight, 0.03-0.1 mg/kg body weight, 0.1-30 mg/kg body weight, 0.1-10 mg/kg body weight, 0.1-3 mg/kg body weight, 0.1-1 mg/kg body weight, 0.1-0.3 mg/kg body weight, 0.3-30 mg/kg body weight, 0.3-10 mg/kg body weight, 0.3-3 mg/kg body weight, 0.3-1 mg/kg body weight, 1-30 mg/kg body weight, 1-10 mg/kg body weight, 1-3 mg/kg body weight, 3-30 mg/kg body weight, 3-10 mg/kg body weight or 10-30 mg/kg body weight. In one embodiment, the amiloride analog is selected from the group consisting of benzamil, phenamil, EIPA, bepridil, KB-R7943, 5-(N-methyl-N-isobutyl)-amiloride, 5-(N,N-hexamethylene)-amiloride and 5-(N,N-dimenthyl)amiloride hydrochloride. . In another embodiment, the amiloride analog has a hydrophobic substitutent at the C_5-NH_2 position and/or on the guanidino group. In another embodiment, the amiloride analog is selected from the amiloride analogs selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

[0086] In other embodiments, the inhibitor is amiloride, an amiloride analog or a salt thereof and is administered as a pharmaceutical composition formulated as a single dose in the range of 0.1-1000 mg/dose, 0.1-300 mg/dose, 0.1-100 mg/dose, 0.1-30 mg/dose, 0.1-10

mg/dose, 0.1-3 mg/dose, 0.1-1 mg/dose, 0.1-0.3 mg/dose, 0.3-1000 mg/dose, 0.3-300 mg/dose, 0.3-100 mg/dose, 0.3-30 mg/dose, 0.3-10 mg/dose, 0.3-3 mg/dose, 0.3-1 mg/dose, 1-1000 mg/dose, 1-300 mg/dose, 1-100 mg/dose, 1-30 mg/dose, 1-10 mg/dose, 1-3 mg/dose, 3-1000 mg/dose, 3-300 mg/dose, 3-100 mg/dose, 3-30 mg/dose, 3-10 mg/dose, 10-1000 mg/dose, 10-300 mg/dose, 10-100 mg/dose, 10-30 mg/dose, 30-1000 mg/dose, 30-300 mg/dose, 30-100 mg/dose, 100-1000 mg/dose, 100-300 mg/dose, or 300-1000 mg/dose. In one embodiment, the amiloride analog is selected from the group consisting of benzamil, phenamil, EIPA, bepridil, KB-R7943, 5-(N-methyl-N-isobutyl)-amiloride, 5-(N,N-hexamethylene)-amiloride and 5-(N,N-dimenthyl)amiloride hydrochloride. In another embodiment, the amiloride analog has a hydrophobic substitutent at the C_5-NH_2 position and/or on the guanidino group. In another embodiment, the amiloride analog is selected from the amiloride analogs selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group. In some embodiments, the pharmaceutical composition formulated for intravenous injection, intracerebral injection, intracerebroventricular injection, intrathecal injection or intramuscular injection.

[0087] The inhibitor may be administered in any suitable form and in any suitable composition to subjects. In some examples, the inhibitor may be configured as a pharmaceutically acceptable salt. The composition may be formulated to include, for example, a fluid carrier/solvent (a vehicle), a preservative, one or more excipients, a coloring agent, a flavoring agent, a salt(s), an anti-foaming agent, and/or the like. The inhibitor may be present at a concentration in the vehicle that provides a therapeutically effective amount of the inhibitor for treatment of ischemia when administered to an ischemic subject.

[0088] In some embodiments, amiloride analogs with higher water solubility or lipid solubility are produced. In certain embodiments, the amiloride analogs contain a water solubilizing group, such as an N,N-dimethyl amino group or a sugar, at the guanidino group to improve water solubility (formula 13-16, FIG. 24). In some embodiments, the amiloride analogs have a water solubility of 5 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM or higher. In other embodiments, the amiloride analogs have a solubility that allows for a 10 mg, 25 mg, 50 mg, 100 mg, 150 mg, 200 mg, 250 mg, 300 mg, 400 mg, or 500 mg dose to be administered intravenously to a human in a single 10 ml injection. In yet other embodiments, the amiloride analogs have a solubility that allows for a 10 mg, 25 mg, 50 mg, 100 mg, 150 mg, 200 mg, 250 mg, 300 mg, 400 mg, or 500 mg dose to be administered intracerebroventicularly to a human in a single 2 ml injection.

SYNTHESIS AND SCREENING OF AMILORIDE ANALOGS

[0089] Another aspect of the present application relates to the synthesis and screening of new amiloride analogs. Synthesis pathway of amiloride analogs are designed based on the desired analog structure. The newly synthesized amiloride analogs are then screened for their inhibitive effect on ASIC family members, such as ASIC1a and ASIC2a. One or more ASIC inhibitors, particularly ASIC1a inhibitors as described above, may be obtained. The inhibitors may be obtained by any suitable approach, such by screening a set of candidate inhibitors (*e.g.*, a library of two or more compounds) and/or by rationale design, among others.

[0090] Screening may involve any suitable assay system that measures interaction between ASIC proteins and the set of candidate inhibitors. Exemplary assay systems may include assays performed biochemically (*e.g.*, binding assays), with cells grown in culture ("cultured cells"), and/or with organisms, among others.

[0091] In some embodiments, a cell-based assay system is used to measure the effect of each candidate inhibitor on ion flux, such as acid-sensitive ion flux, in the cells. In some embodiments, the ion flux is a flux of calcium and/or sodium. In some embodiments, the assay system uses cells expressing an ASIC family member, such as ASIC1a or ASIC2a, or two or more distinct sets of cells expressing two or more distinct ASIC family members, such as ASIC1a and another ASIC family member(s), to determine the selectivity of each inhibitor for these family members. The cells may express each ASIC family member endogenously or through introduction of foreign nucleic acid. In some examples, the assay system may measure ion flux electrophysiologically (such as by patch clamp), using an ion-sensitive or membrane potential-sensitive dye (*e.g.*, a calcium sensitive dye such as Fura-2), or via a gene-based reporter system that is sensitive to changes in membrane potential and/or intracellular ion (*e.g.*, calcium) concentrations, among others. The assay system may be used to test candidate inhibitors for selective and/or specific inhibition of ASIC family members, particularly ASIC1a.

[0092] One or more ASIC inhibitors may be administered to a subject with a nerve injury, such as an ischemic subject to test the efficacy of the inhibitors for treatment of the nerve injury. The ischemic subjects may be people or animals. In some examples, the ischemic subjects may provide an animal model system of ischemia and/or stroke. Exemplary animal model systems include rodents (mice and/or rats, among others) with ischemia induced experimentally. The ischemia may be induced mechanically (*e.g.*, surgically) and/or by administration of a drug, among others. In some examples, the ischemia may be induced by occlusion of a blood vessel, such as by constriction of a mid-cerebral artery.

[0093] Another aspect of the present application relates to a pharmaceutical composition for reducing nervous system injury. The pharmaceutical composition comprises

an effective amount of amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof; and a pharmaceutically acceptable carrier, wherein the pharmaceutical composition is formulated for intravenous, intrathecal or intracerebroventricular injection.

[0094] In some embodiments, the pharmaceutical composition comprises an amiloride analog or a pharmaceutically acceptable salt thereof, wherein the amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

[0095] In other embodiments, the pharmaceutical composition comprises an amiloride analog or a pharmaceutically acceptable salt thereof, wherein the amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

[0096] In other embodiments, the pharmaceutical composition further comprises one or more other ASIC inhibitors. In one embodiment, the one or more other ASIC inhibitors comprise PcTx1 or a PcTx1 derivative.

[0097] Another aspect of the present application relates to a pharmaceutical composition for reducing nervous system injury. The pharmaceutical composition comprises an effective amount of an amiloride analog or a pharmaceutically acceptable salt thereof; and a pharmaceutically acceptable carrier.

[0098] In some embodiments, the pharmaceutical composition is formulated for intravenous, intrathecal, intracerebroventricular or Intramuscular injection.

[0099] In one embodiment, the amiloride analog is a methylated analog of benzamil. In another embodiment, the amiloride analog comprises a ring formed on a guanidine group. In another embodiment, the amiloride analog comprises an acylguanidino group. In yet another embodiment, the amiloride analog comprises a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

[0100] In other embodiments, the pharmaceutical composition further comprises one or more other ASIC inhibitors. In one embodiment, the one or more other ASIC inhibitors comprise PcTx1 or a PcTx1 derivative.

[0101] As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, sweeteners and the like. The pharmaceutically acceptable carriers may be prepared from a wide range of materials including, but not limited to, flavoring agents, sweetening agents and miscellaneous materials such as buffers and absorbents that may be needed in order to prepare a particular therapeutic composition. The use of such media and agents with pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Optionally, the amiloride and/or an amiloride analog may be mixed together into pharmaceutical compositions comprising supplementary active ingredients that are not contraindicated by said amiloride and/or an amiloride analog.

[0102] In some embodiments, the pharmaceutical composition is formulated for intravenous injection. In other embodiments, the pharmaceutical composition comprises amiloride and/or amiloride analog formulated for intravenous injection. In other embodiments, the pharmaceutical composition comprises amiloride and/or amiloride analog

formulated for intracerebroventricular injection. In other embodiments, the pharmaceutical composition comprises amiloride and/or amiloride analog formulated for intrathecal injection. In other embodiments, the pharmaceutical composition comprises amiloride and/or amiloride analog formulated for intramuscular injection. Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the injectable compositions are sterile and are fluid to the extent that easy syringability exists. The injectable composition must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the requited particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0103] Sterile injectable solutions can be prepared by incorporating amiloride and/or amiloride analog in the required amount in an appropriate solvent, followed by filtered

sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active, ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0104] In some embodiments, the pharmaceutical composition is provided in a dry form, and is formulated into a tablet or capsule form. Tablets may be formulated in accordance with conventional procedures employing solid carriers well-known in the art. Hard and soft capsules employed in the present invention can be made from any pharmaceutically acceptable material, such as gelatin or cellulosic derivatives.

[0105] In certain embodiments, the pharmaceutical composition is formulated for immediate release, extended-release, delayed-release or combinations thereof. Extended-release, also known as sustained-release, time-release or timed-release, controlled-release (CR), modified release (MR), or continuous-release (CR or Contin), is a mechanism used in medicine tablets or capsules to dissolve slowly and release the active ingredient over time. The advantages of sustained-release tablets or capsules are that they can often be taken less frequently than instant-release formulations of the same drug, and that they keep steadier levels of the drug in the bloodstream, thus extending the duration of the drug action.

[0106] In one embodiment, the pharmaceutical composition is formulated for extended release by embedding the active ingredient in a matrix of insoluble substance(s) such as acrylics or chitin. A extended release form is designed to release the active ingredient at a predetermined rate by maintaining a constant drug level for a specific period of time. This can be achieved through a variety of formulations, including, but not limited to liposomes and drug-polymer conjugates, such as hydrogels.

[0107] In another embodiment, the pharmaceutical composition is formulated for delayed-release, such that the active ingredient(s) is not immediately released upon administration. A non-limiting example of a delayed release vehicle is an enteric coated oral medication that dissolves in the intestines rather than the stomach.

[0108] In other embodiments, the pharmaceutical composition is formulated for immediate release of a portion of the active ingredient, followed with an extended-release of the remainder of the active ingredient. In one embodiment, the pharmaceutical composition is formulated as a powder that can be ingested for rapid release of the active ingredient. In another embodiment, the pharmaceutical composition is formulated into a liquid, gel, liquid suspension or emulsion form. Said liquid, gel, suspension or emulsion may be ingested by the subject in naked form or contained within a capsule.

[0109] In yet another embodiment, the pharmaceutical composition may be provided as a skin or transdermal patch for the topical administration of controlled and/or sustained quantities of the active ingredient.

EXAMPLES

[0110] The following examples describes selected aspects and embodiments of the present teachings, particularly data describing *in vitro* and *in vivo* effects of ASIC inhibition, and exemplary cystine knot peptides for use as inhibitors. These examples are intended for the purposes of illustration and should not be construed to limit the scope of the present teachings.

Example 1: Neuroprotection in Ischemia by Blocking Calcium-Permeable Acid-Sensing Ion Channels

[0111] This example describes experiments showing a role of ASIC1a in mediating ischemic injury and the ability ASIC1a inhibitors to reduce ischemic injury; *see* FIGS. 2-10. Ca^{2+} toxicity may play a central role in ischemic brain injury. The mechanism by which

toxic Ca^{2+} loading of cells occurs in the ischemic brain has become less clear as multiple human trials of glutamate antagonists have failed to show effective neuroprotection in stroke. Acidosis is a common feature of ischemia and plays a critical role in brain injury. This example demonstrates that acidosis activates Ca^{2+}-permeable acid-sensing ion channels (ASICs), which may induce glutamate receptor-independent, Ca^{2+}-dependent, neuronal injury. Accordingly, cells lacking endogenous ASICs may be resistant to acid injury, while transfection of Ca^{2+}-permeable ASIC1a may establish sensitivity. In focal ischemia, intracerebroventricular injection of ASIC1a blockers or knockout of the ASIC1a gene may protect the brain from ischemic injury and may do so more potently than glutamate antagonism.

[0112] The normal brain requires complete oxidation of glucose to fulfill its energy requirements. During ischemia, oxygen depletion forces the brain to switch to anaerobic glycolysis. Accumulation of lactic acid as a byproduct of glycolysis and protons produced by ATP hydrolysis causes pH to fall in the ischemic brain and aggravates ischemic brain injury.

[0113] Acid-sensing ion channels (ASICs) are a class of ligand-gated channels expressed throughout neurons of mammalian central and peripheral nervous systems. To date, six ASIC subunits have been cloned. Four of these subunits form functional homomultimeric channels that are activated by acidic pH to conduct a sodium-selective, amiloride-sensitive, cation current. Two of the ASIC subunits, ASIC1a and ASIC2a subunits, have been shown to be abundant in the brain.

Experimental Procedures

Neuronal Culture

[0114] Following anesthesia with halothane, cerebral cortices were dissected from E16 Swiss mice or P1 $ASIC1^{+/+}$ and $ASIC1^{-/-}$ mice and incubated with 0.05% trypsin-EDTA for 10 min at 37° C. Tissues were then triturated with fire-polished glass pipettes and plated

on poly-L-ornithine-coated 24-well plates or 25x25 mm glass coverslips at a density of $2.5x10^5$ cells per well or 10^6 cells per coverslip. Neurons were cultured with MEM supplemented with 10% horse serum (for E16 cultures) or Neurobasal medium supplemented with B27 (for P1 cultures) and used for electrophysiology and toxicity studies after 12 days. Glial growth was suppressed by addition of 5-fluoro-2-deoxyuridine and uridine, yielding cultured cells with 90% neurons as determined by NeuN and GFAP staining (data not shown).

Electrophysiology

[0115] ASIC currents were recorded with whole-cell patch-clamp and fast-perfusion techniques. The normal extracellular solution (ECF) contained (in mM) 140 NaCl, 5.4 KCl, 25 HEPES, 20 glucose, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 0.0005 TTX (pH 7.4), 320-335 mOsm. For low pH solutions, various amounts of HCl were added. For solutions with pH<6.0, MES instead of HEPES was used for more reliable pH buffering. Patch electrodes contained (in mM) 140 CsF, 2.0 $MgCl_2$, 1.0 CaC_2, 10 HEPES, 11 EGTA, 4 MgATP (pH 7.3), 300 mOsm. The Na^+-free solution consisted of 10 mM $CaCl_2$, 25 mM HEPES with equiosmotic NMDG or sucrose substituting for NaCl (Chu et al., 2002). A multibarrel perfusion system (SF-77B, Warner Instrument Co.) was employed for rapid exchange of solutions.

Cell Injury Assay--LDH Measurement

[0116] Cells were washed three times with ECF and randomly divided into treatment groups. MK801 (10 μM), CNQX (20 μM), and nimodipine (5 μM) were added in all groups to eliminate potential secondary activation of glutamate receptors and voltage-gated Ca^{2+} channels. Following acid incubation, neurons were washed and incubated in Neurobasal medium at 37° C. LDH release was measured in culture medium using the LDH assay kit (Roche Molecular Biochemicals). Medium (100 μL) was transferred from culture wells to 96-well plates and mixed with 100 μL reaction solution provided by the kit. Optical density was

measured at 492 nm 30 min later, utilizing a microplate reader (Spectra Max Plus, Molecular Devices). Background absorbance at 620 was subtracted. The maximal releasable LDH was obtained in each well by 15 min incubation with 1% Triton X-100 at the end of each experiment.

Ca^{2+} Imaging

[0117] Cortical neurons grown on 25 x 25 mm glass coverslips were washed three times with ECF and incubated with 5 μM fura-2-acetoxymethyl ester for 40 min at 22° C., washed three times, and incubated in normal ECF for 30 min. Coverslips were transferred to a perfusion chamber on an inverted microscope (Nikon TE300). Cells were illuminated using a xenon lamp and observed with a 40x UV fluor oil-immersion objective lens, and video images were obtained using a cooled CCD camera (Sensys KAF 1401, Photometrics). Digitized images were acquired and analyzed in a PC controlled by Axon Imaging Workbench software (Axon Instruments). The shutter and filter wheel (Lambda 10-2) were controlled by the software to allow timed illumination of cells at 340 or 380 nm excitation wavelengths. Fura-2 fluorescence was detected at emission wavelength of 510 nm. Ratio images (340/380) were analyzed by averaging pixel ratio values in circumscribed regions of cells in the field of view. The values were exported to SigmaPlot for further analysis.

Fluorescein-Diacetate Staining and Propidium Iodide Uptake

[0118] Cells were incubated in ECF containing fluorescein-diacetate (FDA) (5 μM) and propidium iodide (PI) (2 μM) for 30 min followed by wash with dye-free ECF. Alive (FDA-positive) and dead (PI-positive) cells were viewed and counted on a microscope (Zeiss) equipped with epifluorescence at 580/630 nm excitation/emission for PI and 500/550 nm for FDA. Images were collected using an Optronics DEI-730 camera equipped with a BQ 8000 sVGA frame grabber and analyzed using computer software (Bioquant, TN).

Transfection of COS-7 Cells

[0119] COS-7 cells were cultured in MEM with 10% HS and 1% PenStrep (GIBCO). At ~50% confluence, cells were cotransfected with cDNAs for ASICs and GFP in pc^{DNA3} vector using FuGENE6 transfection reagents (Roche Molecular Biochemicals). DNA for ASICs (0.75 μg) and 0.25 μg of DNA for GFP were used for each 35 mm dish. GFP-positive cells were selected for patch-clamp recording 48 hr after transfection. For stable transfection of ASIC1a, 500 μg/mL G418 was added to culture medium I week following the transfection. The surviving G418-resistant cells were further plated and passed for >5 passages in the presence of G418. Cells were then checked with patch-clamp and immunofluorescent staining for the expression of ASIC1a.

Oxygen-Glucose Deprivation

[0120] Neurons were washed three times and incubated with glucose-free ECF at pH 7.4 or 6.0 in an anaerobic chamber (Model 1025, Forma Scientific) with an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 at 35° C. Oxygen-glucose deprivation (OGD) was terminated after 1 hr by replacing the glucose-free ECF with Neurobasal medium and incubating the cultures in a normal cell culture incubator. With HEPES-buffered ECF used, 1 hr OGD slightly reduced pH from 7.38 to 7.28 (n=3) and from 6.0 to 5.96 (n=4).

Focal Ischemia

[0121] Transient focal ischemia was induced by suture occlusion of the middle cerebral artery (MCAO) in male rats (SD, 250-300 g) and mice (with congenic C57B16 background, ~25 g) anesthetized using 1.5% isoflurane, 70% N_2O, and 28.5% O_2 with intubation and ventilation. Rectal and temporalis muscle temperature was maintained at 37° C ± 0.5° C. with a thermostatically controlled heating pad and lamp. Cerebral blood flow was monitored by transcranical LASER doppler. Animals with blood flow not reduced below 20% were excluded.

[0122] Animals were killed with chloral hydrate 24 hr after ischemia. Brains were rapidly removed, sectioned coronally at 1 mm (mice) or 2 mm (rats) intervals, and stained by immersion in vital dye (2%) 2,3,5-triphenyltetrazolium hydrochloride (TTC). Infarction area was calculated by subtracting the normal area stained with TTC in the ischemic hemisphere from the area of the nonischemic hemisphere. Infarct volume was calculated by summing infarction areas of all sections and multiplying by slice thickness. Rat intraventricular injection was performed by stereotaxic technique using a microsyringe pump with cannula inserted stereotactically at 0.8 mm posterior to bregma, 1.5 mm lateral to midline, and 3.8 mm ventral to the dura. All manipulations and analyses were performed by individuals blinded to treatment groups.

Results

(a) Acidosis activates ASICs in mouse cortical neurons

[0123] FIGS. 3 and 4 shows exemplary data related to the electrophysiology and pharmacology of ASICs in cultured mouse cortical neurons. FIGS. 3A and 3B are graphs illustrating the pH dependence of ASIC currents activated by a pH drop from 7.4 to the pH values indicated. Dose-response curves were fit to the Hill equation with an average $pH_{0.5}$ of 6.18 ± 0.06 (n=10). FIGS. 3C and 3D are graphs illustrating the current-voltage relationship of ASICs (n=5). The amplitudes of ASIC current at various voltages were normalized to that recorded at -60 mV. FIGS. 4A and 4B are graphs illustrating a dose-dependent blockade of ASIC currents by amiloride. IC_{50}=16.4 ± 4.1 μM, N=8. FIGS. 4C and 4D are graphs illustrating a blockade of ASIC currents by PcTX venom. **p<0.01.

[0124] ASIC currents in cultured mouse cortical neurons were recorded (see FIG. 3). At a holding potential of -60 mV, a rapid reduction of extracellular pH (pH_e) to below 7.0 evoked large transient inward currents with a small steady-state component in the majority of neurons (FIG. 3A). The amplitude of inward current increased in a sigmoidal fashion as pH_e

decreased, yielding a $pH_{0.5}$ of 6.18 ± 0.06 (n=10, FIG. 3B). A linear I-V relationship and a reversal close to the Na^+ equilibrium potential were obtained (n=6, FIGS. 3C and 3D). These data demonstrate that lowering pH_e may activate typical ASICs in mouse cortical neurons.

[0125] The effect of amiloride, a nonspecific blocker of ASICs, on the acid-activated currents was tested (*see* FIG. 4). As shown in FIG. 4, amiloride dose-dependently blocked ASIC currents in cortical neurons with an IC_{50} of 16.4 ± 4.1 μM (n=8, FIGS. 4A and 4B). The effect of PcTX venom on acid-activated current in cortical neurons is shown in FIG. 4C and 4D. At 100 ng/mL, PcTX venom reversibly blocked the peak amplitude of ASIC current by 47% ± 7% (n=15, FIGS. 4C and 4D), indicating significant contributions of homomeric ASIC1a to total acid-activated currents. Increasing PcTX concentration did not induce further reduction in the amplitude of ASIC current in the majority of cortical neurons (n=8, data not shown), indicating coexistence of PcTX-insensitive ASICs (*e.g.*, heteromeric ASIC1a/2a) in these neurons.

(b) ASIC response is potentiated by modeled ischemia

[0126] FIG. 5 shows exemplary data indicating that modeled ischemia may enhance activity of ASICs. FIG. 5A is a series of exemplary traces showing an increase in amplitude and a decrease in desensitization of ASIC currents following 1 hr OGD. FIG. 5B is a graph of summary data illustrating an increase of ASIC current amplitude in OGD neurons. N=40 and 44, *p<0.05. FIG. 5C is a series of exemplary traces and summary data showing decreased ASIC current desensitization in OGD neurons. N=6, **p<0.01. FIG. 5D is a pair of exemplary traces showing lack of acid-activated current at pH 6.0 in $ASIC1^{-/-}$ neurons, in control condition, and following 1 hr OGD (n=12 and 13).

[0127] Since acidosis may be a central feature of brain ischemia, it was determined to test whether ASICs may be activated in ischemic conditions and whether ischemia may modify the properties of these channels; *see* FIG. 5. ASIC currents in neurons following 1 hr

oxygen glucose deprivation (OGD) were recorded. Briefly, one set of cultures was washed three times with glucose-free extracellular fluid (ECF) and subjected to OGD, while control cultures were subjected to washes with glucose containing ECF and incubation in a conventional cell culture incubator. OGD was terminated after 1 hr by replacing glucose-free ECF with Neurobasal medium and incubating cultures in the conventional incubator. ASIC current was then recorded 1 hr following the OGD when there was no morphological alteration of neurons. OGD treatment induced a moderate increase of the amplitude of ASIC currents (1520 ± 138 pA in control group, N=44; 1886 ± 185 pA in neurons following 1 hr OGD, N=40, $p<0.05$, FIGS. 5A and 5B). More importantly, OGD induced a dramatic decrease in ASIC desensitization as demonstrated by an increase in time constant of the current decay (814.7 ± 58.9 ms in control neurons, N=6; 1928.9 ± 315.7 ms in neurons following OGD, N=6, $p<0.01$, FIGS. 5A and 5C). In cortical neurons cultured from $ASIC1^{-/-}$ mice, reduction of pH from 7.4 to 6.0 did not activate any inward current (n=52), similar to a previous study in hippocampal neurons (Wemmie et al., 2002). In these neurons, 1 hr OGD did not activate or potentiate acid-induced responses (FIG. 5D, n=12 and 13).

(c) Acidosis Induces Glutamate-Independent Ca^{2+} Entry via ASIC1a

[0128] FIGS. 6 and 7 show exemplary data suggesting that ASICs in cortical neurons may be Ca^{2+} permeable, and that Ca^{2+} permeability may be ASIC1a dependent. FIG. 6A shows exemplary traces obtained with Na^+-free ECF containing 10 mM Ca^{2+} as the only charge carrier. Inward currents were recorded at pH 6.0. The average reversal potential is ~-17 mV after correction of liquid junction potential (n=5). FIG. 6B shows representative traces and summary data illustrating blockade of Ca^{2+}-mediated current by amiloride and PcTX venom. The peak amplitude of Ca^{2+}-mediated current decreased to 26% ± 2% of control value by 100 μM amiloride (n=6, $p<0.01$) and to 22% ± 0.9% by 100 ng/mL PcTX venom (n=5, $p<0.01$). FIG. 7A shows exemplary 340/380 nm ratios as a function of pH,

illustrating an increase of $[Ca^{2+}]_i$ by pH drop to 6.0. Neurons were bathed in normal ECF containing 1.3 mM $CaCl_2$ with blockers for voltage-gated Ca^{2+} channels (5 μM nimodipine and 1 μM ω-conotoxin MVIIC) and glutamate receptors (10 μM MK801 and 20 μM CNQX). The inset of FIG. 7A shows exemplary inhibition of acid-induced increase of $[Ca^{2+}]_i$ by 100 μM amiloride. FIG. 7B shows exemplary summary data illustrating inhibition of acid-induced increase of $[Ca^{2+}]_i$ by amiloride and PcTX venom. N=6-8, **p<0.01 compared with pH 6.0 group. FIG. 7C shows exemplary 340/380 nm ratios as a function of pH and NMDA presence/absence, demonstrating a lack of acid-induced increase of $[Ca^{2+}]_i$ in $ASIC1^{-/-}$ neurons; neurons had a normal response to NMDA (n=8). FIG. 7D shows exemplary traces illustrating a lack of acid-activated current at pH 6.0 in $ASIC1^{-/-}$ neurons.

[0129] The Ca^{2+} permeability of ASICs in cortical neurons was determined using a standard ion-substitution protocol (Jia *et al.,* Neuron, 1996, 17:945-956) and the Fura-2 fluorescent Ca^{2+}-imaging technique (Chu *et al.,* 2002, J. Neurophysiol. 87:2555-2561). With bath solutions containing 10 mM Ca^{2+} (Na^+ and K^+-free) as the only charge carrier and at a holding potential of -60 mV, we recorded inward currents larger than 50 pA in 15 out of 18 neurons, indicating significant Ca^{2+} permeability of ASICs in the majority of cortical neurons (FIG. 6A). Consistent with activation of homomeric ASIC1a channels, currents carried by 10 mM Ca^{2+} were largely blocked by both the nonspecific ASIC blocker amiloride and the ASIC1a-specific blocker PcTX venom (FIG. 6B). The peak amplitude of Ca^{2+}-mediated current was decreased to 26% ± 2% of control by 100 μM amiloride (n=6, p<0.01) and to 22% ± 0.9% by 100 ng/mL PcTX venom (n=5, p<0.01). Ca^{2+} imaging, in the presence of blockers of other major Ca^{2+} entry pathways (MK801 10 μM and CNQX 20 μM for glutamate receptors; nimodipine 5 μM and ω-conotoxin MVIIC 1 μM for voltage-gated Ca^{2+} channels), demonstrated that 18 out of 20 neurons responded to a pH drop with detectable increases in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (FIG. 7A). In general, $[Ca^{2+}]_i$

remains elevated during prolonged perfusion of low pH solutions. In some cells, the $[Ca^{2+}]_i$ increase lasted even longer than the duration of acid perfusion (FIG. 7A). Long-lasting Ca^{2+} responses suggest that ASIC response in intact neurons may be less desensitized than in whole-cell recordings or that Ca^{2+} entry through ASICs may induce subsequent Ca^{2+} release from intracellular stores. Preincubation of neurons with 1 μM thapsigargin partially inhibited the sustained component of Ca^{2+} increase, suggesting that Ca^{2+} release from intracellular stores may also contribute to acid-induced intracellular Ca^{2+} accumulation (n=6, data not shown). Similar to the current carried by Ca^{2+} ions (FIG. 6B), both peak and sustained increases in $[Ca^{2+}]_i$ were largely inhibited by amiloride and PcTX venom (FIGS. 7A and 7B, n=6-8), consistent with involvement of homomeric ASIC1a in acid-induced $[Ca^{2+}]_i$ increase. Knockout of the ASICI gene eliminated the acid-induced $[Ca^{2+}]_i$ increase in all neurons without affecting NMDA receptor-mediated Ca^{2+} response (FIG. 7C, n=8). Patch-clamp recordings demonstrated lack of acid-activated currents at pH 6.0 in 52 out of 52 of the $ASIC1^{-/-}$ neurons, consistent with absence of ASIC1a subunits. Lowering pH to 5.0 or 4.0, however, activated detectable current in 24 out of 52 $ASIC1^{-/-}$ neurons, indicating the presence of ASIC2a subunits in these neurons (FIG. 7D). Further electrophysiological studies demonstrated that $ASIC1^{-/-}$ neurons have normal responses for various voltage-gated channels and NMDA, GABA receptor-gated channels (data not shown).

(d) ASIC blockade protects acidosis-induced, glutamate-independent neuronal injury

[0130] FIG. 8 shows exemplary data suggesting that acid incubation may induce glutamate receptor-independent neuronal injury protected by ASIC blockade. FIGS. 8A and 8B show graphs presenting exemplary data for time-dependent LDH release induced by 1 hr (FIG. 8A) or 24 hr incubation (FIG. 8B) of cortical neurons in pH 7.4 (solid bars) or 6.0 ECF (open bars). N=20-25 wells, *$p<0.05$, and **$p<0.01$, compared to pH 7.4 group at the same time points (acid-induced neuronal injury with fluorescein diacetate (FDA) also was analyzed

by staining of cell bodies of alive neurons and propidium iodide (PI) staining of nuclei of dead neurons). FIG. 8C shows a graph illustrating inhibition of acid-induced LDH release by 100 μM amiloride or 100 ng/mL PcTX venom (n=20-27, *p<0.05, and **p<0.01). MK801, CNQX, and nimodipine were present in ECF for all experiments (FIGS. 8A-C).

[0131] Acid-induced injury was studied on neurons grown on 24-well plates incubated in either pH 7.4 or 6.0 ECF containing MK801, CNQX, and nimodipine; *see* FIG. 8. Cell injury was assayed by the measurement of lactate dehydrogenase (LDH) release (Koh and Choi, J. Neurosci., 1987, 20:83-90) at various time points (FIGS. 8A and 8B) and by fluorescent staining of alive/dead cells. Compared to neurons treated at pH 7.4, 1 hr acid incubation (pH 6.0) induced a time-dependent increase in LDH release (FIG. 8A). After 24 hr, 45.7% ± 5.4% of maximal LDH release was induced (n=25 wells). Continuous treatment at pH 6.0 induced greater cell injury (FIG. 8B, n=20). Consistent with the LDH assay, alive/dead staining with fluorescein diacetate and propidium iodide showed similar increases in cell death by 1 hr acid treatment (data not shown). One hour incubation with pH 6.5 ECF also induced significant but less LDH release than with pH 6.0 ECF (n=8 wells, data not shown).

[0132] The effect of amiloride and PcTX venom on acid-induced LDH release were tested to determine whether activation of ASICs is involved in acid-induced glutamate receptor-independent neuronal injury. Addition of either 100 μM amiloride or 100 ng/mL PcTX venom 10 min before and during the 1 hr acid incubation significantly reduced LDH release (FIG. 8C). At 24 hr, LDH release was decreased from 45.3% ± 3.8% to 31.1% ± 2.5% by amiloride and to 27.9% ± 2.6% by PcTX venom (n=20-27, p<0.01). Addition of amiloride or PcTX venom in pH 7.4 ECF for 1 hr did not affect baseline LDH release, although prolonged incubation (*e.g.*, 5 hr) with amiloride alone increased LDH release (n=8, data not shown).

(e) Activation of homomeric ASIC1a is responsible for acidosis-induced injury

[0133] FIG. 9 is a series of graphs presenting exemplary data indicating that ASIC1a may be involved in acid-induced injury in vitro. FIG. 9A shows exemplary data illustrating inhibition of acid-induced LDH release by reducing $[Ca^{2+}]_e$ (n=11-12, **p<0.01 compared with pH 6.0, 1.3 Ca^{2+}). FIG. 9B shows exemplary data illustrating acid incubation induced increase of LDH release in ASIC1a-transfected but not nontransfected COS-7 cells (n=8-20). Amiloride (100 μM) inhibited acid-induced LDH release in ASIC1a-transfected cells. *p<0.05 for 7.4 versus 6.0 and 6.0 versus 6.0+amiloride. FIG. 9C shows exemplary data illustrating a lack of acid-induced injury and protection by amiloride and PcTX venom in $ASIC1^{-/-}$ neurons (n=8 in each group, p>0.05). FIG. 9D shows exemplary data illustrating acid-induced increase of LDH release in cultured cortical neurons under OGD (n=5). LDH release induced by combined 1 hr OGD/acidosis was not inhibited by trolox and L-NAME (n=8-11). OGD did not potentiate acid-induced LDH release in $ASIC1^{-/-}$ neurons. **p<0.01 for pH 7.4 versus pH 6.0 and *p<0.05 for pH 6.0 versus 6.0+PcTX venom. MK801, CNQX, and nimodipine were present in ECF for all experiments (FIG. 9A-D).

[0134] Neurons were treated with pH 6.0 ECF in the presence of normal or reduced $[Ca^{2+}]_e$ to determine whether Ca^{2+} entry plays a role in acid-induced injury (*see* FIG. 9). Reducing Ca^{2+} from 1.3 to 0.2 mM inhibited acid-induced LDH release (from 40.0% ± 4.1% to 21.9% ± 2.5%), as did ASIC1a blockade with PcTX venom (n=11-12, p<0.01; FIG. 9A). Ca^{2+}-free solution was not tested, as a complete removal of $[Ca^{2+}]_e$ may activate large inward currents through a Ca^{2+}-sensing cation channel, which may otherwise complicate data interpretation. Inhibition of acid injury by both amiloride and PcTX, nonspecific and specific ASIC1a blockers, and by reducing $[Ca^{2+}]_e$ suggests that activation of Ca^{2+}-permeable ASIC1a may be involved in acid-induced neuronal injury.

[0135] Acid injury of nontransfected and ASIC1a transfected COS-7 cells was studied to provide additional evidence that activation of ASIC1a is involved in acid injury. COS-7 is a cell line commonly used for expression of ASICs due to its lack of endogenous channels. Following confluence (36-48 hr after plating), cells were treated with either pH 7.4 or 6.0 ECF for 1 hr. LDH release was measured 24 hr after acid incubation. Treatment of nontransfected COS-7 cells with pH 6.0 ECF did not induce increased LDH release when compared with pH 7.4-treated cells (10.3% ± 0.8% for pH 7.4, and 9.4% ± 0.7% for pH 6.0, N=19 and 20 wells; $p>0.05$, FIG. 9B). However, in COS-7 cells stably transfected with ASIC1a, 1 hr incubation at pH 6.0 significantly increased LDH release from 15.5% ± 2.4% to 24.0% ± 2.9% (n=8 wells, $p<0.05$). Addition of amiloride (100 μM) inhibited acid-induced LDH release in these cells (FIG. 9B).

[0136] Acid injury of CHO cells transiently transfected with cDNAs encoding GFP alone or GFP plus ASIC1a was also studied. After the transfection (24-36 hr), cells were incubated with acidic solution (pH 6.0) for 1 hr, and cell injury was assayed 24 hr following the acid incubation. One hour acid incubation largely reduced surviving GFP-positive cells in GFP/ASIC1a group but not in the group transfected with GFP alone (data not shown).

[0137] Cell toxicity experiments on cortical neurons cultured from $ASIC^{+/+}$ and $ASIC1^{-/-}$ mice were performed to further demonstrate an involvement of ASIC1a in acidosis-induced neuronal injury. Again, 1 hr acid incubation of $ASIC^{+/+}$ neurons at 6.0 induced substantial LDH release that was reduced by amiloride and PcTX venom (n=8-12). One hour acid treatment of $ASIC1^{-/-}$ neurons, however, did not induce significant increase in LDH release at 24 hr (13.8% ± 0.9% for pH 7.4 and 14.2% ± 1.3% for pH 6.0, N=8, $p>0.05$), indicating resistance of these neurons to acid injury (FIG. 9C). In addition, knockout of the ASIC1 gene also eliminated the effect of amiloride and PcTX venom on acid-induced LDH release (FIG. 9C, n=8 each), further suggesting that the inhibition of acid-induced injury of

cortical neurons by amiloride and PcTX venom (FIG. 8C) was due to blockade of ASIC1 subunits. In contrast to acid incubation, 1 hr treatment of ASIC1$^{-/-}$ neurons with 1 mM NMDA+10 μM glycine (in Mg^{2+}-free [pH 7.4] ECF) induced 84.8% ± 1.4% of maximal LDH release at 24 hr (n=4, FIG. 9C), indicating normal response to other cell injury processes.

(f) Modeled ischemia enhances acidosis-induced glutamate-independent neuronal injury via ASICs

[0138] As the magnitude of ASIC currents may be potentiated by cellular and neurochemical components of brain ischemia-cell swelling, arachidonic acid, and lactate and, more importantly, the desensitization of ASIC currents may be reduced dramatically by modeled ischemia (*see* FIGS. 5A and 5C), activation of ASICs in ischemic conditions is expected to produce greater neuronal injury. To test this hypothesis, neurons were subjected to 1 hr acid treatment under oxygen and glucose deprivation (OGD). MK801, CNQX, and nimodipine were added to all solutions to inhibit voltage-gated Ca^{2+} channels and glutamate receptor-mediated cell injury associated with OGD. One hour incubation with pH 7.4 ECF under OGD conditions induced only 27.1% ± 3.5% of maximal LDH release at 24 hr (n=5, FIG. 9D). This finding is in agreement with a previous report that 1 hr OGD does not induce substantial cell injury with the blockade of glutamate receptors and voltage-gated Ca^{2+} channels (Aarts et al., 2003). However, 1 hr OGD, combined with acidosis (pH 6.0), induced 73.9% ± 4.3% of maximal LDH release (n=5, FIG. 9D, $p<0.01$), significantly larger than acid-induced LDH release in the absence of OGD (*see* FIG. 8A, $p<0.05$). Addition of the ASIC1a blocker PcTX venom (100 ng/mL) significantly reduced acid/OGD-induced LDH release to 44.3% ± 5.3% (n=5, $p<0.05$, FIG. 9D).

[0139] The same experiment was performed with cultured neurons from the ASIC1$^{-/-}$ mice. Unlike in ASICI containing neurons, however, 1 hr treatment with combined OGD and

acid only slightly increased LDH release in ASIC1$^{-/-}$ neurons (from 26.1% ± 2.7% to 30.4% ± 3.5%, N=10-12, FIG. 9D). This finding suggests that potentiation of acid-induced injury by OGD may be due largely to OGD potentiation of ASIC1-mediated toxicity.

[0140] It has been demonstrated that activation of a Ca^{2+}-permeable nonselective cation conductance activated by reactive oxygen/nitrogen species resulting in glutamate receptor-independent neuronal injury (Aarts *et al*, Cell, 2003, 115:863-877) . The prolonged OGD-induced cell injury may be reduced dramatically by agents either scavenging free radicals directly (*e.g.*, trolox) or reducing the production of free radicals (*e.g.*, L-NAME). To determine whether combined short duration OGD and acidosis induced neuronal injury may involve a similar mechanism, the effect of trolox and L-NAME on OGD/acid-induced LDH release was tested. As shown in FIG. 9D, neither trolox (500 μM) nor L-NAME (300 μM) had significant effect on combined 1 hr OGD/acidosis-induced neuronal injury (n=8-11). Additional experiments also demonstrated that the ASIC blockers amiloride and PcTX venom had no effect on the conductance of TRPM7 channels (Aarts *et al. supra*). Together, these findings strongly suggest that activation of ASICs but not TRPM7 channels may be largely responsible for combined 1 hr OGD/acidosis-induced neuronal injury in our studies.

(g) Activation of ASIC1a in ischemic brain injury in vivo

FIG. 10 shows data illustrating neuroprotection by ASICI blockade and ASICI gene knockout in brain ischemia in vivo. FIG. 10A shows a graph of exemplary data obtained from TTC-stained brain sections illustrating the stained volume ("infarct volume") in brains from aCSF (n=7), amiloride (n=11), or PcTX venom (n=5) injected rats. *p<0.05 and **p<0.01 compared with aCSF injected group. FIG. 10B shows a graph of exemplary data illustrating reduction in infarct volume in brains from ASIC1$^{-/-}$ mice (n=6 for each group). *p<0.05 and **p<0.01 compared with +/+ group. FIG. 10C shows a graph of exemplary data illustrating reduction in infarct volume in brains from mice *i.p.* injected with 10 mg/kg memantine

(Mem) or *i.p.* injection of memantine accompanied by *i.c.v.* injection of PcTX venom (500 ng/mL). **p<0.01 compared with aCSF injection and between memantine and memantine plus PcTX venom (n=5 in each group). FIG. 10D shows a graph of exemplary data illustrating reduction in infarct volume in brains from either $ASIC1^{+/+}$ (wt) or $ASIC1^{-/-}$ mice *i.p.* injected with memantine (n=5 in each group). *p<0.05, and **p<0.01.

[0141] The protective effect of amiloride and PcTX venom in a rat model of transient focal ischemia (Longa et al., Stroke, 1989, 20:84-91) was tested to determine whether activation of ASIC1a is involved in ischemic brain injury *in vivo*. Ischemia (100 min) was induced by transient middle cerebral artery occlusion (MCAO). A total of 6 μl artificial CSF (aCSF) alone, aCSF-containing amiloride (1 mM), or PcTX venom (500 ng/mL) was injected intracerebroventricularly 30 min before and after the ischemia. The volume for cerebral ventricular and spinal cord fluid for 4-week-old rats is estimated to be ~60 μl. Assuming that the infused amiloride and PcTX were uniformly distributed in the CSF, a concentration of ~100 μM for amiloride and ~50 ng/mL for PcTX were expected, which is a concentration found effective in cell culture experiments. Infarct volume was determined by TTC staining (Bederson et al., Stroke, 1986, 17:1304-1308) at 24 hr following ischemia. Ischemia (100 min) produced an infarct volume of 329.5 ± 25.6 mm^3 in aCSF-injected rats (n=7) but only 229.7 ± 41.1 mm^3 in amiloride-injected (n=11, p<0.05) and 130.4 ± 55.0 mm^3 (~60% reduction) in PcTX venom-injected rats (n=5, p<0.01) (FIG. 10A).

[0142] $ASIC1^{-/-}$ mice were used to further demonstrate the involvement of ASIC1a in ischemic brain injury *in vivo*. Male $ASIC1^{+/+}$, $ASIC1^{+/-}$, and $ASIC1^{-/-}$ mice (~25 g, with congenic C57B16 background) were subjected to 60 min MCAO as previously described (Stenzel-Poore *et al.*, Lancet, 2003, 362:1028-1037). Consistent with the protection by pharmacological blockade of ASIC1a (above), -/- mice displayed significantly smaller (~61% reduction) infarct volumes (32.9 ± 4.7 mm^3, N=6) as compared to +/+ mice (84.6 ± 10.6

mm^3, N=6, $p<0.01$).+/- mice also showed reduced infarct volume (56.9.+-.6.7 mm^3, N=6, $p<0.05$) (FIG. 10B).

[0143] To determine whether blockade of ASIC1a channels or knockout of the ASIC1 gene would provide additional protection *in vivo* in the setting of glutamate receptor blockade, memantine (10 mg/kg) was injected intraperitoneally (*i.p.*) into C57B16 mice immediately following 60 min MCAO and accompanied by intracerebroventricular injection (*i.c.v.*) of a total volume of 0.4 µl aCSF alone or aCSF containing PcTX venom (500 ng/mL) 15 min before and following ischemia. In control mice with *i.p.* injection of saline and *i.c.v.* injection of aCSF, 60 min MCAO induced an infarct volume of 123.6 ± 5.3 mm^3 (n=5, FIG. 10C). In mice with intraperitoneal injection of memantine and intracerebroventricular injection of aCSF, the same duration of ischemia induced an infarct volume of 73.8.+-.6.9 mm^3 (n=5, $p<0.01$). However, in mice injected with memantine and PcTX venom, an infarct volume of only 47.0 ± 1.1 mm^3 was induced (n=5, $p<0.01$ compared with both control and memantine groups, FIG. 10C). These data suggest that blockade of homomeric ASIC1a may provide additional protection in *in vivo* ischemia in the setting of NMDA receptor blockade. Additional protection was also observed in $ASIC1^{-/-}$ mice treated with pharmacologic NMDA blockade (FIG. 10D). In $ASIC^{+/+}$ mice *i.p.* injected with saline or 10 mg/kg memantine, 60 min MCAO induced an infarct volume of 101.4 ± 9.4 mm^3 or 61.6 ± 12.7 mm^3, respectively (n=5 in each group, FIG. 10D). However, in $ASIC1^{-/-}$ mice injected with memantine, the same ischemia duration induced an infarct volume of 27.7 ± 1.6 mm^3 (n=5), significantly smaller than the infarct volume in $ASIC1^{+/+}$ mice injected with memantine ($p<0.05$).

[0144] Taken together, these data demonstrate that activation of Ca^{2+}-permeable ASIC1a is a novel, glutamate-independent biological mechanism underlying ischemic brain injury. .

Example 2: Time Window of PcTX Neuroprotection

[0145] This example describes exemplary experiments that measure the neuroprotective effect of PcTX venom at different times after onset of stroke in rodents; *see* FIG. 11. Briefly, brain ischemia (stroke) was induced in rodents by mid-cerebral artery occlusion (MCAO). At the indicated times after induction, artificial cerebrospinal fluid (aCSF), PcTX venom (0.5 μL, 500 ng/mL total protein), or inactivated (boiled) venom was infused into the lateral ventricles of each rodent. As shown in FIG. 11, administration of PcTX venom provided a 60% reduction in stroke volume both at one hour and at three hours after stroke onset. Furthermore, substantial stroke volume reduction still may be maintained if treatment is withheld for five hours after the onset of the MCAO. Accordingly, neuroprotection due to ASIC inhibition may have an extended therapeutic time window after stroke onset, allowing stroke subjects to benefit from treatment performed hours after the stroke began. This effect of ASIC blockade on stroke neuroprotection is far more robust than that of calcium channel blockade of the NMDA receptor (a major target for experimental stroke therapeutics) using a glutamate antagonist. No glutamate antagonist, thus far, has such a favorable profile as shown here for ASIC1a-selective inhibition.

Example 3: Exemplary Cystine Knot Peptides

[0146] This example describes exemplary cystine knot peptides, including full-length PcTx1 and deletion derivatives of PcTx, which may be screened in cultured cells, tested in ischemic animals (*e.g.*, rodents such as mice or rats), and/or administered to ischemic human subjects.

[0147] FIG. 12 shows the primary amino acid sequence (SEQ ID NO:1), in one-letter code, of an exemplary cystine knot peptide, PcTx1, indicated at 50, with various exemplary peptide features shown relative to amino acid positions 1-40. Peptide 50 may include six cysteine residues that form cystine bonds 52, 54, 56 to create a cystine knot motif 58. The

peptide also may include one or more beta sheet regions 60 and a positively charged region 62. An N-terminal region 64 and a C-terminal region 66 may flank the cystine knot motif.

[0148] FIG. 13 shows a comparison of the PcTx1 peptide 50 of FIG. 12 aligned with various exemplary deletion derivatives of the peptide. These derivatives may include an N-terminal deletion 70 (SEQ ID NO:2), a partial C-terminal deletion 72 (SEQ ID NO:3), a full C-terminal deletion 74 (SEQ ID NO:4), and an N/C terminal deletion 76 (SEQ ID NO:5). Other derivatives of PcTx1 may include any deletion, insertion, or substitution of one or more amino acids, for example, while maintaining sequence similarity or identity of at least about 25% or about 50% with the original PcTx1 sequence.

[0149] Each PcTx1 derivative may be tested for its ability to inhibit ASIC proteins selectively and/or for an effect, if any, on ischemia. Any suitable test system(s) may be used to perform this testing including any of the cell-based assay systems and/or animal model systems described elsewhere in the present teachings. The PcTx1 derivative also or alternatively may be tested in ischemic human subjects.

Example 4: Selectivity of PcTX Venom for ASIC1a

[0150] This example describes experiments that measure the selectivity of PcTX venom (and thus PcTx1 toxin) for ASIC1a alone, relative to other ASIC proteins or combinations of ASIC proteins expressed in cultured cells. COS-7 cells expressing the indicated ASIC proteins were treated with PcTX venom (25 ng/mL on ASIC1a expressing cells and 500 ng/mL on ASIC2a, ASIC3 or ASIC1a+2a expressing cells). Channel currents were measured at the pH of half maximal channel activation (pH 0.5). As shown in FIG. 14, PcTX venom largely blocked the currents mediated by ASIC1a homomeric channels at a protein concentration of 25 ng/mL, with no effect on the currents mediated by homomeric ASIC2a, ASIC3, or heteromeric ASIC1a/ASIC2a at 500 ng/mL (n=3-6). At 500 ng/mL, PcTX venom also did not affect the currents mediated by other ligand-gated channels (*e.g.*

NMDA and GABA receptor-gated channels) and voltage-gated channels (*e.g.* Na+, Ca2+, and K+ channels) (n=4-5). These experiments indicate that PcTX venom and thus PcTx1 peptide is a specific blocker for homomeric ASIC1a. Using this cell-based assay system, the potency and selectivity of ASIC inhibition may be measured for various synthetic peptides or other candidate inhibitors (*e.g., see* Example 3).

Example 5: Nasal Administration of PcTX Venom is Neuroprotective

[0151] This example describes exemplary data indicating the efficacy of nasally administered PcTX venom for reducing ischemia-induced injury in an animal model system of stroke. Cerebral ischemia was induced in male mice by mid-cerebral artery occlusion. One hour after occlusion was initiated animals were treated as controls or were treated with PcTX venom (50 μL of 5 ng/mL (total protein) PcTx venom introduced intranasally). As shown in FIG. 15, nasal administration of PcTX venom resulted in a 55% reduction in ischemia-induced injury (ischemic damage), as defined by infarct volume, relative to control treatment. Nasal administration may be via a spray that is deposited substantially in the nasal passages rather than inhaled into the lungs and/or may be via an aerosol that is at least partially inhaled into the lungs. In some examples, nasal administration may have a number of advantages over other routes of administration, such as more efficient delivery to the brain and/or adaptability for self-administration by an ischemic subject.

Example 6: Inhibition of ASIC1a Channel By Amiloride And Amiloride Analogs

[0152] As shown in FIG. 16, amiloride and amiloride analogs benzamil, phenamil and EIPA block ASIC1a current in a dose-dependent manner. Similarly, amiloride and amiloride analogs benzamil and EIPA block ASIC2a current in a dose-dependent manner (FIG. 17). Table 1 summarizes inhibition of the ASIC1a channel by amiloride and amiloride analogs. Amiloride was an effective blocker of this channel with an IC50 of 7.7μM.

Table 1. Inhibition of the ASIC1a channel by amiloride and amiloride analogs.

Knockout 1a	39.1 ± 3.8 (n=4)	
Knockout 2a	5.14 ± 0.79 (n=5)	
Neuron (-60 mV)	43.3 ± 1.43 (n=6)	EIPA
Neuron (-20 mV)	32.2 ± 6.3 (n=3)	
CHO 1a	111 ± 30 (n=5)	
CHO 2a	31 (n=1)	
Knockout 1a	35.9 ± 2.1 (n=5)	
Knockout 2a	20.1 ± 2.2 (n=2)	
Neuron (-60 mV)	82.9 ± 5.2 (n=8)	Bepridil
Neuron (-60 mV)	100 ± 11 (n=10)	KB-R7943
Neuron (-60 mV)	24.3 ± 17.2 (n=2)	5-(N-Methyl-N-isobutyl)-amiloride
Neuron (-60 mV)	15.0 ± 11.7 (n=3)	5-(N,N-hexamethylene)amiloride
Neuron (-60 mV)	14.8 ± 7.1 (n=2)	5-(N, N-Dimenthyl)amiloride hydrochloride

Example 7: Reduction of Infarct Volume in Mice by Intracerebroventricular Injection of Amiloride And Amiloride Analogs

[0153] Mice were subjected to 60 minutes of middle cerebral artery occlusion (MCAO) as described above. Amiloride or an amiloride analog benzamil, bepridel, EIPA or KB-R7943 was administered by intracerebroventricular injection one hour after MCAO. The animals were evaluated one day after ischemia induction. As shown in FIG. 18,

intracerebroventricular injection of amiloride or an amiloride analog benzamil, bepridel, EIPA or KB-R7943 effectively reduce infarct volume.

Example 8: Reduction of Infarct Volume in Mice by Intravenous Injection of Amiloride

[0154] Mice were subjected to 60 minutes of middle cerebral artery occlusion (MCAO) as described above. Amiloride was administered by intravenous injection 1, 3 or 5 hours after MCAO. The animals were evaluated one day after ischemia induction. As shown in FIG. 19, intravenous injection of amiloride effectively reduce infarct volume. The effective CNS penetration of amiloride may be explained by the fact that blood-brain-barrier is compromised following brain ischemia/reperfusion. FIG. 20 shows that intravenous injection of amiloride has a prolonged therapeutic window of 5h.

Example 9: Structure Activity Relationships for Hydrophobic Amiloride Analogs on Various Channels

[0155] As shown in Table 1, substituting the C-5 amino group in amiloride with alkyl groups led to a decrease in potency at the ASIC1a channel . The same substitution increases potency to the ASIC3 channel (Kuduk *et al.*, Bioorg. Med. Chem. Lett., 2009, 19:2514-2518). The reverse result was obtained when substituting hydrophobic groups onto the guanidino part of the structure. Indeed, the benzyl substituted guanidino analog, benzamil, was the most potent ASIC1a blocking compound tested (IC50 = 4.9 μM). Taken together, these results showed that amiloride is an effective blocker of ASUC1a with an IC_{50} of 7.7 μM. They also provide structure activity relationships (see FIG. 21) for designing amiloride analogs that would inhibit the ASIC1a channel. Accordingly, in some embodiments, amiloride analogs are generated by introducing changes in the guanidine portion of the amiloride structure. Since amiloride is only a very weak inhibitor of the Na^+/Ca^{2+} ion exchanger (IC_{50} = 1.1 mM). The amiloride analogs are likely be a very weak inhibitor of the Na^+/Ca^{2+} ion exchanger as well. In some embodiments, the amiloride analogs are designed

to have increased selectivity for ASIC1a over the ASIC3 channel. In other embodiments, a ring structure, such as *a cyclic guanidine group,* is introduced into the amiloride structure to increase *inhibitory potency of ASIC1a currents.* It is also possible that one or more of the N-H groups of amiloride will form H-bonds either internally with the 3-amino group or with the ion channel.

[0156] The *in vivo* results in mice showed that efficacy can be achieved with a plasma concentration of 32.5μM (iv dose of 50 μl x 1mM) and a total brain concentration of 12.5μM (icv dose of 1 μl x 500 μM). It is thus estimated that only a 10-fold increase in potency is necessary to achieve efficacious concentrations that are suitable for an acute therapeutic for stroke in humans. Therefore, novel analogs are screened for an increased ASIC1a IC50 potency from the 4 to 8 μM for amiloride and benzamil to <1 μM.

[0157] In some embodiments, the amiloride analogs comprise methylated analogs of benzamil (formula 1-5 of FIG. 21) and amidino analog of benzamil (formula 6 of FIG.21). In other embodiments, the amiloride analogs contain a ring formed on the guanidine group. In other embodiments, the amiloride analogs contain an acylguanidino group for increased inhibitory potency of ASIC1a currents.

[0158] Amiloride is soluble in water at 1 mM and is effective in treating ischemia in a mouse model at a dose of 50 ul per injection. The equivalent dose on a mg/kg basis in a 65 kg human would be close to 40 mg and require an injection volume of over 160 ml. Similarly benzamil has a reported solubility in 0.9% saline of 0.4mg/ml (1.7 mM), which permits administration of only 5 mg benzamil dihydrochloride in a 10 ml injection.. Accordingly, amiloride analogs with higher water solubility are desired. In some embodiments, the amiloride analogs contain a water solubilizing group, such as an N,N-dimethyl amino group or a sugar, at the guanidino group to improve water solubility. In some embodiments, the amiloride analogs have a water solubility of 5 mM, 10 mM, 20 mM,

30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM or higher. In other embodiments, the amiloride analogs have a solubility that allows for a 10 mg, 25 mg, 50 mg, 100 mg, 150 mg, 200 mg, 250 mg, 300 mg, 400 mg, or 500 mg dose to be administered intravenously to a human in a single 10 ml injection. In yet other embodiments, the amiloride analogs have a solubility that allows for a 10 mg, 25 mg, 50 mg, 100 mg, 150 mg, 200 mg, 250 mg, 300 mg, 400 mg, or 500 mg dose to be administered intracerebroventicularly to a human in a single 2 ml injection.

[0159] The disclosure set forth above may encompass one or more distinct inventions, with independent utility. Each of these inventions has been disclosed in its preferred form(s). These preferred forms, including the specific embodiments thereof as disclosed and illustrated herein, are not intended to be considered in a limiting sense, because numerous variations are possible. The subject matter of the inventions includes all novel and nonobvious combinations and subcombinations of the various elements, features, functions, and/or properties disclosed herein. The following claims particularly point out certain combinations and subcombinations regarded as novel and nonobvious. Inventions embodied in other combinations and subcombinations of features, functions, elements, and/or properties may be claimed in applications claiming priority from this or a related application. Such claims, whether directed to a different invention or to the same invention, and whether broader, narrower, equal, or different in scope to the original claims, also are regarded as included within the subject matter of the inventions of the present disclosure.

WHAT IS CLAIMED IS:

1. A method for reducing nerve injury in a subject, comprising:

administering to said subject a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog, or a pharmaceutically acceptable salt thereof.

2. The method of Claim 1, wherein said active ingredient comprises amiloride or a pharmaceutically acceptable salt thereof.

3. The method of Claim 1, wherein said active ingredient comprises an amiloride analog or a pharmaceutically acceptable salt thereof.

4. The method of Claim 3, wherein said amiloride analog is selected from the group consisting of benzamil, phenmil, 5-(N-ethyl-N-isobutyl)-amiloride (EIPA), bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

5. The method of Claim 4, wherein said amiloride analog is benzamil.

6. The method of Claim 3, wherein said amiloride analog is a methylated analog of benzamil.

7. The method of Claim 3, wherein said amiloride analog comprises a ring formed on a guanidine group.

8. The method of Claim 3, wherein said amiloride analog comprises an acylguanidino group.

9. The method of Claim 3, wherein said amiloride analog comprises a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

10. The method of Claim 1, wherein said pharmaceutical composition is administered intravenously.

11. The method of Claim 1, wherein said pharmaceutical composition is administered intrathecally.

12. The method of Claim 1, wherein said pharmaceutical composition is administered intracerebroventricularly.

13. The method of Claim 2, wherein said amiloride, amiloride analog or a pharmaceutically acceptable salt thereof is given in a dose range of 0.1 mg - 10 mg /kg body weight.

14. The method of Claim 1, wherein said pharmaceutical composition is administered within one hour of the onset of an ischemic event.

15. The method of Claim 1, wherein said pharmaceutical composition is administered within five hours of the onset of an ischemic event.

16. The method of Claim 1, wherein said pharmaceutical composition is administered between one hour and five hours of the onset of an ischemic event.

17. The method of Claim 1, wherein said nerve injury is a nervous system injury.

18. The method of Claim 1, wherein said nerve injury is brain injury.

19. A method for treating brain injury in a subject, comprising:

administering to said subject a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog, or a pharmaceutically acceptable salt thereof.

20. The method of Claim 19, wherein said pharmaceutical composition is administered intravenously, intrathecally or intracerebroventricularly.

21. The method of Claim 19, wherein said amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

22. The method of Claim 19, wherein said amiloride analog is benzamil.

23. The method of Claim 19, wherein said amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group,

wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

24. A method for reducing nervous system injury caused by a change of ion flux into neurons, comprising:

administering to a subject in need of such treatment a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof.

25. The method of Claim 24, wherein said pharmaceutical composition is administered intravenously, intrathecally, intracerebroventricularly or intramuscularly.

26. The method of Claim 24, wherein said amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

27. The method of Claim 24, wherein said amiloride analog is benzamil.

28. The method of Claim 24, wherein said amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group,

wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

29. A method for reducing nervous system injury, comprising:

administering to a subject in need of such treatment a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof.

30. The method of Claim 29, wherein said pharmaceutical composition is administered intravenously, intrathecally, intracerebroventricularly or intramuscularly.

31. The method of Claim 29, wherein said amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

32. The method of Claim 29, wherein said amiloride analog is benzamil.

33. The method of Claim 29, wherein said amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group,

wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

34. A pharmaceutical composition for reducing nervous system injury, comprising:

an effective amount of amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof; and

a pharmaceutically acceptable carrier, wherein the pharmaceutical composition is formulated for intravenous, intrathecal or intracerebroventricular injection.

35. The pharmaceutical composition of Claim 34, wherein said amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

36. The method of Claim 34, wherein said amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

37. A pharmaceutical composition for reducing nervous system injury, comprising:

an effective amount of an amiloride analog or a pharmaceutically acceptable salt thereof; and

a pharmaceutically acceptable carrier.

38. The method of Claim 37, wherein wherein the pharmaceutical composition is formulated for intravenous, intrathecal, intracerebroventricular or intramuscular injection.

39. The pharmaceutical composition of Claim 37, wherein said amiloride analog is a methylated analog of benzamil.

40. The pharmaceutical composition of Claim 37, wherein said amiloride analog comprises a ring formed on a guanidine group.

41. The pharmaceutical composition of Claim 37, wherein said amiloride analog comprises an acylguanidino group.

42. The pharmaceutical composition of Claim 37, wherein said amiloride analog comprises a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

AMENDED CLAIMS

received by the International Bureau on 15 October 2014 (15.10.2014)

1. A method for reducing infarct volume in a subject, comprising: administering to said subject a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog, or a pharmaceutically acceptable salt thereof.

2. The method of Claim 1, wherein said active ingredient comprises amiloride or a pharmaceutically acceptable salt thereof.

3. The method of Claim 1, wherein said active ingredient comprises an amiloride analog or a pharmaceutically acceptable salt thereof.

4. The method of Claim 3, wherein said amiloride analog is selected from the group consisting of benzamil, phenmil, 5-(N-ethyl-N-isobutyl)-amiloride (EIPA), bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

5. The method of Claim 4, wherein said amiloride analog is benzamil.

6. The method of Claim 3, wherein said amiloride analog is a methylated analog of benzamil.

7. The method of Claim 3, wherein said amiloride analog comprises a ring formed on a guanidine group.

8. The method of Claim 3, wherein said amiloride analog comprises an acylguanidino group.

9. The method of Claim 3, wherein said amiloride analog comprises a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

10. The method of Claim 1, wherein said pharmaceutical composition is administered intravenously.

11. The method of Claim 1, wherein said pharmaceutical composition is administered intrathecally.

12. The method of Claim 1, wherein said pharmaceutical composition is administered intracerebroventricularly.

13. The method of Claim 2, wherein said amiloride, amiloride analog or a pharmaceutically acceptable salt thereof is given in a dose range of 0.1 mg - 10 mg /kg body weight.

14. The method of Claim 1, wherein said pharmaceutical composition is administered within one hour of the onset of an ischemic event.

15. The method of Claim 1, wherein said pharmaceutical composition is administered within five hours of the onset of an ischemic event.

16. The method of Claim 1, wherein said pharmaceutical composition is administered between one hour and five hours of the onset of an ischemic event.

17. The method of Claim 1, wherein said nerve injury is a nervous system injury.

18. The method of Claim 1, wherein said nerve injury is brain injury.

19. A method for treating brain injury by reducing infarct volume in a subject, comprising: administering to said subject a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog, or a pharmaceutically acceptable salt thereof.

20. The method of Claim 19, wherein said pharmaceutical composition is administered intravenously, intrathecally or intracerebroventricularly.

21. The method of Claim 19, wherein said amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

22. The method of Claim 19, wherein said amiloride analog is benzamil.

23. The method of Claim 19, wherein said amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

24. A method for reducing nervous system injury caused by a change of ion flux into neurons by reducing infarct volume in a subject, comprising: administering to a subject in need of such treatment a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof.

25. The method of Claim 24, wherein said pharmaceutical composition is administered intravenously, intrathecally, intracerebroventricularly or intramuscularly.

26. The method of Claim 24, wherein said amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

27. The method of Claim 24, wherein said amiloride analog is benzamil.

28. The method of Claim 24, wherein said amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

29. A method for reducing infarct volume in a subject, comprising: administering to a subject in need of such treatment a therapeutically effective amount of a pharmaceutical composition comprising amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof.

30. The method of Claim 29, wherein said pharmaceutical composition is administered intravenously, intrathecally, intracerebroventricularly or intramuscularly.

31. The method of Claim 29, wherein said amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

32. The method of Claim 29, wherein said amiloride analog is benzamil.

33. The method of Claim 29, wherein said amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

34. A pharmaceutical composition for reducing infarct volume in a subject, comprising: an effective amount of amiloride, an amiloride analog or a pharmaceutically acceptable salt thereof; and a pharmaceutically acceptable carrier, wherein the pharmaceutical composition is formulated for intravenous, intrathecal or intracerebroventricular injection.

35. The pharmaceutical composition of Claim 34, wherein said amiloride analog is selected from the group consisting of benzamil, phenmil, EIPA bepridil, KB-R7943, 5-(N-methyl-N-isobutyl) amiloride, 5-(N,N-hexamethylene) amiloride and 5-(N,N-dimenthyl) amiloride hydrochloride.

36. The pharmaceutical composition of Claim 34, wherein said amiloride analog is selected from the group consisting of methylated analogs of benzamil, amiloride analogs containing a ring formed on a guanidine group, amiloride analogs containing an acylguanidino group, and amiloride analogs containing a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

37. A pharmaceutical composition for reducing infarct volume in a subject, comprising: an effective amount of an amiloride analog or a pharmaceutically acceptable salt thereof; and a pharmaceutically acceptable carrier.

38. The pharmaceutical composition of Claim 37, wherein the pharmaccutical composition is formulated for intravenous, intrathecal, intracerebroventricular or intramuscular injection.

39. The pharmaceutical composition of Claim 37, wherein said amiloride analog is a methylated analog of benzamil.

40. The pharmaceutical composition of Claim 37, wherein said amiloride analog comprises a ring formed on a guanidine group.

41. The pharmaceutical composition of Claim 37, wherein said amiloride analog comprises an acylguanidino group.

42. The pharmaceutical composition of Claim 37, wherein said amiloride analog comprises a water solubilizing group formed on a guanidine group, wherein the water solubilizing group is a N,N-dimethyl amino group or a sugar group.

STATEMENT UNDER ARTICLE 19 (1)

Applicant is submitting the Amendment under Article 19 to amend Claims 1-42 by replacing pages 56-60. Applicant respectfully requests the International Preliminary Examining Authority's acceptance of the Amendment.

The changes are as follows:

Claim 1 amended; Claims 2 to 18 unchanged; Claim 19 amended; Claims 20 to 23 unchanged; Claim 24 amended; Claims 25 to 28 unchanged; Claim 29 amended; Claims 30 to 33 unchanged; Claim 34 amended; Claim 35 unchanged; Claim 36 amended; Claim 37 amended; Claim 38 amended; Claims 39-42 unchanged.

The basis for the amendments is as follows:

(i) Basis for the amendment: Claim 1 has been amended at line 1 and now indicates that the method is for reducing infarct volume in a subject. The basis for this amendment can be found at least at paragraphs [0039]-[0041], [0140], [0142] and Figs. 18-20 in the specification as originally filed.

(ii) Basis for the amendment: Claim 19 has been amended at line 1 and now indicates that the method is for treating brain injury by reducing infarct volume in a subject. The basis for this amendment can be found at least at paragraphs [0039]-[0041], [0140], [0142] and Figs. 18-20 in the specification as originally filed.

(iii) Basis for the amendment: Claim 24 has been amended at line 2 and now indicates that the method is for reducing nervous system injury caused by a change of ion flux into neurons by reducing infarct volume in a subject. The basis for this amendment can be found at least at paragraphs [0039]-[0041], [0140], [0142] and Figs. 18-20 in the specification as originally filed.

(iv) Basis for the amendment: Claim 29 has been amended at line 1 and now indicates that the method is for reducing infarct volume in a subject. The basis for this amendment can

be found at least at paragraphs [0039]-[0041], [0140], [0142] and Figs. 18-20 in the specification as originally filed.

(v) Basis for the amendment: Claim 34 has been amend at line 1-2 and now indicates that the pharmaceutical composition is for reducing infarct volume in a subject. The basis for this amendment can be found at least at paragraphs [0039]-[0041], [0140], [0142] and Figs. 18-20 in the specification as originally filed.

(vi) Basis for the amendment: Claim 36 has been amended to now indicate that it is drawn to the pharmaceutical composition of Claim 34. Basis for the amendment is found in Claim 34 as originally filed.

(vii) Basis for the amendment: Claim 37 has been amend at line 1-2 and now indicates that the pharmaceutical composition is for reducing infarct volume in a subject. The basis for this amendment can be found at least at paragraphs [0039]-[0041], [0140], [0142] and Figs. 18-20 in the specification as originally filed.

(viii) Basis for the amendment: Claim 38 has been amended to now indicate that it is drawn to the pharmaceutical composition of Claim 37. It has also been amended to remove the duplication of the term "wherein." Basis for the amendment is found in Claim 37 as originally filed.

Should anything further be needed in order to record the above changes, please contact the undersigned listed below.

Fig. 1

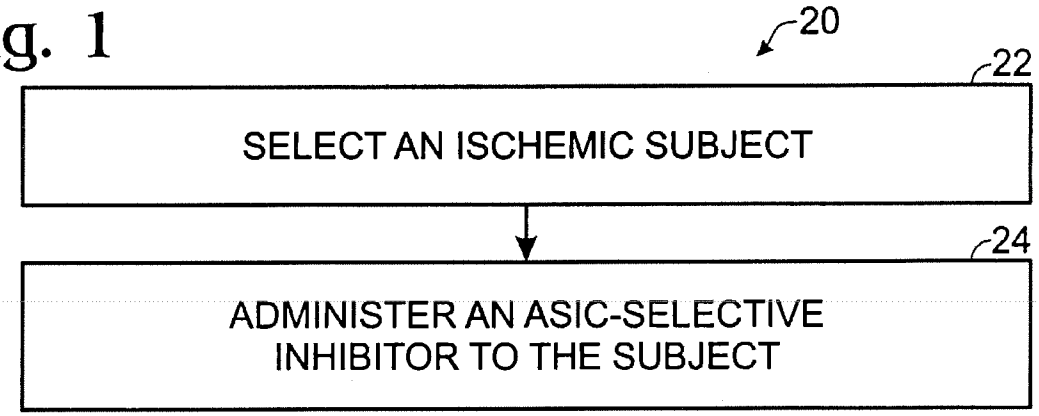


Fig. 2

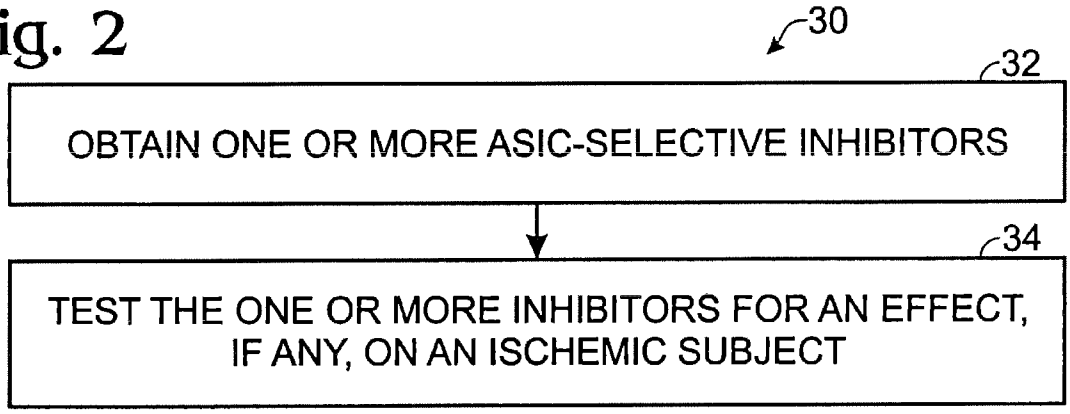


Fig. 3A

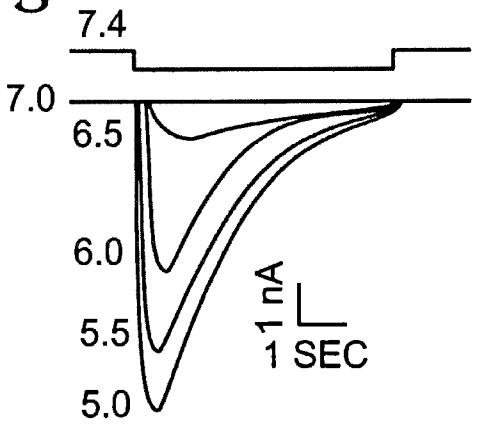


Fig. 3B

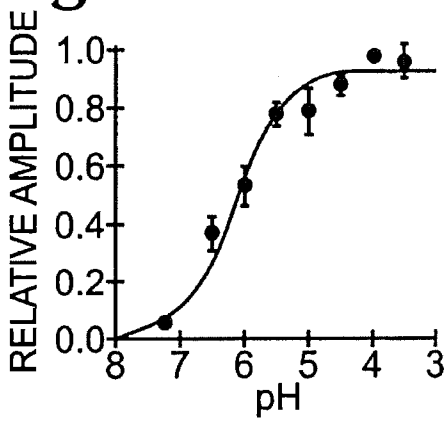


Fig. 3C

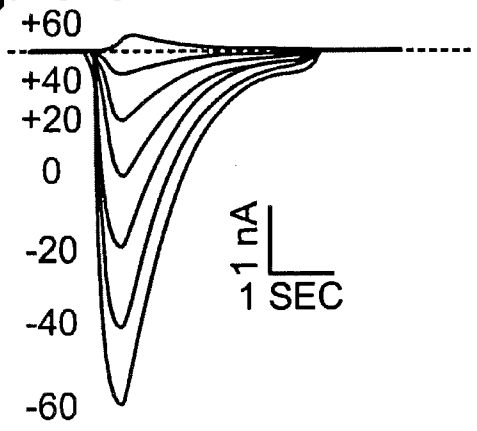


Fig. 3D

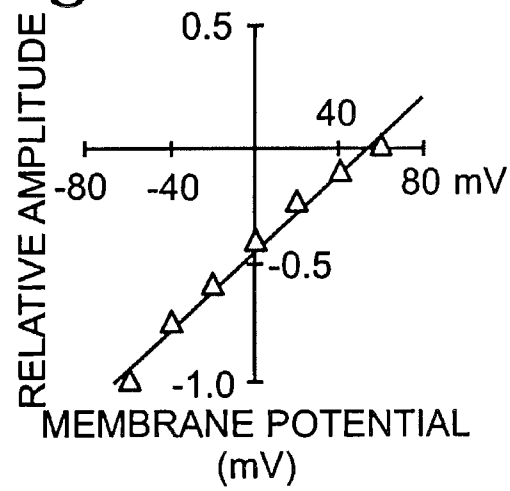


Fig. 4A

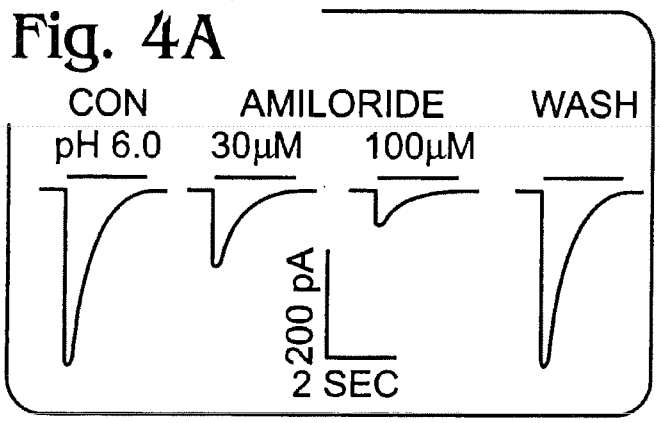


Fig. 4B

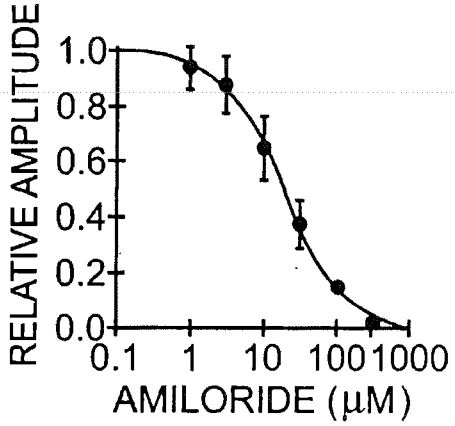


Fig. 4C

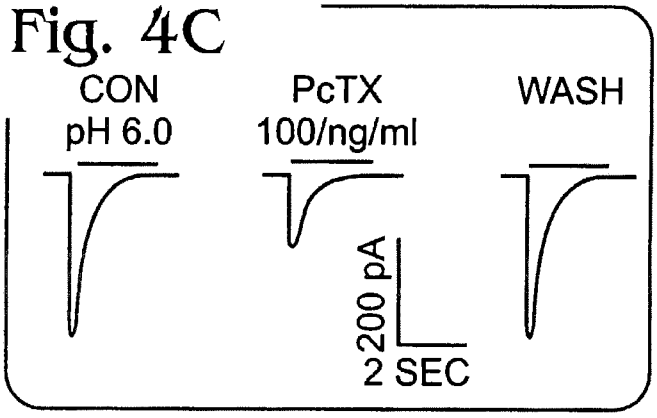


Fig. 4D

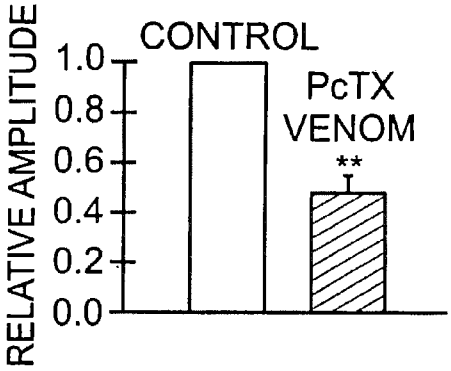


Fig. 5A

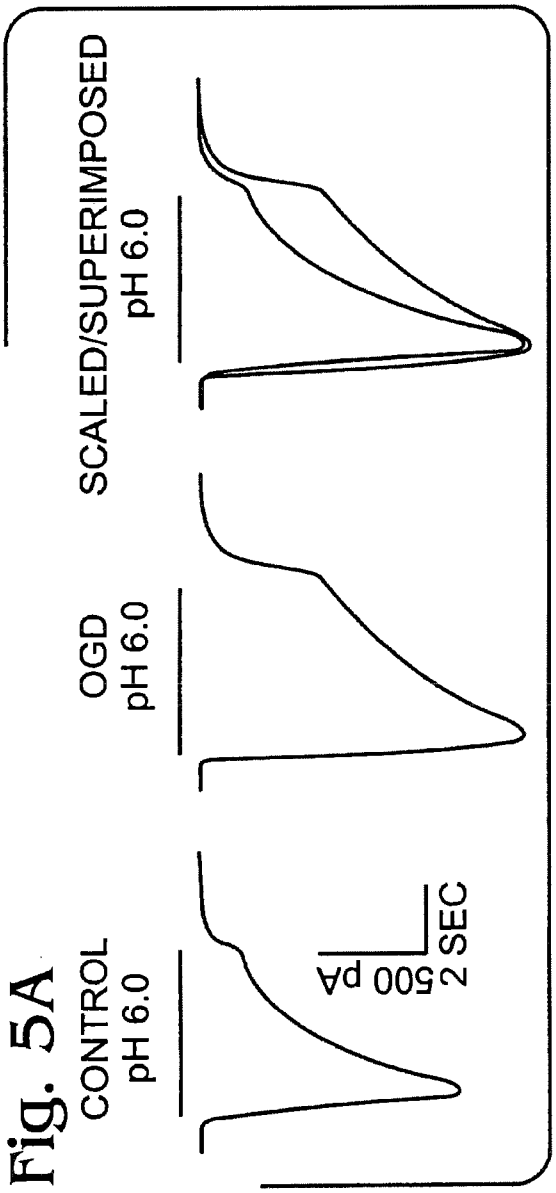


Fig. 5B

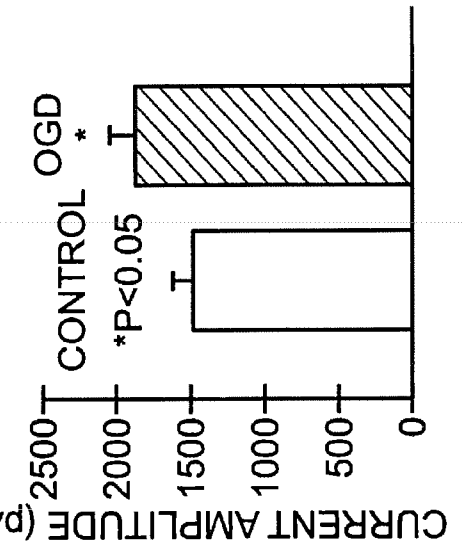


Fig. 5C

Fig. 5D

Fig. 6B

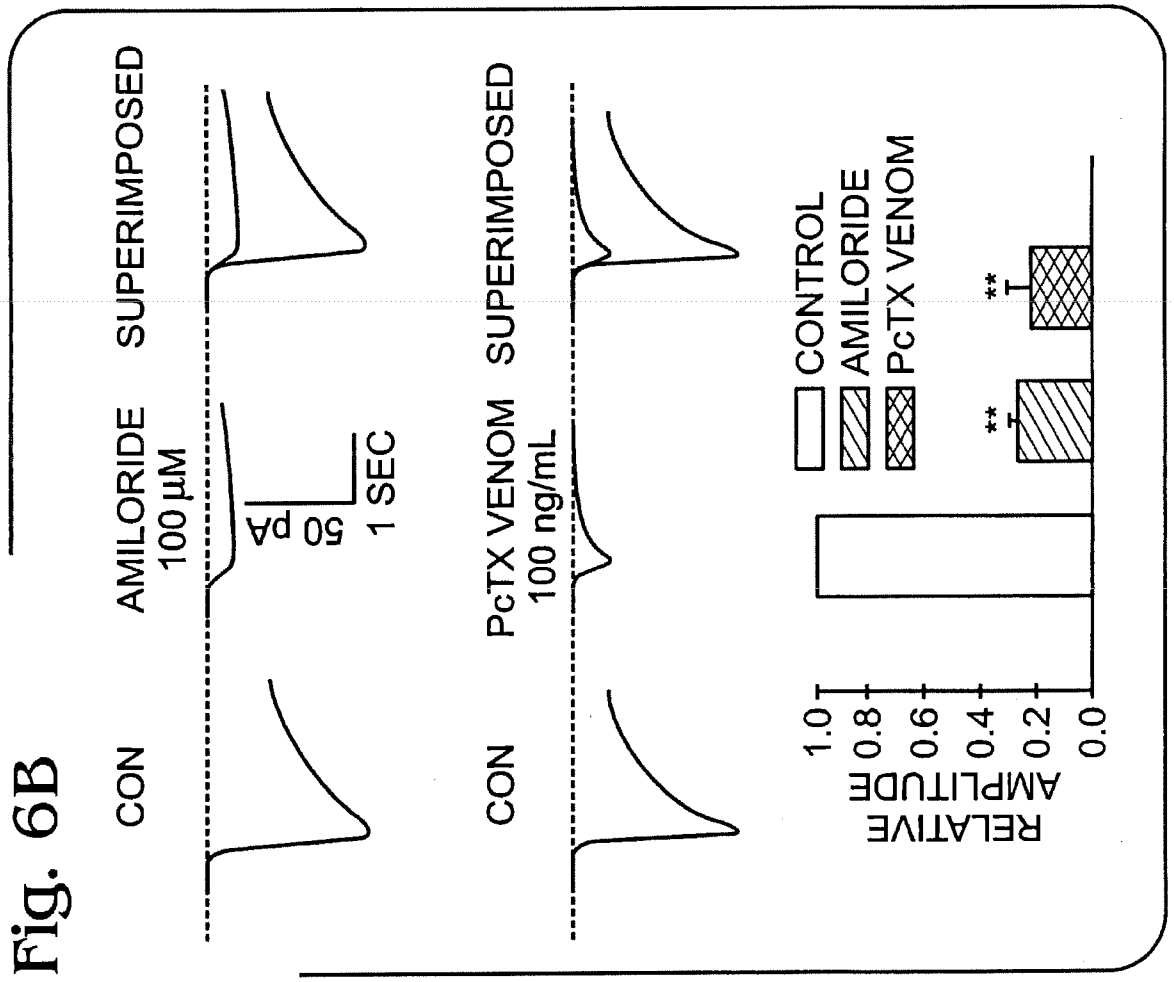

CON
AMILORIDE
100 µM
SUPERIMPOSED
50 pA
1 SEC
CON
PcTX VENOM
100 ng/mL
SUPERIMPOSED
CONTROL
AMILORIDE
PcTX VENOM
RELATIVE AMPLITUDE
1.0
0.8
0.6
0.4
0.2
0.0
**
**

Fig. 6A

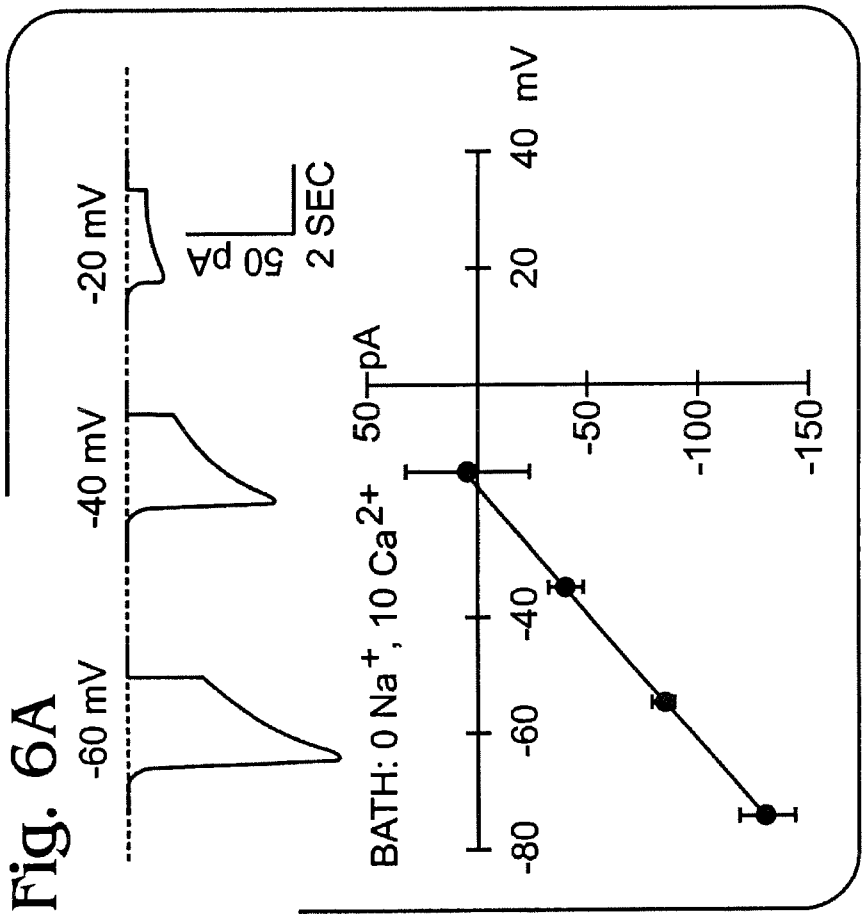

-60 mV
-40 mV
-20 mV
50 pA
2 SEC
BATH: 0 Na+, 10 Ca2+
50
pA
-50
-100
-150
-80
-60
-40
20
40
mV

Fig. 7A

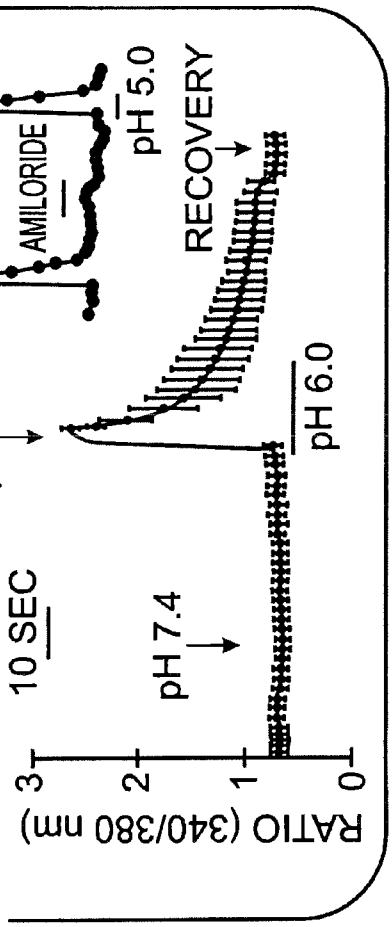


Fig. 7B

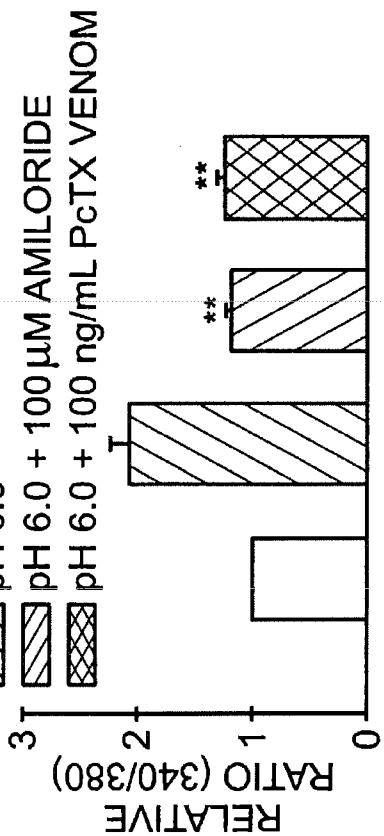


Fig. 7C

Fig. 7D

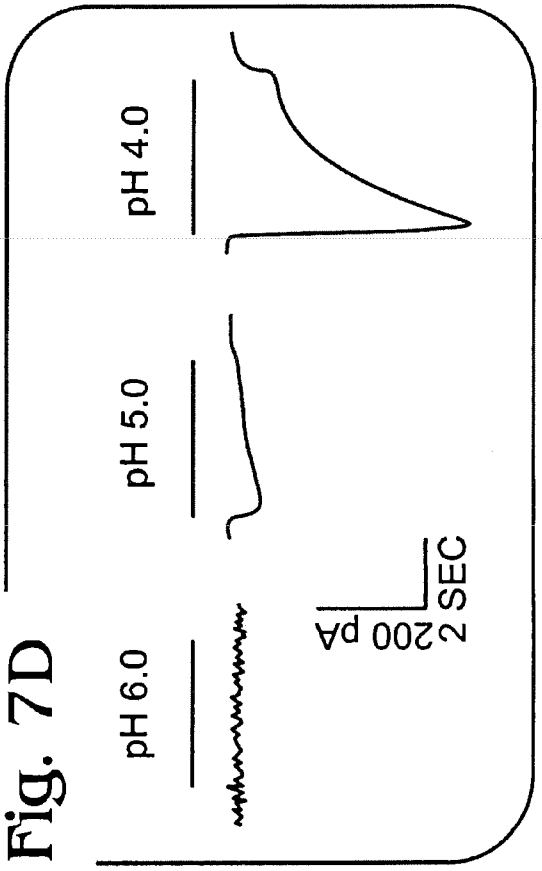


Fig. 8A

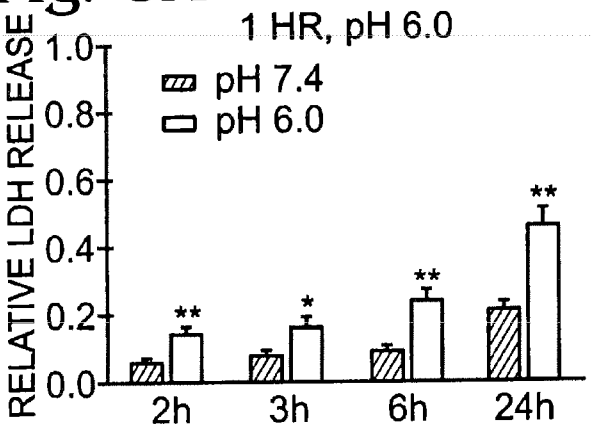


Fig. 8B

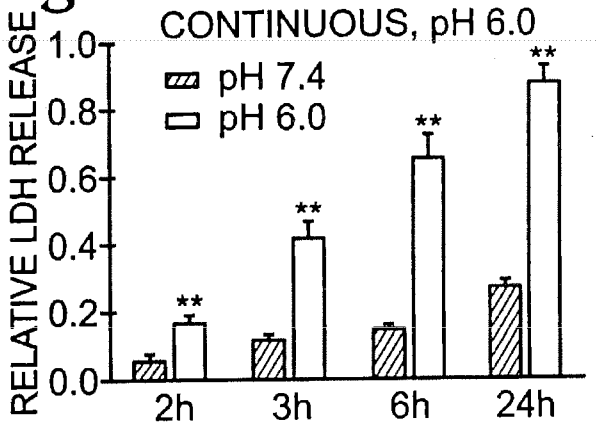


Fig. 8C

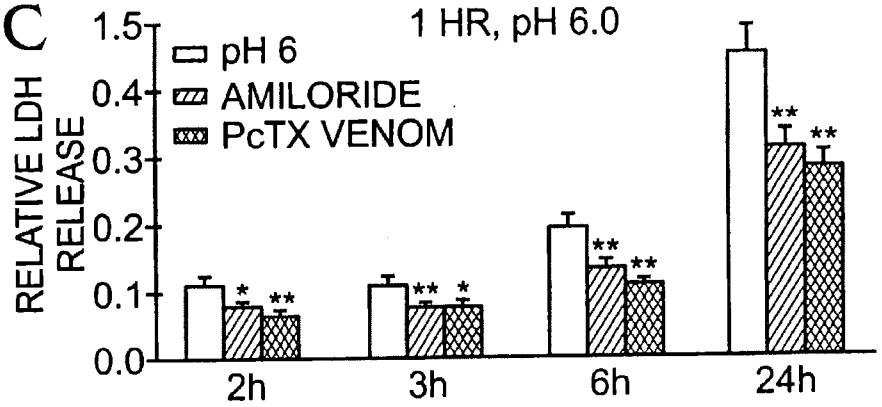


Fig. 9A

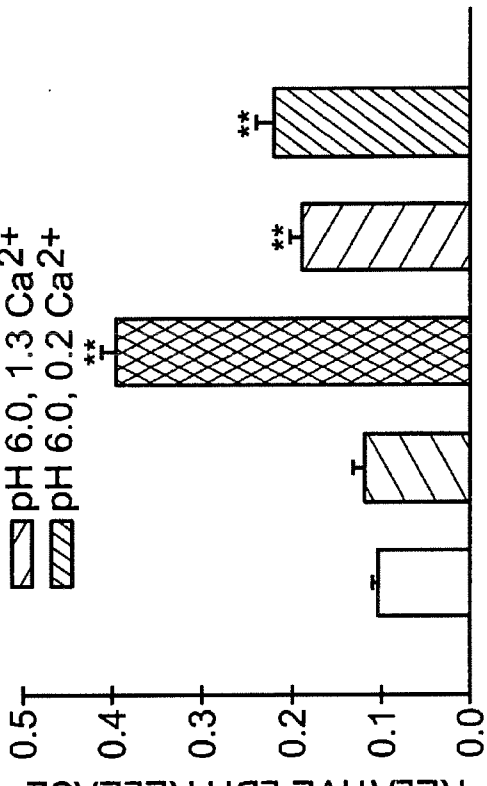


Fig. 9B

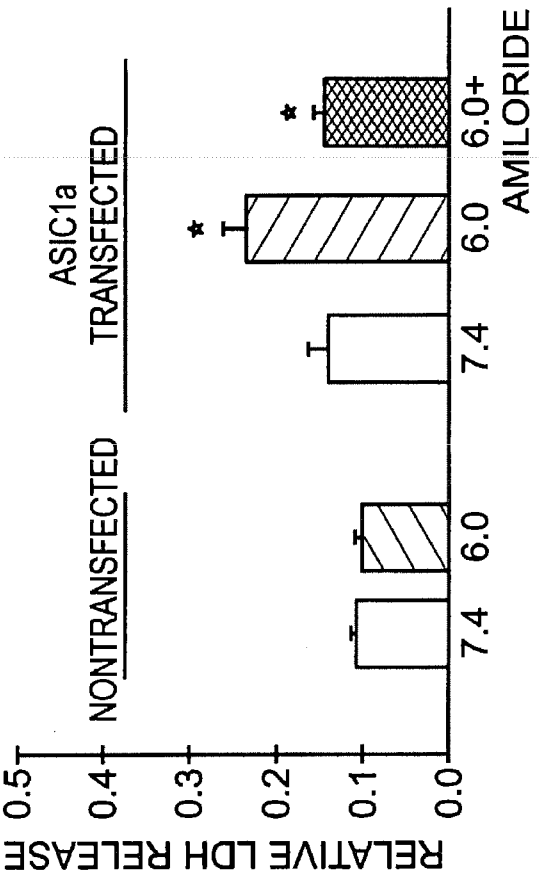


Fig. 9C

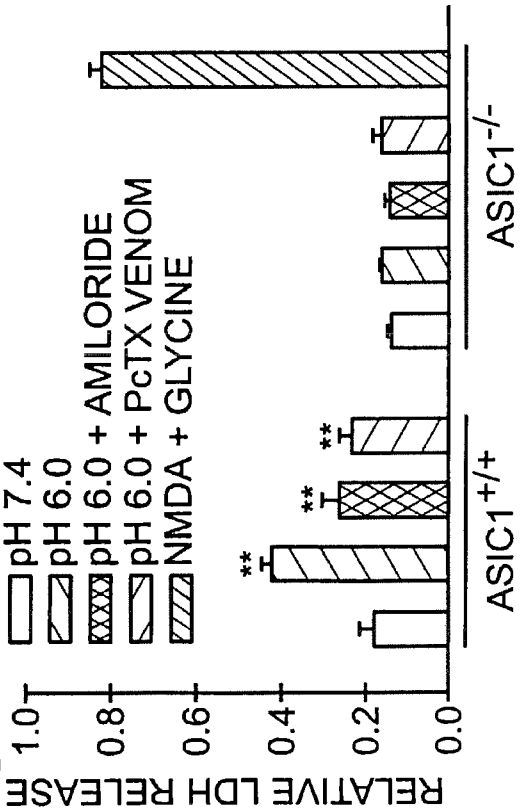


Fig. 9D

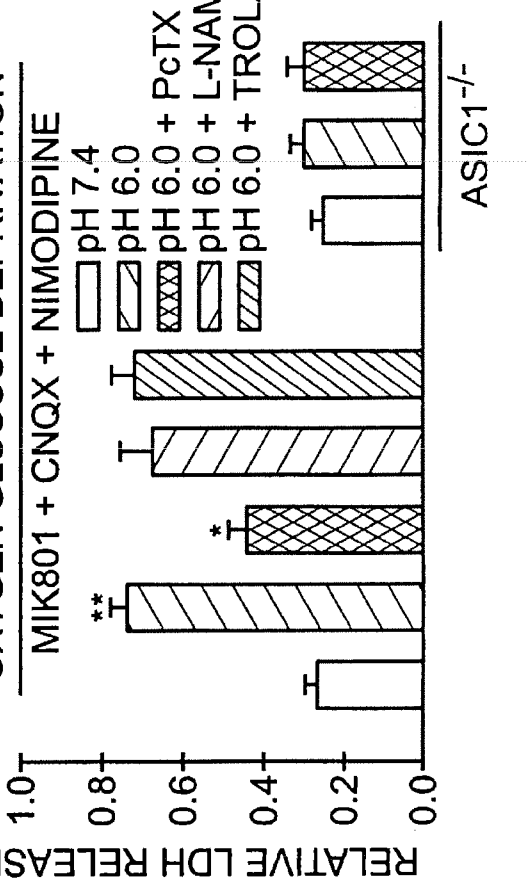


Fig. 10A

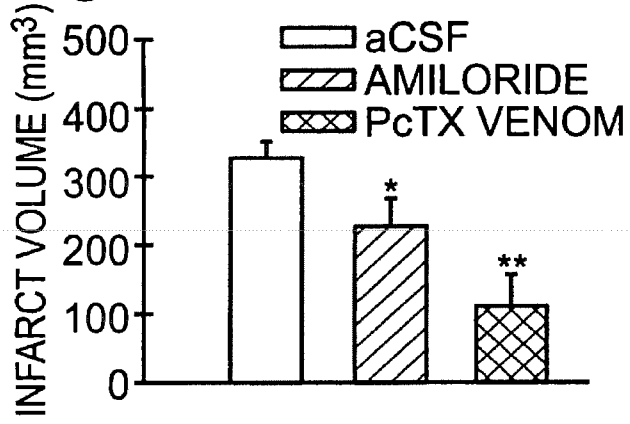


Fig. 10B

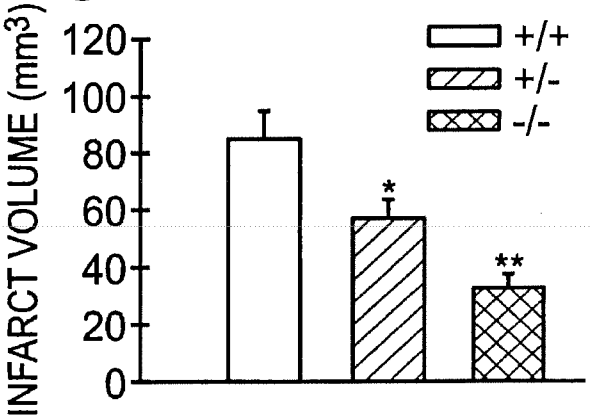


Fig. 10C

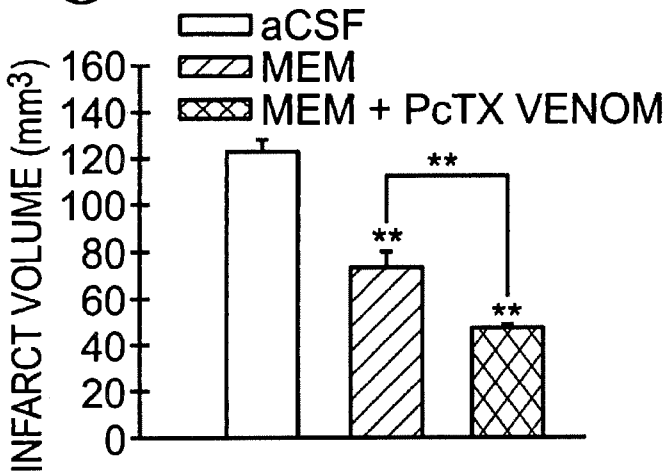


Fig. 10D

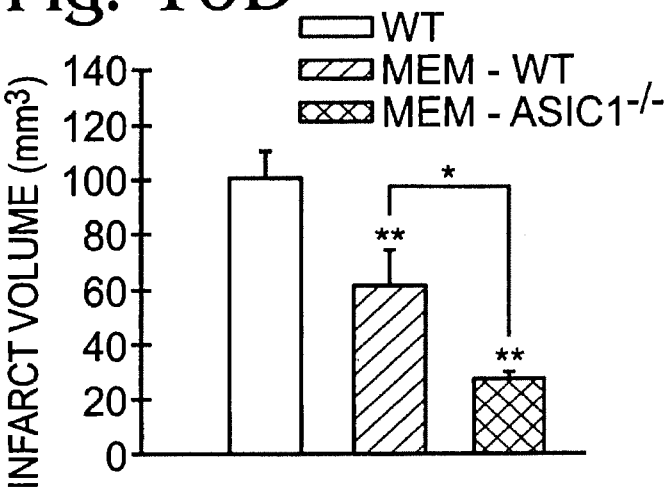


Fig. 11

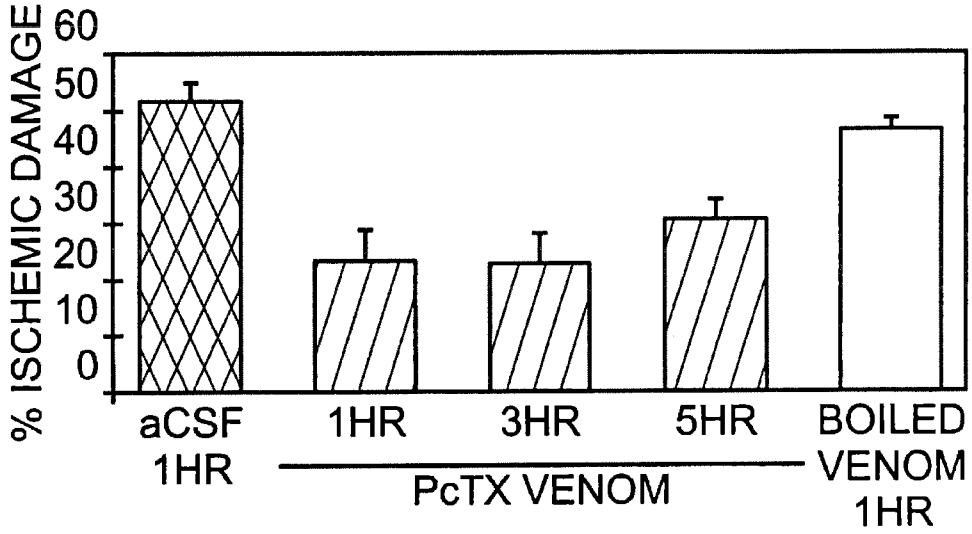


Fig. 12

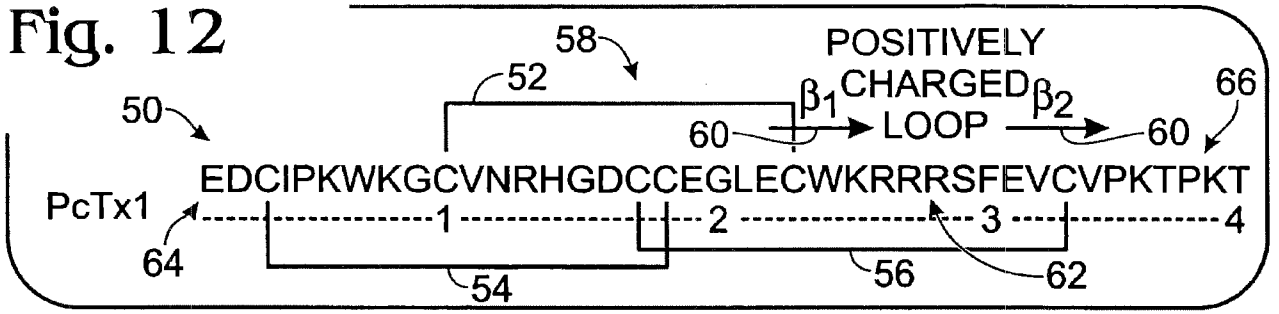


Fig. 13

	VARIANT	IDENTIFIER	POSITION 1 2 3 4
50	FULL LENGTH PcTx1	(SEQ ID:1):	EDCIPKWKGCVNRHGDCCEGLECWKRRRSFEVCVPKTPKT
70	N-TERMINAL DEL.	(SEQ ID:2):	CIPKWKGCVNRHGDCCEGLECWKRRRSFEVCVPKTPKT
72	C-TERMINAL DEL.	(SEQ ID:3):	EDCIPKWKGCVNRHGDCCEGLECWKRRRSFEVCVPKT
74	FULL C-TERMINAL DEL.	(SEQ ID:4):	EDCIPKWKGCVNRHGDCCEGLECWKRRRSFEVC
76	N & C -TERMINAL DEL.	(SEQ ID:5):	CIPKWKGCVNRHGDCCEGLECWKRRRSFEVC

Fig. 14

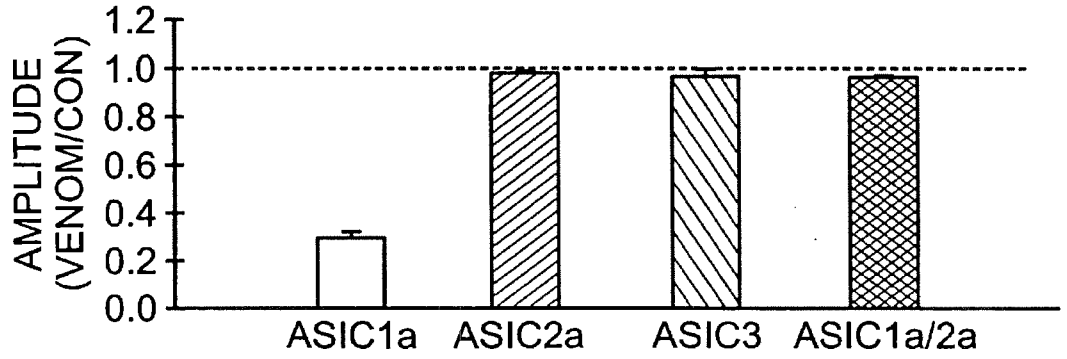
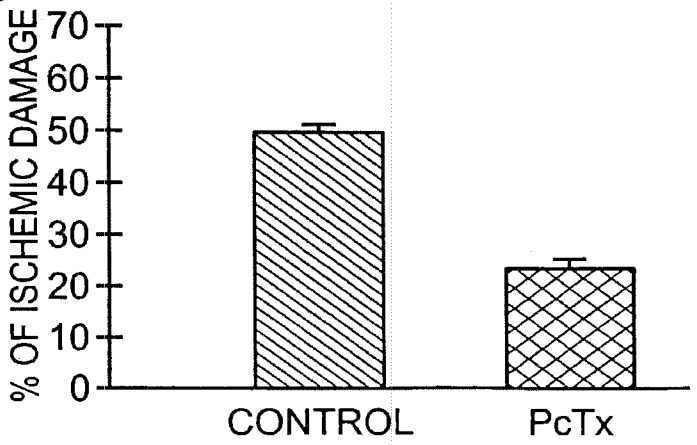


Fig. 15



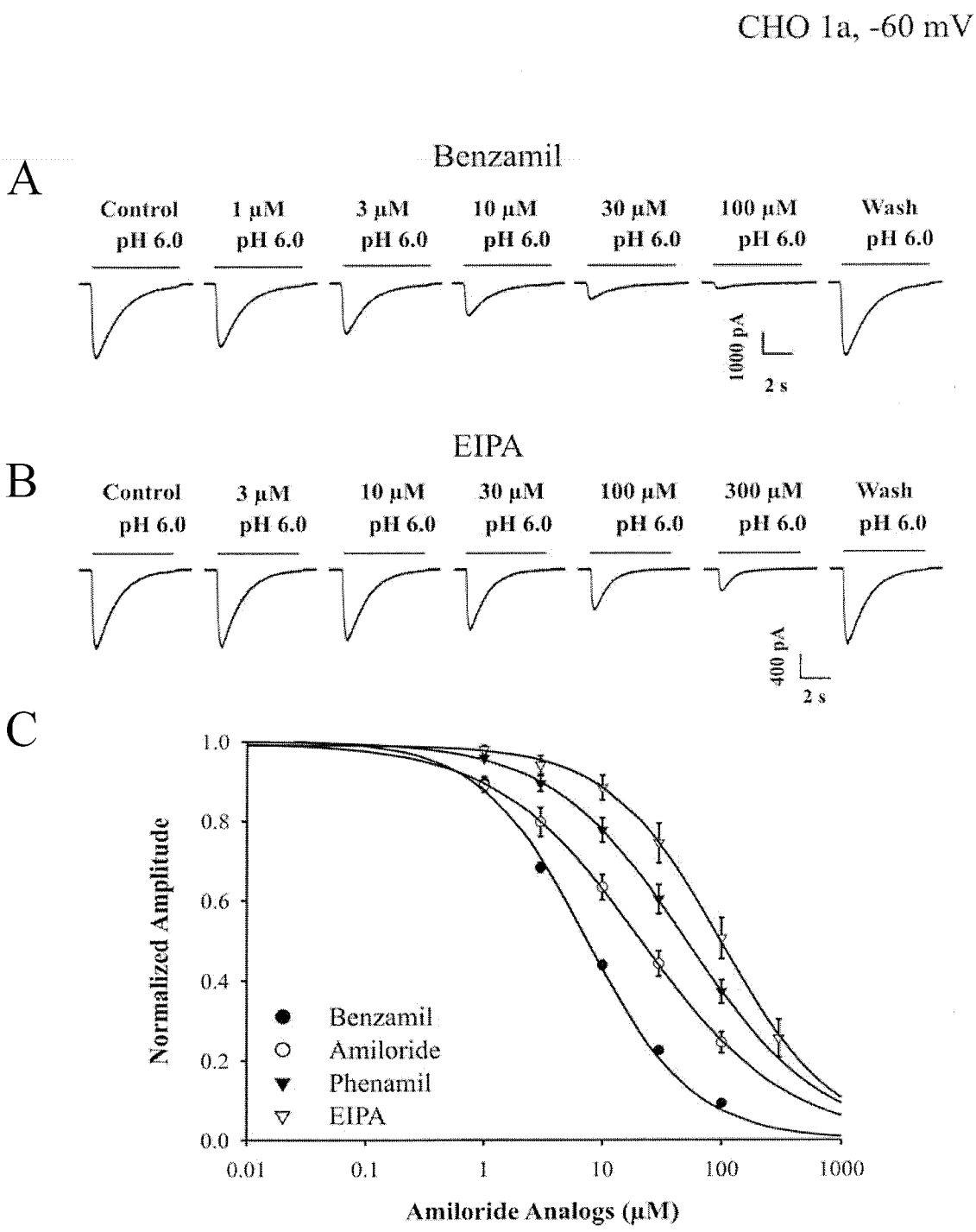

CHO 1a, -60 mV
A
Benzamil
Control pH 6.0
1 µM pH 6.0
3 µM pH 6.0
10 µM pH 6.0
30 µM pH 6.0
100 µM pH 6.0
Wash pH 6.0
1000 pA
2 s
B
EIPA
Control pH 6.0
3 µM pH 6.0
10 µM pH 6.0
30 µM pH 6.0
100 µM pH 6.0
300 µM pH 6.0
Wash pH 6.0
400 pA
2 s
C
Normalized Amplitude
1.0
0.8
0.6
0.4
0.2
0.0
Benzamil
Amiloride
Phenamil
EIPA
0.01
0.1
1
10
100
1000
Amiloride Analogs (µM)

FIG. 16

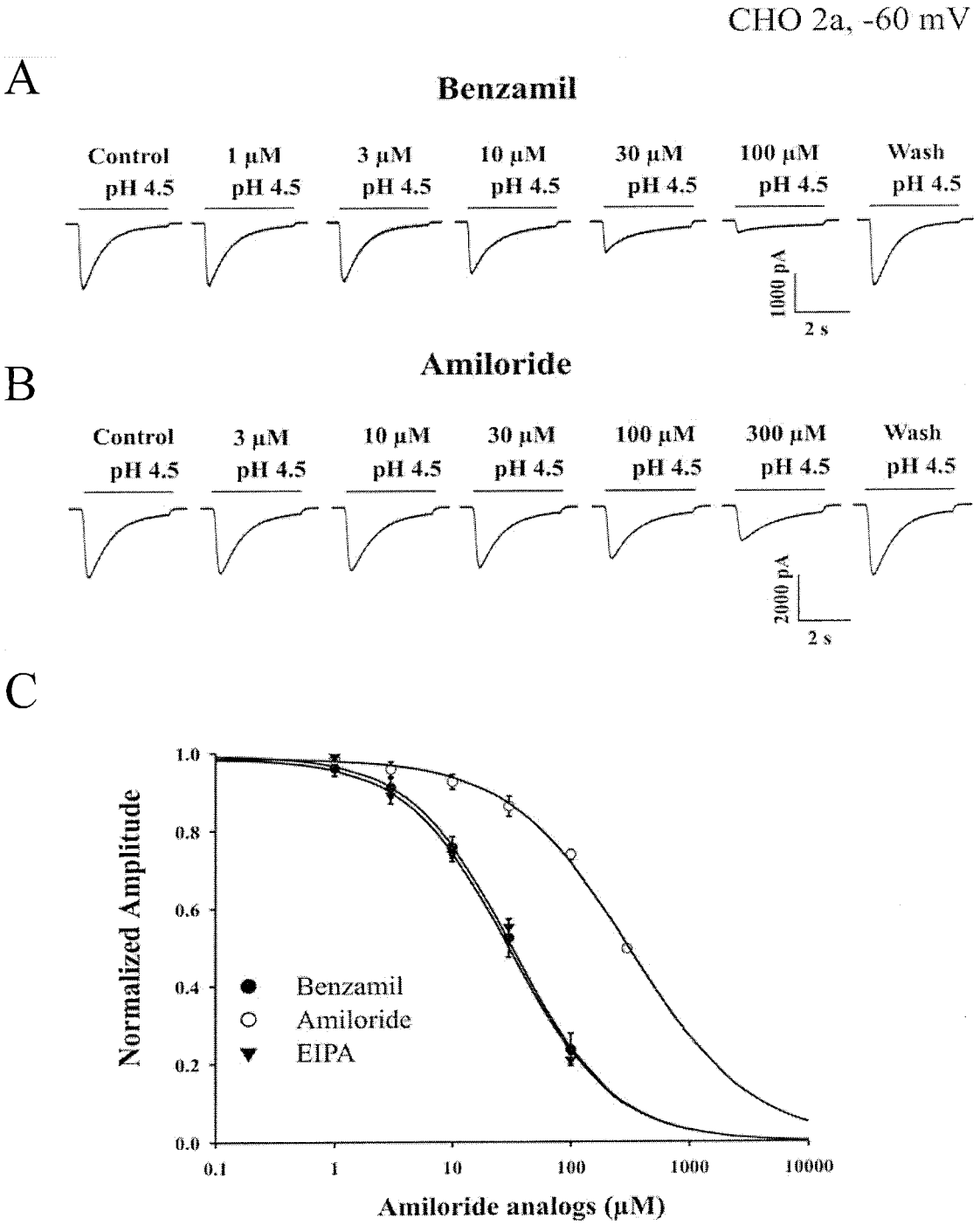

CHO 2a, -60 mV
A
Benzamil
Control pH 4.5
1 µM pH 4.5
3 µM pH 4.5
10 µM pH 4.5
30 µM pH 4.5
100 µM pH 4.5
Wash pH 4.5
1000 pA
2 s
B
Amiloride
Control pH 4.5
3 µM pH 4.5
10 µM pH 4.5
30 µM pH 4.5
100 µM pH 4.5
300 µM pH 4.5
Wash pH 4.5
2000 pA
2 s
C
Normalized Amplitude
1.0
0.8
0.6
0.4
0.2
0.0
Benzamil
Amiloride
EIPA
0.1
1
10
100
1000
10000
Amiloride analogs (µM)

FIG. 17

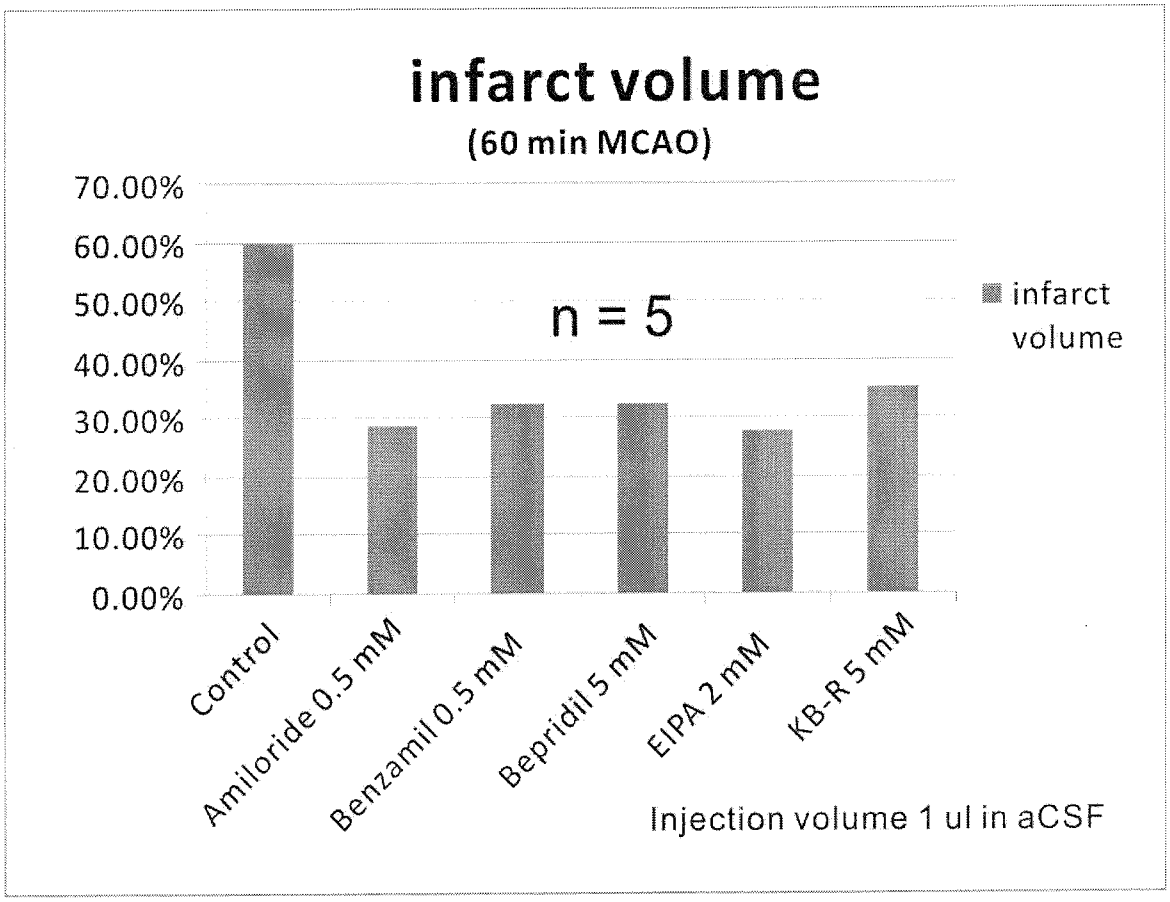

infarct volume
(60 min MCAO)
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
n = 5
infarct volume
Control
Amiloride 0.5 mM
Benzamil 0.5 mM
Bepridil 5 mM
EIPA 2 mM
KB-R 5 mM
Injection volume 1 ul in aCSF

FIG. 18

Saline 50 ul Amiloride 1 mM x 50 ul

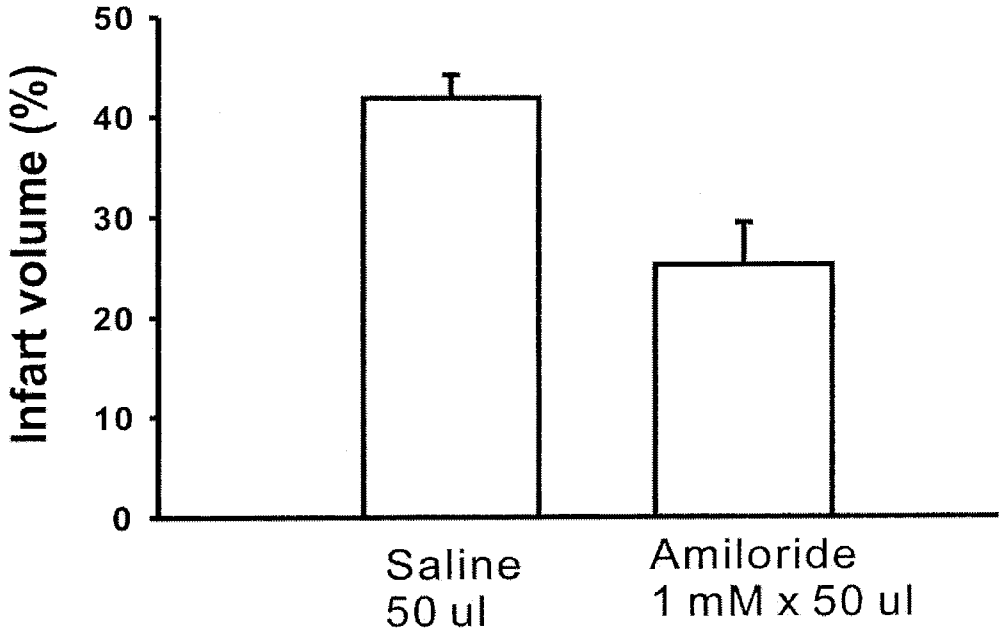


FIG. 19

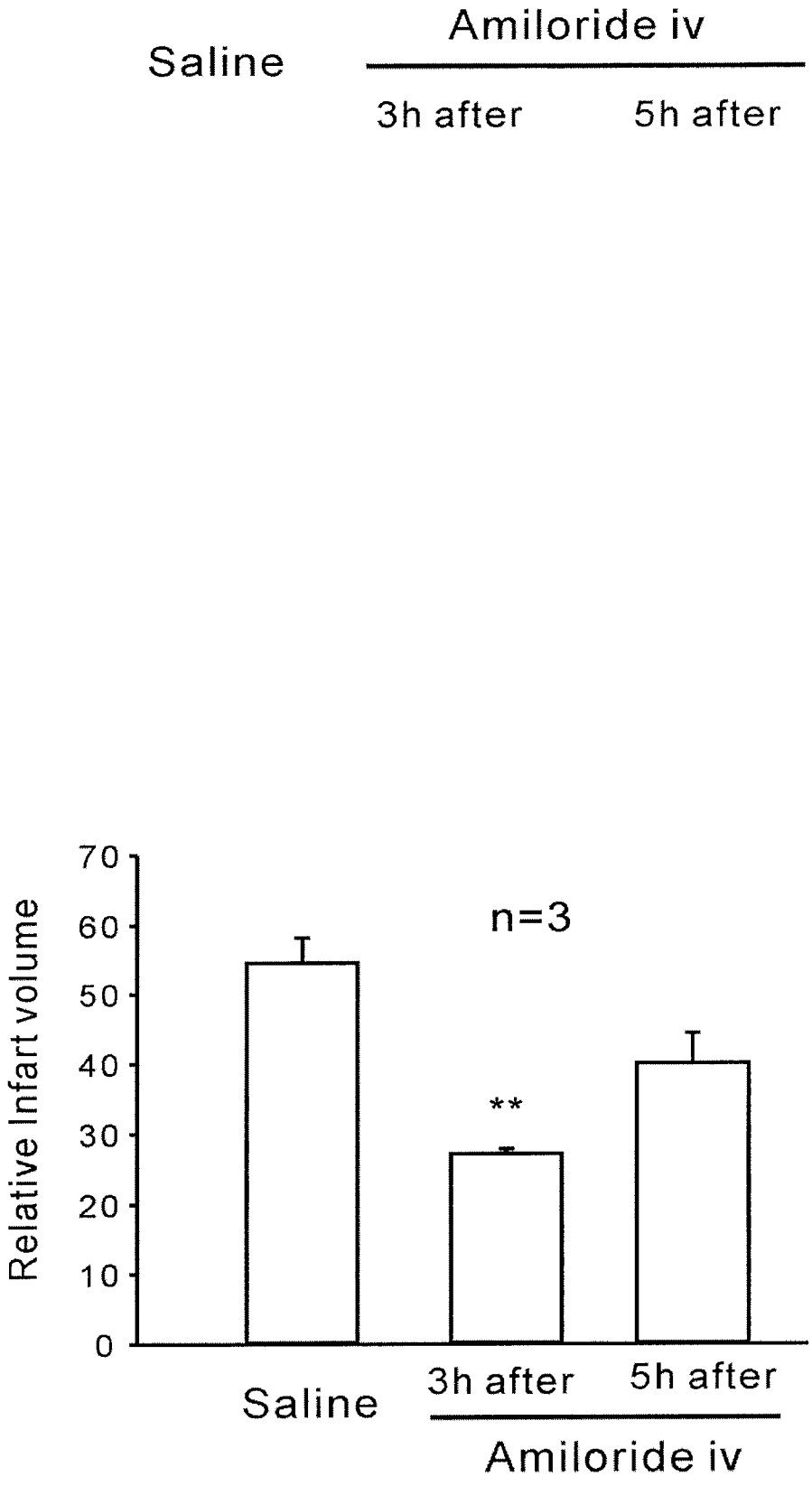

Saline
Amiloride iv
3h after
5h after
Relative Infart volume
70
60
50
40
30
20
10
0
n=3
**
Saline
3h after
5h after
Amiloride iv

FIG. 20

Structure Activity Relationships (SAR) for hydrophobic amiloride analogs on various channels

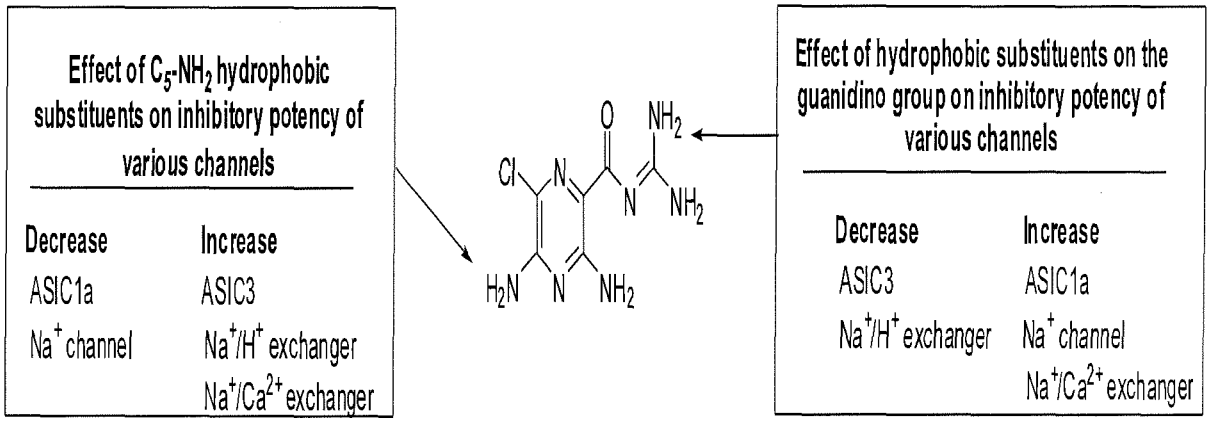


FIG. 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/056011

A. CLASSIFICATION OF SUBJECT MATTER

A61K 31/4965(2006.01)i, A61K 31/496(2006.01)i, A61K 31/495(2006.01)i, A61P 25/00(2006.01)i

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 31/4965; A61K 31/495; A61P 25/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: nerve injury, stroke, ischemia, amiloride, benzamil, phenamil, bepridil, KB-R7943

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DURHAM-LEE, J. C. et al., 'Amiloride improves locomotor recovery after spinal cord injury', Journal of Neurotrauma, 2011, Vol. 28, pages 1319-1326 See abstract and page 1320.	34,37-38
Y		35-36,39-42
Y	KLEYMAN, T. R. et al., 'Amiloride and its analogs as tools in the study of ion transport', The Journal of Membrane Biology, 1988, Vol. 105, pages 1-21 See abstract and tables 1-5.	35-36,39-41
Y	BENOS, D. J. et al., 'Effect of amiloride and some of its analogues on cation transport in isolated frog skin and thin lipid membranes', The Journal of General Physiology, 1976, Vol. 68, pages 43-63 See abstract and table III.	42
A	ROBERTSON, N. J. et al., 'Methyl-isobutyl amiloride reduces brain Lac/NAA, cell death and microglial activation in a perinatal asphyxia model', Journal of Neurochemistry, 26 December 2012 (E-pub.), Vol. 124, pages 645-657 See abstract.	34-42

☒ 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	Date of mailing of the international search report
26 May 2014 (26.05.2014)	**26 May 2014 (26.05.2014)**

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/056011

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2009-0062309 A1 (DELGADO-ALMEIDA, A.) 5 March 2009 See abstract and paragraph [0082].	34-42

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/056011

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-33
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1-33 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required to search (PCT Article 17(2)(a)(i), PCT Rule 39.1(iv)).

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/056011

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2009-0062309 A1	05/03/2009	None	

(19)中华人民共和国国家知识产权局

(12)发明专利申请

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PCT/US2013/056011 2013.08.21
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WO2015/026339 EN 2015.02.26
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(51)Int.Cl.
A61K 31/4965(2006.01)
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A61K 31/495(2006.01)
A61P 25/00(2006.01)

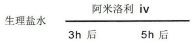
权利要求书3页 说明书27页 附图18页

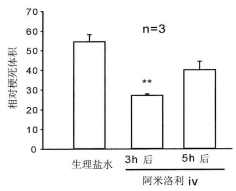
(54)发明名称

用于减轻神经系统损伤的组合物及该组合物的制造方法和用途

(57)摘要

本申请公开了可用于减轻受治者的神经损伤或神经系统损伤的包含阿米洛利和/或阿米洛利类似物的组合物。公开了此组合物的配方。本申请进一步涉及通过向受治者施用治疗有效量的含有阿米洛利、阿米洛利类似物或它们的药学上可接受的盐的药物组合物，以治疗神经损伤或神经系统损伤的方法。





CN 105592849 A

1.一种用于减轻受治者神经损伤的方法，包括：向所述受治者施用治疗有效量的包含阿米洛利、阿米洛利类似物或它们的药学上可接受的盐的药物组合物。

2.权利要求1所述的方法，其中，所述活性组分包括阿米洛利或其药学上可接受的盐。

3.权利要求1所述的方法，其中，所述活性组分包括阿米洛利类似物或其药学上可接受的盐。

4.权利要求3所述的方法，其中，所述阿米洛利类似物选自苯扎米尔、非那米尔、5-(N-乙基-N-异丁基)-阿米洛利(EIPA)、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。

5.权利要求4所述的方法，其中，所述阿米洛利类似物为苯扎米尔。

6.权利要求3所述的方法，其中，所述阿米洛利类似物为苯扎米尔的甲基化类似物。

7.权利要求3所述的方法，其中，所述阿米洛利类似物包含形成于胍基团上的环。

8.权利要求3所述的方法，其中，所述阿米洛利类似物包含酰胍基。

9.权利要求3所述的方法，其中，所述阿米洛利类似物包含形成于胍基团上的水增溶性基团，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

10.权利要求1所述的方法，其中，所述药物组合物通过静脉给药。

11.权利要求1所述的方法，其中，所述药物组合物通过鞘内给药。

12.权利要求1所述的方法，其中，所述药物组合物通过侧脑室给药。

13.权利要求2所述的方法，其中，所述阿米洛利、阿米洛利类似物或它们的药学上可接受的盐以0.1mg-10mg/kg体重的剂量范围给药。

14.权利要求1所述的方法，其中，在局部缺血事件开始的一小时内施用所述药物组合物。

15.权利要求1所述的方法，其中，在局部缺血事件开始的五小时内施用所述物组合物。

16.权利要求1所述的方法，其中，在局部缺血事件开始的一小时与五小时之间施用所述物组合物。

17.权利要求1所述的方法，其中，所述神经损伤为神经系统损伤。

18.权利要求1所述的方法，其中，所述神经损伤为脑损伤。

19.一种治疗受治者脑损伤的方法，包括：向所述受治者施用治疗有效量的包含阿米洛利、阿米洛利类似物或它们的药学上可接受的盐的药物组合物。

20.权利要求19所述的方法，其中，所述药物组合物通过静脉、鞘内或侧脑室给药。

21.权利要求19所述的方法，其中，所述阿米洛利类似物选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。

22.权利要求19所述的方法，其中，所述阿米洛利类似物为苯扎米尔。

23.权利要求19所述的方法，其中，所述阿米洛利类似物选自苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

24.一种减轻由进入神经元的离子流的变化而导致的神经系统损伤的方法，包括：向需要此治疗的受治者施用治疗有效量的包含阿米洛利、阿米洛利类似物或它们的药学上可接

受的盐的药物组合物。

25.权利要求24所述的方法，其中，所述药物组合物通过静脉、鞘内、侧脑室或肌肉给药。

26.权利要求24所述的方法，其中，所述阿米洛利类似物选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。

27.权利要求24所述的方法，其中，所述阿米洛利类似物为苯扎米尔。

28.权利要求24所述的方法，其中，所述阿米洛利类似物选自苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

29.一种减轻神经系统损伤的方法，包括：向需要此治疗的受治者施用治疗有效量的包含阿米洛利、阿米洛利类似物或它们的药学上可接受的盐的药物组合物。

30.权利要求29所述的方法，其中，所述药物组合物通过静脉、鞘内、侧脑室或肌肉给药。

31.权利要求29所述的方法，其中，所述阿米洛利类似物选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。

32.权利要求29所述的方法，其中，所述阿米洛利类似物为苯扎米尔。

33.权利要求29所述的方法，其中，所述阿米洛利类似物选自苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

34.一种用于减轻神经系统损伤的药物组合物，包含：有效量的阿米洛利、阿米洛利类似物或它们的药学上可接受的盐；以及药学上可接受的载体，其中将所述药物组合物配制为用于静脉、鞘内或侧脑室注射。

35.权利要求34所述的药物组合物，其中，所述阿米洛利类似物选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。

36.权利要求34所述的方法，其中，所述阿米洛利类似物选自苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

37.一种用于减轻神经系统损伤的药物组合物，包含：有效量的阿米洛利类似物或其药学上可接受的盐；以及药学上可接受的载体。

38.权利要求37所述的方法，其中，其中将所述药物组合物配制为用于静脉、鞘内、侧脑室或肌肉注射。

39.权利要求37所述的药物组合物，其中，所述阿米洛利类似物为苯扎米尔的甲基化类似物。

40.权利要求37所述的药物组合物,其中,所述阿米洛利类似物包含形成于胍基团上的环。

41.权利要求37所述的药物组合物,其中,所述阿米洛利类似物包含酰胍基。

42.权利要求37所述的药物组合物,其中,所述阿米洛利类似物包含形成于胍基团上的水增溶性基团,其中所述水增溶性基团为N,N-二甲基氨基或糖基。

用于减轻神经系统损伤的组合物及该组合物的制造方法和用途

技术领域

[0001] 本申请涉及神经病学领域。特别地，本申请针对包含能够用于减轻受治者的神经损伤或神经系统损伤的阿米洛利(amiloride)和/或阿米洛利类似物的组合物。

背景技术

[0002] 神经损伤可由多种条件引起，如退行性神经疾病、中风、局部缺血、神经系统的化学和机械损伤。许多类型的神经损伤会引发进入神经元的离子流的变化，这反过来导致了神经细胞的死亡。因此，各种离子通道可以是用以调解该变化的离子流的备选，因而减轻了神经损伤的程度。

发明内容

[0003] 本申请的一个方面涉及一种用于减轻受治者的神经损伤的方法。所述方法包括向所述受治者施用治疗有效量的含有选自阿米洛利和阿米洛利类似物的活性组分的药物组合物。在一些实施方式中，所述药物组合物是通过静脉、鞘内或侧脑室给药的。

[0004] 在一些实施方式中，所述活性组分包括阿米洛利或其药学上可接受的盐。

[0005] 在另外的实施方式中，所述活性组分包括阿米洛利类似物或其药学上可接受的盐。在相关实施方式中，所述阿米洛利类似物选自苯扎米尔(benzamil)、非那米尔(phenmil)、5-(N-乙基-N-异丁基)-阿米洛利(EIPA)、苄普地尔(bepridil)、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。在另外的相关实施方式中，所述阿米洛利类似物为苯扎米尔。在另外的相关实施方式中，所述阿米洛利类似物为苯扎米尔的甲基化类似物。在另外的相关实施方式中，所述阿米洛利类似物包含形成于胍基团上的环。在另外的相关实施方式中，所述阿米洛利类似物包含酰胍基。在另外的相关实施方式中，所述阿米洛利类似物包含形成于胍基团上的水增溶性基团，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

[0006] 在一些实施方式中，所述阿米洛利、阿米洛利类似物或它们的药学上可接受的盐以0.1mg-10mg/kg体重的剂量范围给药。

[0007] 在一些实施方式中，在局部缺血事件开始的一小时内、在局部缺血事件开始的五小时内或在局部缺血事件开始的一小时与五小时之间施用所述药物组合物。

[0008] 在一些实施方式中，所述神经损伤为脑损伤。

[0009] 本申请的另一个方面涉及一种用于治疗受治者脑损伤的方法。所述方法包括向所述受治者施用治疗有效量的包含阿米洛利、阿米洛利类似物或它们的药学上可接受的盐的药物组合物。在一些实施方式中，所述药物组合物是通过静脉、鞘内或侧脑室给药的。

[0010] 在一些实施方式中，所述阿米洛利类似物选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。在相关实施方式中，所述阿米洛利类似物为苯扎米尔。在另外的实施

方式中，所述阿米洛利类似物选自苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

[0011] 本申请的另一个方面涉及一种用于减轻由进入神经元的离子流的变化而导致的神经系统损伤的方法。所述方法包括向需要此治疗的受治者施用治疗有效量的包含阿米洛利、阿米洛利类似物或它们的药学上可接受的盐的药物组合物。

[0012] 在一些实施方式中，通过静脉、鞘内、侧脑室或肌肉施用所述药物组合物。

[0013] 在另外的实施方式中，所述阿米洛利类似物选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。在相关实施方式中，所述阿米洛利类似物为苯扎米尔。在另外的实施方式中，所述阿米洛利类似物选自苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

[0014] 本申请的另一个方面涉及一种用于减轻神经系统损伤的方法。所述方法包括向需要此治疗的受治者施用治疗有效量的包含阿米洛利、阿米洛利类似物或它们的药学上可接受的盐的药物组合物。

[0015] 在一些实施方式中，通过静脉、鞘内、侧脑室或肌肉施用所述药物组合物。

[0016] 在另外的实施方式中，所述阿米洛利类似物选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。在相关实施方式中，所述阿米洛利类似物为苯扎米尔。在另外的实施方式中，所述阿米洛利类似物选自苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

[0017] 本申请的另一个方面涉及一种用于减轻神经系统损伤的药物组合物。所述药物组合物包含有效量的阿米洛利、阿米洛利类似物或它们的药学上可接受的盐；以及药学上可接受的载体，其中将所述药物组合物配制为用于静脉、鞘内或侧脑室注射。

[0018] 在一些实施方式中，所述药物组合物包含阿米洛利类似物或其药学上可接受的盐，其中所述阿米洛利类似物选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。

[0019] 在另外的实施方式中，所述药物组合物包含阿米洛利类似物或其药学上可接受的盐，其中所述阿米洛利类似物选自苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

[0020] 本申请的另一个方面涉及一种用于减轻神经系统损伤的药物组合物。所述药物组合物包含有效量的阿米洛利类似物或其药学上可接受的盐；以及药学上可接受的载体。

[0021] 在一些实施方式中，将所述药物组合物配制为用于静脉、鞘内、侧脑室或肌肉注射。

[0022] 在一实施方式中，所述阿米洛利类似物为苯扎米尔的甲基化类似物。在另外的实施方式中，所述阿米洛利类似物包含形成于胍基团上的环。在另外的实施方式中，所述阿米

洛利类似物包含酰胍基。在再一实施方式中，所述阿米洛利类似物包含形成于胍基团上的水增溶性基团，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

附图说明

[0023] 图1为图示了减轻局部缺血性受治者神经损伤的示例性方法的流程图。

[0024] 图2为图示了鉴别用于治疗局部缺血性相关的神经损伤的药物的示例性方法的流程图。

[0025] 图3为展示了关于培养的小鼠皮层神经元的酸敏感离子通道(ASIC)蛋白的电生理学和药理学的示例性数据的一系列图表。

[0026] 图4为另外的展示了关于培养的小鼠皮层神经元的ASIC蛋白质的电生理学和药理学的示例性数据的系列图表。

[0027] 图5为根据本发明的各个方面的一系列展示了示例性数据的图表和曲线，所述示例性数据显示模拟的局部缺血可提高ASIC蛋白质的活性。

[0028] 图6和7为一系列展示了示例性数据的图表和曲线，所述示例性数据显示皮层神经元中的ASIC蛋白质可为Ca^{2+}可渗透的，且Ca^{2+}的渗透性可取决于ASIC1a。

[0029] 图8为一系列展示了示例性数据的图表，所述示例性数据显示酸孵育可导致通过ASIC阻滞保护的谷氨酸受体-独立神经元的损伤。

[0030] 图9为一系列展示了示例性数据的图表，所述示例性数据显示ASIC1a可能参与了体外的酸诱导损伤。

[0031] 图10为一系列包含数据的图表，所述数据显示了通过ASIC1a阻滞及通过ASIC1基因敲除的体内脑局部缺血的神经保护。

[0032] 图11为描绘了作为时间与治疗类型的函数的动物模型系统的因中风而产生的局部缺血性损害的百分比的示例性数据的图表。

[0033] 图12为具有所示的多种示例性肽特征的示例性胱氨酸结肽(cystine knot peptide)PcTx1的主要氨基酸序列(primary amino acid)的视图。

[0034] 图13为图12的胱氨酸结肽与该肽的多种示例性缺失衍生物的比较图。

[0035] 图14为描绘了作为细胞内表达的ASIC家族成员的函数的细胞内测得的钙流的幅度的示例性图表。

[0036] 图15为展示了关于鼻腔给药的PcTx毒素在动物模型系统中减轻局部缺血性损伤的效力的示例性数据的图表。

[0037] 图16为显示了用苯扎米尔(A组)或5-(N-乙基-N-异丁基)阿米洛利(EIPA)(B组)，以及凭借阿米洛利和阿米洛利类似物而剂量依赖地阻滞在CHO细胞中表达的ASIC 1a电流(C组)治疗的CHO细胞中代表性的ASIC 1a电流的曲线的组合图。

[0038] 图17为显示了用苯扎米尔(A组)或阿米洛利(B组)，以及凭借阿米洛利和阿米洛利类似物而剂量依赖地阻滞在CHO细胞中表达的ASIC 2a电流(C组)治疗的CHO细胞中代表性的ASIC 2a电流的曲线的组合图。

[0039] 图18为显示了通过侧脑室注射阿米洛利或阿米洛利类似物减少了小鼠梗死体积的图表。

[0040] 图19为显示了MCAO后60分钟通过静脉注射生理盐水或阿米洛利减少了小鼠皮层

组织中的梗死体积的组合图。

[0041] 图20为显示了MCAO后3小时或5小时通过静脉注射生理盐水或阿米洛利减少了小鼠皮层组织中的梗死体积的组合图。

[0042] 图21显示了疏水性的阿米洛利类似物在各种通道上的结构活性关系(SAR)。

具体实施方式

[0043] 本申请提供了用于减轻神经损伤的方法和组合物。神经损伤可由退行性神经疾病、中风、局部缺血、外伤、神经系统的化学和机械损伤所导致。如在本文中所用的,术语“神经系统”包括中枢神经系统和周围神经系统。术语“中枢神经系统”或“CNS”包括脊椎动物的脑和脊髓的所有细胞和组织。术语“周围神经系统”指的是除了脑和脊髓之外的神经系统部分的所有细胞和组织,如调节自主运动的运动神经元,包括交感神经系统和副交感神经系统并管理非自主功能的自主神经系统,以及控制胃肠系统的肠神经系统。因此,术语“神经系统”包括但不仅限于:神经元细胞,神经胶质细胞,星形胶质细胞,脑脊液(CSF)中的细胞,组织间隙中的细胞,脊髓的保护层中的细胞,硬脑膜外细胞(例如,硬脑膜外面的细胞),临近神经组织或与之联络或受其支配的非神经组织中的细胞,神经外膜、神经束膜、神经内膜、精索、束等中的细胞。

[0044] 在一些实施方式中,所述神经损伤为神经系统损伤。在另外的实施方式中,所述神经损伤为脑损伤。在一些实施方式中,所述神经损伤为由进入神经元的离子流的变化而导致的神经系统损伤。例如,中风/脑局部缺血是发病和死亡的主要原因。突触后的谷氨酸受体的过激活及随后的Ca^{2+}毒性在局部缺血性脑损伤中扮演了关键性的角色。本申请证实了可渗透Ca^{2+}的酸敏感离子通道(ASIC)的激活参与了酸中毒诱导的、与谷氨酸受体无关的局部缺血性脑损伤,并提供了靶向ASIC家族成员(ASICs)的神经保护的新方向。本申请进一步提供了新的ASICs抑制剂,其具有对于同源的ASICs通道的更强的效力,以及更高的水溶性。在一些实施方式中,本申请提供了通过抑制ASIC1a通道而减轻神经损伤的药物组合物和方法。

[0045] 本申请的一个方面涉及一种用于减轻受治者的神经损伤的方法。所述方法包括向所述受治者施用治疗有效量的含有选自阿米洛利和阿米洛利类似物的活性组分的药物组合物。在一些实施方式中,通过静脉、鞘内或侧脑室施用所述药物组合物。

[0046] 在一些实施方式中,所述活性组分包括阿米洛利或其药学上可接受的盐。在另外的实施方式中,所述活性组分包括阿米洛利类似物或其药学上可接受的盐。在相关实施方式中,所述阿米洛利类似物选自苯扎米尔、非那米尔、5-(N-乙基-N-异丁基)-阿米洛利(EIPA)、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。在另外的相关实施方式中,所述阿米洛利类似物为苯扎米尔。在另外的相关实施方式中,所述阿米洛利类似物为苯扎米尔的甲基化类似物。在另外的相关实施方式中,所述阿米洛利类似物包含形成于胍基团上的环。在另外的相关实施方式中,所述阿米洛利类似物包含酰胍基。在另外的相关实施方式中,所述阿米洛利类似物包含形成于胍基团上的水增溶性基团,其中所述水增溶性基团为N,N-二甲基氨基或糖基。

[0047] 在一些实施方式中,所述阿米洛利、阿米洛利类似物或它们的药学上可接受的盐以0.1mg-10mg/kg体重的剂量范围给药。在另外的实施方式中,在局部缺血事件开始的一小

时内、在局部缺血事件开始的五小时内或在局部缺血事件开始的一小时与五小时之间施用所述药物组合物。

[0048] 本申请的另一个方面涉及一种用于治疗受治者脑损伤的方法。所述方法包括向所述受治者施用治疗有效量的包含阿米洛利、阿米洛利类似物或它们的药学上可接受的盐的药物组合物。在一些实施方式中，通过静脉、鞘内或侧脑室施用所述药物组合物。

[0049] 在一些实施方式中，所述阿米洛利类似物选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。在相关实施方式中，所述阿米洛利类似物为苯扎米尔。在另外的相关实施方式中，所述阿米洛利类似物选自苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

[0050] 本申请的另一个方面涉及一种用于减轻由进入神经元的离子流的变化而导致的神经系统损伤的方法。所述方法包括向需要此治疗的受治者施用治疗有效量的包含阿米洛利、阿米洛利类似物或它们的药学上可接受的盐的药物组合物。

[0051] 本申请的另一个方面提供了一种用于治疗局部缺血或减轻由局部缺血导致的损伤的组合物。方法包括通过静脉或鞘内向需要此治疗的受治者施用治疗有效量的选自阿米洛利、阿米洛利类似物和它们的盐的活性组分。本申请的方法可比其它的局部缺血治疗方法具有一个或多个优点。其中，这些优点可包括：(1)较小的局部缺血导致的损伤，(2)更少的治疗副作用(例如，归因于更明确的治疗标靶的选择)，和/或(3)供有效治疗的更长的时间窗。

[0052] 图1显示了包括可在用于减轻局部缺血的受治者的神经损伤的方法中进行的示例性步骤22、24的流程图20。所述步骤可以任何适宜的次数和任何适宜的组合进行。在所述方法中，可选择局部缺血的受治者(或受治者们)以供治疗，以22示出。之后可向所述局部缺血的受治者(们)施用ASIC选择性抑制剂，以24示出。可以治疗有效量向所述局部缺血的受治者施用所述抑制剂，以减轻受治者局部缺血导致的损伤，例如，降低由中风导致的脑损伤的程度。

[0053] 图1的针对局部缺血性治疗的功效的潜在性的解释可由本发明的数据所提供(例如，参见实施例1)。特别地，局部缺血的破坏效果并不等同于酸中毒，即通过局部缺血的组织/细胞的酸化可能并不足以产生局部缺血导致的损伤。换句话说，在很多情况下，局部缺血导致的损伤可能是由经ASIC家族成员，尤其是ASIC1a介导的进入细胞的钙流所导致的。因此，选择性抑制ASIC1a的通道活性可减少此有害的钙流，因而减轻了局部缺血导致的损伤。

[0054] 图2显示了包括可在鉴别用于治疗局部缺血的药物的方法中进行的示例性步骤32、34的流程图30。所述步骤可以任何适宜的次数和任何适宜的组合进行。在所述方法中，可获得一种或多种ASIC选择性抑制剂，以32示出。之后可在局部缺血的受治者上测试所述抑制剂对局部缺血导致的损伤的效果，以34示出。

[0055] 神经损伤

[0056] 本申请针对用于减轻受治者的神经损伤的药物组合物和方法。如在本文中所用的，术语“神经损伤”指的是由物理事件或外伤、挫伤或压迫或手术损伤、包括失血性或局部

缺血性损害的血管药理学损害，或者由神经退行性的或任何其它神经系统的疾病，或任何其它的引发神经系统组织或细胞的损伤或不利状况的因素所导致的神经系统组织或细胞的急性或慢性损伤或不利状况。在一些实施方式中，所述神经损伤由认知失调、精神失调、神经递质介导的失调或神经元失调所导致。神经损伤包括对神经系统的损伤(即神经系统损伤)和脑损伤。

[0057] 如在本文中所用的，术语“认知失调”是指和意指相信涉及或确实涉及神经元的结构和/或功能的进行性丧失(包括神经元的死亡)，或相信与之相关或确实与之相关的疾病或状况，其中所述失调的首要特征可为认知(例如，记忆、注意力、感知和/或思维)障碍。这些失调包括病原体引发的认知障碍，例如，HIV相关的认知障碍或莱姆病(Lyme diseas)相关的认知障碍。在一些实施方式中，所述认知失调为退行性认知失调。所述退行性认知失调的实例包括阿尔茨海默氏症(Alzheimer's Disease)、亨廷顿氏舞蹈症(Huntington's Disease)、帕金森氏症(Parkinson's Disease)、肌萎缩性侧索硬化症(amyotrophic lateral sclerosis,ALS)、自闭症、轻度认知障碍(MCI)、中风、创伤性脑损伤(TBI)、年龄相关的记忆障碍(AAMI)和癫痫。

[0058] 如在本文中所用的，术语“精神失调”是指和意指相信导致了或确实导致了反常的思维和感知的精神疾病或状况。精神失调的特征在于现实性的缺失，这可伴随着妄想、幻觉(在外部刺激的缺失下的具有真实感知性质的有意识的和清醒状态的感知，在其中它们是生动的、具体的，并位于外部的目标空间)、人格改变和/或思维紊乱。其它的常见症状包括异常的和古怪的举止，以及社交困难和在进行日常生活活动方面的障碍。示例性的精神失调为精神分裂症、双相型精神障碍(bipolar disorders)、精神病(psychosis)、焦虑症、抑郁症和慢性疼痛。

[0059] 如在本文中所用的，术语“神经递质介导的失调”是指和意指相信涉及或确实涉及神经递质(如组织胺、谷氨酸、5-羟色胺、多巴胺、去甲肾上腺素)的异常水平或胺类的G蛋白偶联受体(aminergic G protein-coupled receptors)的受损功能，或相信与之相关或确实与之相关的疾病或状况。示例性的神经递质介导的失调包括脊髓损伤、糖尿病性神经病、过敏性疾病和涉及老化保护活动的疾病(如与年龄相关的掉发(脱发)、与年龄相关的体重减轻和与年龄相关的视觉障碍(白内障))。异常的神经递质水平与多种疾病和状况有关，所述疾病和状况包括但不仅限于：阿尔茨海默氏症、帕金森氏症、自闭症、格林-巴利综合征(Guillain-Barre syndrome)、轻度认知障碍、精神分裂症、焦虑症、多发性硬化症(multiple sclerosis)、中风、外伤性脑损伤、脊髓损伤、糖尿病性神经病、纤维肌痛(fibromyalgia)、双相型精神障碍、精神病、抑郁症和各种过敏性疾病。

[0060] 如在本文中所用的，术语“神经元失调”是指和意指相信涉及或确实涉及神经元细胞死亡和/或神经元功能受损或神经元功能降低，或相信与之相关或确实与之相关的疾病或状况。示例性的神经元适应症包括神经退行性疾病和失调，如阿尔茨海默氏症，亨廷顿氏舞蹈症，肌萎缩性侧索硬化症(ALS)，帕金森氏症，犬认知功能障碍综合征(canine cognitive dysfunction syndrome,CCDS)，路易体症(Lewy body disease)，门克斯病(Menkes disease)，威尔森病(Wilson disease)，克雅氏病(Creutzfeldt-Jakob disease)，基底节钙化症(Fahr disease)，涉及脑循环的急性或慢性失调(如局部缺血性或出血性中风，或其它脑出血性损害)，与年龄相关的记忆障碍(AAMI)，轻度认知障碍(MCI)，

与损伤相关的轻度认知障碍(MCI),脑震荡后综合征,创伤后应激障碍,辅助化疗,外伤性脑损伤(TBI),神经元死亡介导的眼部疾病,黄斑变性(macular degeneration),与年龄相关的黄斑变性,自闭症(包括自闭症谱系障碍(autism spectrum disorder)、阿斯伯格综合征(Asperger syndrome)和雷特综合征(Rett syndrome)),撕脱伤,脊髓损伤,重症肌无力,格林-巴利综合征,多发性硬化症,糖尿病性神经病,纤维肌痛,与脊髓损伤、精神分裂症、双相型精神障碍、精神病、焦虑症或抑郁症相关的神经病,以及慢性疼痛。

[0061] 在一些实施方式中,所述神经损伤或神经系统损伤由进入神经元或神经系统组织的离子流的变化所导致。如在本文中所用的,术语"神经系统组织"是指包括神经细胞、神经纤维网(neuropil)、神经胶质(glia)、神经炎性细胞(neural inflammatory cell)以及与"神经系统组织"相接触的内皮细胞的动物组织。"神经细胞"可以是本领域技术人员所知的任何类型的神经细胞,包括但不仅限于神经元。如在本文中所用的,术语"神经元"表示源自动物的神经系统的任何部分的外胚层胚胎起源细胞。神经元表达了已充分特征化的神经元特异性标记物,包括神经丝蛋白(neurofilament protein)、NeuN(神经元核标记物,Neuronal Nuclei marker)、MAP2和III级微管蛋白(class III tubulin)。作为神经元被包括的是,例如,海马(hippocampal)神经元、皮层(cortical)神经元、中脑多巴胺能(midbrain dopaminergic)神经元、脊髓运动(spinal motor)神经元、感觉(sensory)神经元、肠(enteric)神经元、交感(sympathetic)神经元、副交感(parasympathetic)神经元、隔膜胆碱能(septal cholinergic)神经元、中枢神经系统和小脑神经元。本发明中所用的"神经胶质细胞"包括但不仅限于:星形胶质细胞、雪旺细胞(Schwan cell)和少突神经胶质细胞(oligodendrocytes)。本发明中所用的"神经炎性细胞"包括但不仅限于:包括巨噬细胞和小神经胶质细胞的骨髓起源细胞。

[0062] 在一些实施方式中,本申请的药物组合物和方法涉及减轻由局部缺血或与局部缺血相关状况导致的神经损伤。在本文中所用的局部缺血为流入器官和/或组织的血流减少。所述血流减少可能是由多种机制所导致,其包括但不仅限于:向器官和/或组织供血的一个或多个血管的部分或完全阻塞(梗阻)、变窄(缩窄)和/或泄漏/破裂。局部缺血可能由血栓形成、栓塞、动脉粥样硬化、高血压、出血、动脉瘤、手术、外伤、药物治疗等产生。因而所述血流减少可能会是慢性的、短暂的、急性的或偶发性的。

[0063] 任何的器官或组织可能会经历血流减少,并需要针对局部缺血的治疗。示例性的器官和/或组织包括但不仅限于:脑、动脉、心脏、肠和眼(例如,视神经)。其中,局部缺血导致的损伤(即各种类型的局部缺血所引起的疾病和/或损害)包括但不仅限于:缺血性脊髓病、缺血性视神经病变、缺血性结肠炎、冠心病和/或心脏疾病(例如,心绞痛、心脏麻痹(heart attack)等)。因而局部缺血导致的损伤可损害和/或杀死细胞和/或组织,例如,在躯体受影响的位置产生坏死(梗死)组织、发炎和/或组织重塑。根据本申请的各个方面的治疗可降低此损伤的发生率、程度和/或严重性。

[0064] 与局部缺血相关的状况可以是局部缺血的任何后果。所述后果可大体上与局部缺血的发作同时发生(例如,所述局部缺血的直接后果),和/或可大体上在局部缺血的发作之后和/或甚至在局部缺血结束之后发生(例如,所述局部缺血的间接的、随之产生的后果,局部缺血结束后组织的这样的再灌注)。示例性的与局部缺血相关的状况可包括以上所列的症状(和/或状况)的任意组合。或者(或加之),所述症状可包括局部的和/或全身的酸中毒

(pH降低)、缺氧(氧降低)、自由基的生成,等等。

[0065] 在一些实施方式中,所述与局部缺血相关的状况为中风。在本文中所用的中风为脑的一部分(或全部)供血减少所引起的脑局部缺血。中风产生的症状可能是突发性的(如意识丧失)或可在几小时或几天内逐步发作。此外,所述中风可能是严重的缺血性侵袭(完全中风)或除此之外更加轻微的、短暂的缺血性侵袭。中风产生的症状可包括,例如,轻偏瘫(hemiparesis)、半身不遂、单侧麻木、单侧虚弱、单侧瘫痪、暂时四肢无力、四肢发麻、神志不清、说话困难、理解话语困难、单眼或双眼视力困难、视觉模糊、视觉丧失、行走困难、头晕、易跌倒、丧失协调性、突发剧烈头痛、呼吸噪音和/或意识丧失。或者(或加之),所述症状可更易于或仅能通过检测和/或仪器被查出,例如,缺血性血液测试(例如,测试变化的白蛋白,特别是蛋白质异构体、受损的蛋白质等)、心电图、脑电图、运动负荷测试、脑CT或MRI扫描,等等。

[0066] 酸碱平衡对于生物系统来说是很重要的。正常的脑功能依赖于葡萄糖的完全氧化,生成最终产物CO_2和H_2O以供其能量需求。局部缺血期间,由于缺乏供氧而引发的无氧糖酵解的增加导致了乳酸蓄积。乳酸的蓄积,伴随着自ATP水解的H^+释放的增加,导致了组织的pH的下降。细胞外的pH(pH_o)在局部缺血期间通常会降至6.5,且其在严重局部缺血期间或血糖过多的状况下可降至6.0之下。

[0067] 神经损伤的受治者

[0068] 本申请的方法和药物组合物可被用于存在神经损伤或神经损伤历史和/或在治疗开始后及治疗仍旧起效的一段时期存在发展中的神经损伤的显著变化的任何的受治者。在一些实施方式中,所述受治者为局部缺血的受治者。在本文中所用的局部缺血的受治者为存在局部缺血、与局部缺血相关的状况、局部缺血历史和/或在治疗开始后及治疗仍旧起效的一段时期存在发展中的局部缺血的显著变化的任何人(人类受治者)或动物(动物受治者)。

[0069] 所述受治者可以是动物。如在本文中所用的,术语“动物”是指除人类之外的任何的动物。示例性的可能适宜的动物包括具有血流的任何的动物,如啮齿动物(小鼠、大鼠等)、犬、猫、鸟、绵羊、山羊、除人类之外的灵长类等。所述动物可因其自身的缘故被治疗,例如,兽医的目的(如宠物的治疗)。或者,所述动物可提供神经损伤(如局部缺血)的动物模型,以便于测试供人类使用的候选药物,如以确定候选物的效力、功效窗口、副反应等。

[0070] 可以任何适当的标准选择供治疗的局部缺血的受治者。示例性的标准可包括局部缺血的任何的可检测的症状、局部缺血历史、增加局部缺血风险(或诱导局部缺血)的事件(如外科手术、外伤、服用药物等)等。局部缺血历史可包括一次或多次既往的局部缺血的发作。在一些实施例中,选择以供治疗的受治者可能会存在治疗开始前的至少约一、二或三小时出现的局部缺血的发病,或治疗开始前的不到约一天、十二小时或六小时出现的多次局部缺血的发作(如短暂的缺血性侵袭)。

[0071] ASIC抑制剂、阿米洛利和阿米洛利类似物

[0072] ASIC家族成员的抑制剂,如在本文中所用的,为降低(部分地、大体上或完全阻滞)所述ASIC家族的一个或多个成员(除了别的以外,即ASIC1a、ASIC1b、ASIC2a、ASIC2b、ASIC3和ASIC4)的活性的物质。在一些实施例中,所述抑制剂可降低一个或多个成员的通道活性,如所述成员使离子(除了别的以外,例如,钠、钙和/或钾离子)流动而透过细胞膜(进入和/

或离开细胞)的能力。其中,所述物质可以是化合物(小于约10kDa的小分子、肽、核酸、脂质等)、两种以上的化合物的复合物,和/或混合物。另外,所述物质可通过任何适当的机制来抑制ASIC家族成员,除了别的以外,其包括竞争性、非竞争性、无竞争性和/或混合的抑制作用。

[0073] 所述抑制剂可以是抑制酸敏感离子通道1a(ASIC1a)的ASIC1a抑制剂。ASIC1a,如在本文中所用的,是指来自任何物种的ASIC1a蛋白或通道。例如,在Waldmann,R.,et al.1997,Nature 386,pp.173-177中描述了示例性的人类ASIC1a蛋白/通道,其以引用的方式并入本申请中。

[0074] 表述"ASIC1a抑制剂"可指的是在合理的药理学判断范围内,潜在的或实际的在药学上用作ASIC1a的抑制剂,并包含含有药学活性剂并作为ASIC1a抑制剂而被描述、宣传或认可的参考物质的产品。

[0075] ASIC1a抑制剂可以是在ASIC家族内有选择性的。如在本文中所用的,ASIC1a的选择性抑制作用为在各自接触同样(次最大)浓度的抑制剂之后进行比较时(例如在培养细胞中),对于ASIC1a比对于另外的ASIC家族成员明显更强的抑制作用。所述抑制剂可相对于至少一种另外的ASIC家族成员(ASIC1b、ASIC2a、ASIC2b、ASIC3、ASIC 4等)选择性地和/或相对于所有另外的ASIC家族成员选择性地抑制ASIC1a。选择性抑制剂的抑制作用的强度可通过相对于不同的ASIC家族成员,抑制作用发生时的抑制剂的浓度(例如,IC_{50}(产生最大抑制作用的50%的抑制剂的浓度)或K_i值(抑制常数或解离常数))来描述。ASIC1a选择性抑制剂可在低于用以抑制至少一种另外的或所有另外的ASIC家族成员的至少约二倍、四倍或十倍(浓度的二分之一、四分之一或十分之一或更低)的浓度下抑制ASIC1a活性。因此,ASIC1a选择性抑制剂可具有低于用以抑制至少一种另外的ASIC家族成员和/或用以抑制所有另外的ASIC家族成员的至少约二倍、四倍或十倍(二分之一、四分之一或十分之一或更低)的用以抑制ASIC1a的IC_{50}和/或K_i。

[0076] 除了存在选择性之外,ASIC1a选择性抑制剂还可以是对ASIC1a特异性的。如在本文中所用的,ASIC1a特异性抑制作用为相对于所有另外的ASIC家族成员(如ASIC2a和ASIC3a),大体上专属于ASIC1a的抑制作用。ASIC1a特异性抑制剂可在低于用以抑制所有另外的ASIC家族成员的至少约二十倍(浓度的5%以下)的抑制剂浓度下抑制ASIC1a。因此,ASIC1a特异性抑制剂可具有相对于所有另外的ASIC家族成员低至少约二十倍(百分之五以下)的针对ASIC1a的IC_{50}和/或K_i,以致于,例如,另外的ASIC家族成员的抑制作用至少大体上(或完全)检测不到。在一些实施方式中,相比于市售可用的阿米洛利相关的ASIC1a抑制剂,如阿米洛利、苯扎米尔、非那米尔、5-(N-乙基-N-异丁基)-阿米洛利(EIPA)、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐,所述ASIC1a选择性抑制剂具有对于同源的ASIC1a通道的更强的效力,以及更高的水溶性。

[0077] 可以使用任何适宜的ASIC抑制剂或抑制剂的组合。例如,可用ASIC1a选择性抑制剂和非选择性ASIC抑制剂,或用ASIC1a选择性抑制剂和对于非ASIC通道蛋白(如非ASIC的钙通道)的抑制剂来治疗受治者。在一些实施例中,可用ASIC1a选择性抑制剂和NMDA受体的抑制剂(如谷氨酸拮抗剂)来治疗受治者。

[0078] 所述抑制剂可以是肽或包含肽。所述肽可具有任意适宜数量的氨基酸亚单位,通

常为至少约十个至低于约一千个亚单位。在一些实施例中，所述肽可具有胱氨酸结模体(cystine knot motif)。胱氨酸结，如本文中所用的，通常包含六个以上的胱氨酸的排列。含有这些胱氨酸的肽可产生“结(knot)”，所述“结”包括(1)由两个双硫键和他们连接的主链部分形成的环，以及(2)穿过所述环的第三双硫键。在一些实施例中，所述肽可以是来自蛛形纲动物(arachnid)和/或芋螺(cone snail)物种的芋螺毒素(conotoxin)。例如，所述肽可以是PcTx1(psalmotoxin 1)，来自狼蛛(tarantula)(千里达老虎尾蜘蛛(Psalmopoeus cambridgei(Pc)))的毒素。

[0079] 在一些实施例中，所述肽可在结构上与PcTx1相关，从而所述肽与PcTx1因一个或多个氨基酸的至少一个缺失、嵌入和/或替换而不同。例如，所述肽可具有至少约25％或至少约50％的序列一致性，以及与PcTx1的至少约25％或至少约50％的序列相似性(见下文)。可能适于作为抑制剂的肽的进一步的特征将在以下实施例3中描述。

[0080] 供一致性和相似性评分的比较和产生的氨基酸序列的对齐方法是本领域公知的。示例性的可能适宜的对齐方法包括：Smith&Waterman对齐方法(Best Fit)、Needleman&Wunsch同源性比对算法(GAP)、Pearson&Lipman相似性法(Tfasta和Fasta)，等等。可能适宜的这些和其它的方法的计算机算法包括但不仅限于：CLUSTAL、GAP、BESTFIT、BLASTP、FASTA和TFASTA。

[0081] 如在本文中所用的，两个肽在上下文中的“序列一致性”或“一致性”涉及当以最大限度的对应性来对齐时，对应的相同的肽序列中的残基的百分比。在一些实施例中，不相同的肽残基位置可通过保守性氨基酸替换而不同，其中氨基酸残基被替换为具有类似的化学性质(例如，电荷和疏水性)的其它的氨基酸残基，因而预期对分子的功能特性产生较小的(或不产生)影响。其中序列在保守性替换方面不同，可将序列一致性百分比向上调，而产生序列的“相似性”，这会校正所述替换的保守性。例如，各个保守性替换可被评分为部分而非全部的错配，因而校正了所述序列一致性百分比以提供相似性评分。评分保守性替换以获得相似性评分是本领域公知的，且可通过任何适宜的方法，例如，根据Meyers and Miller, Computer Applic.Biol Sci.,4:11-17(1988)的算法(例如，在程序PC/GENE(Intelligenetics,Mountain View,Calif.,USA)中执行时)来计算。

[0082] 阿米洛利(含胍基团的吡嗪衍生物)已被用于轻微高血压的治疗，其很少报道有副作用。阿米洛利通过直接阻滞上皮钠通道(ENaC)起效，因而抑制肾的最近的远曲小管、连接小管和集合管中的钠的再吸收。这促进了钠和水从身体的流失，但不会消耗钾。如在本文中所用的，术语“阿米洛利”是指阿米洛利和阿米洛利的盐，如阿米洛利盐酸盐。

[0083] 阿米洛利类似物，如在本文中所用的，是指具有与阿米洛利类似的生物活性但存在稍有变化的化学结构的化合物。阿米洛利类似物的实例包括但不仅限于苯扎米尔、非那米尔、5-(N-乙基-N-异丁基)-阿米洛利(EIPA)、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。其它的实例包括：在C_5-NH_2位置处和/或在胍基上(如图21所示)具有疏水性取代基的阿米洛利类似物，以及苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。在一些实施方式中，所述阿米洛利类似物并不阻滞人类的Na^+/Ca^{2+}离子交换器。在另外的实施方式中，所述阿米洛利类似物为Na^+/Ca^{2+}离子交换

器的弱抑制剂，并帮助维持较低水平的细胞内Ca^{2+}。在另外的实施方式中，所述阿米洛利类似物为Na^{+}/Ca^{2+}离子交换器的非常弱的抑制剂，具有1.1mM以下的IC_{50}。在一些另外的实施方式中，所述阿米洛利类似物并不阻滞ASIC2a和/或ASIC3通道。在一些实施方式中，所述阿米洛利类似物具有比ASIC3通道和/或ASIC2通道更高的针对ASIC1a的选择性。

[0084] 如在本文中所用的，术语“阿米洛利类似物”是指阿米洛利类似物和阿米洛利类似物的盐，如5-(N,N-二甲基)阿米洛利盐酸盐。

[0085] 在一些实施方式中，阿米洛利和/或阿米洛利类似物与其它的ASIC抑制剂，如PcTx1及其衍生物结合使用。

[0086] 抑制剂的给药

[0087] 给药(或施用)，如在本文中所用的，包括以任何适宜的次数，在任何适宜的条件下，受治者接触抑制剂的任何的路径。给药可以是自主给药或通过他人，如医疗人员(例如，医生、护士等)给药。给药可通过注射(除了别的以外，例如，静脉、肌肉、皮下、脑内、侧脑室、硬膜外和/或鞘内)、口服(例如，采用胶囊、锭剂、流体组分等)、吸入(例如，鼻内和/或口内吸入的气雾剂(小于约10微米的平均液滴直径))、经皮肤(例如，采用皮肤贴剂)和/或粘膜(除了别的以外，例如，经过口腔、鼻腔和/或肺粘膜)吸收等来进行。粘膜给药可以，例如，利用喷剂(如鼻喷剂)、吸入的气雾剂等来实现。喷剂可以是表面喷剂(液滴平均直径大于约50微米)和/或空间喷剂(液滴平均直径为约10-50微米)。在一些实施例中，局部缺血可使局部缺血受治者的血脑障壁产生变化，由此提高了功效，导入(例如，通过注射和/或吸收)受治者血流的抑制剂借此可抵达脑部。给药可进行一次或多次，并可以与局部缺血诊断有关的任何适宜的次数进行，以提供治疗。因此，给药可在局部缺血被发现之前(例如，预防性地)、轻微的局部缺血发作之后、慢性局部缺血期间、完全中风之后等进行。在一些实施方式中，阿米洛利或阿米洛利类似物通过静脉给药。在另外的实施方式中，阿米洛利或阿米洛利类似物通过脑内给药。在另外的实施方式中，阿米洛利或阿米洛利类似物通过侧脑室给药。在另外的实施方式中，阿米洛利或阿米洛利类似物通过肌肉给药。在另外的实施方式中，阿米洛利或阿米洛利类似物通过鞘内给药。

[0088] 可施用治疗有效量(或简称“有效量”)的抑制剂。抑制剂的治疗有效量或有效量，如在本文中所用的，为当向受治者给药时，在大量的受治者中降低了受治者的局部缺血导致的损伤的程度、发生率和/或范围的所述抑制剂的任意的量。因此，治疗有效量可在，例如，将各种量的所述抑制剂给药至测试的受治者的临床研究中确定(并且，通常会与对照组的受治者相比较)。抑制剂的治疗有效量可通过一次注射或以0.1-50mL每针的体积的多次注射来给药。

[0089] 在一些实施方式中，所述抑制剂为阿米洛利、阿米洛利类似物或它们的盐，并以以下范围内的一日量(作为单次剂量或多次剂量)给药：0.01-30mg/kg体重、0.01-10mg/kg体重、0.01-3mg/kg体重、0.01-1mg/kg体重、0.01-0.3mg/kg体重、0.01-0.1mg/kg体重、0.01-0.03mg/kg体重、0.03-30mg/kg体重、0.03-10mg/kg体重、0.03-3mg/kg体重、0.03-1mg/kg体重、0.03-0.3mg/kg体重、0.03-0.1mg/kg体重、0.1-30mg/kg体重、0.1-10mg/kg体重、0.1-3mg/kg体重、0.1-1mg/kg体重、0.1-0.3mg/kg体重、0.3-30mg/kg体重、0.3-10mg/kg体重、0.3-3mg/kg体重、0.3-1mg/kg体重、1-30mg/kg体重、1-10mg/kg体重、1-3mg/kg体重、3-30mg/kg体重、3-10mg/kg体重或10-30mg/kg体重。在一个实施方式中，所述阿米洛利类似物

选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。在另外的实施方式中，所述阿米洛利类似物具有在C_5-NH_2位置处和/或在胍基上的疏水性取代基。在另外的实施方式中，所述阿米洛利类似物选自以下组中的阿米洛利类似物：苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

[0090] 在另外的实施方式中，所述抑制剂为阿米洛利、阿米洛利类似物或它们的盐，并作为配制为以下范围内的单次剂量的药物组合物给药：0.1-1000mg/剂、0.1-300mg/剂、0.1-100mg/剂、0.1-30mg/剂、0.1-10mg/剂、0.1-3mg/剂、0.1-1mg/剂、0.1-0.3mg/剂、0.3-1000mg/剂、0.3-300mg/剂、0.3-100mg/剂、0.3-30mg/剂、0.3-10mg/剂、0.3-3mg/剂、0.3-1mg/剂、1-1000mg/剂、1-300mg/剂、1-100mg/剂、1-30mg/剂、1-10mg/剂、1-3mg/剂、3-1000mg/剂、3-300mg/剂、3-100mg/剂、3-30mg/剂、3-10mg/剂、10-1000mg/剂、10-300mg/剂、10-100mg/剂、10-30mg/剂、30-1000mg/剂、30-300mg/剂、30-100mg/剂、100-1000mg/剂、100-300mg/剂或300-1000mg/剂。在一个实施方式中，所述阿米洛利类似物选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。在另外的实施方式中，所述阿米洛利类似物具有在C_5-NH_2位置处和/或在胍基上的疏水性取代基。在另外的实施方式中，所述阿米洛利类似物选自以下组中的阿米洛利类似物：苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。在一些实施方式中，配制所述药物组合物以用于静脉注射、脑内注射、侧脑室注射、鞘内注射或肌肉注射。

[0091] 所述抑制剂可以任意适宜的形式和以任意适宜的组合物被给药至受治者。在一些实施例中，所述抑制剂可被配制为药学上可接受的盐。可配制所述组合物以包括，例如，液体载体/溶剂(媒介)、防腐剂、一种或多种赋形剂、着色剂、调味剂、盐、消泡剂等。所述抑制剂可在媒介中以在给药至局部缺血的受治者时提供用于治疗局部缺血的治疗有效量的所述抑制剂的浓度而存在。

[0092] 在一些实施方式中，制得了具有更高水溶性或脂溶性的阿米洛利类似物。在某些实施方式中，所述阿米洛利类似物在胍基处包含水增溶性基团(如N,N-二甲基氨基或糖基)以改善水溶性(式13-16，图24)。在一些实施方式中，所述阿米洛利类似物具有5mM、10mM、20mM、30mM、40mM、50mM、60mM、70mM、80mM、90mM、100mM或更高的水溶性。在另外的实施方式中，所述阿米洛利类似物具有允许在单次10mL注射液中将10mg、25mg、50mg、100mg、150mg、200mg、250mg、300mg、400mg或500mg的剂量静脉给药至人体的溶解度。在再另外的实施方式中，所述阿米洛利类似物具有允许在单次2mL注射液中将10mg、25mg、50mg、100mg、150mg、200mg、250mg、300mg、400mg或500mg的剂量通过侧脑室给药至人体的溶解度。

[0093] 阿米洛利类似物的合成与筛选

[0094] 本申请的另一个方面涉及新的阿米洛利类似物的合成与筛选。阿米洛利类似物的合成路线是基于所需的类似物结构而设计的。之后筛选新合成的阿米洛利类似物对于ASIC家族成员(如ASIC1a和ASIC2a)的抑制效果。可获得一种或多种ASIC抑制剂，特别是如上所

述的ASIC1a抑制剂。可以任意适宜的方式获得所述抑制剂，其中，如通过筛选一组候选抑制剂(例如，两个以上化合物的库)和/或通过的合理设计。

[0095] 筛选可包括任意适宜的测定ASIC蛋白与所述组的候选抑制剂之间的相互作用的检测系统。其中，示例性的检测系统可包括利用培养基中生长的细胞(“培养的细胞”)和/或利用有机体的生化测定(例如，结合法(binding assay))。

[0096] 在一些实施方式中，基于细胞的测定系统被用于测定在细胞中各个候选抑制剂对离子流(例如，酸敏感离子流)的效果。在一些实施方式中，所述离子流为钙和/或钠的流。在一些实施方式中，所述测定系统采用表达ASIC家族成员(如ASIC1a或ASIC2a)的细胞或表达两种以上的不同的ASIC家族成员(如ASIC1a和另外的ASIC家族成员)的两种以上的不同组的细胞，以确定各个抑制剂对此家族成员的选择性。所述细胞可内源性地或通过引入外来的核酸来表达各个ASIC家族成员。在一些实施例中，除了别的以外，所述测定系统可利用离子敏感或膜电势敏感染料(例如，钙敏感染料，如Fura-2)，或凭借对膜电势和/或细胞内离子(例如，钙)浓度的变化敏感的基于基因的报告系统，来电生理学地测定离子流(如通过膜片钳)。所述测定系统可被用于测试候选抑制剂对ASIC家族成员(尤其是ASIC1a)的选择性和/或特异性抑制作用。

[0097] 可向具有神经损伤的受治者(如局部缺血的受治者)施用一种或多种ASIC抑制剂，以测试用于治疗神经损伤的所述抑制剂的效力。所述局部缺血的受治者可以是人或动物。在一些实施例中，所述局部缺血的受治者可提供局部缺血和/或中风的动物模型系统。示例性的动物模型系统包括具有实验引发的局部缺血的啮齿动物(除了别的以外，小鼠和/或大鼠)。除了别的以外，所述局部缺血可以是机械性(例如，外科手术)和/或通过药物的给药而引发的。在一些实施例中，所述局部缺血可由血管阻塞，如由大脑中动脉阻塞而引发。

[0098] 本申请的另一个方面涉及用于减轻神经系统损伤的药物组合物。所述药物组合物包含有效量的阿米洛利、阿米洛利类似物或它们的药学上可接受的盐；以及药学上可接受的载体，其中将所述药物组合物配制为用于静脉、鞘内或侧脑室注射。

[0099] 在一些实施方式中，所述药物组合物包含阿米洛利类似物或其药学上可接受的盐，其中所述阿米洛利类似物选自苯扎米尔、非那米尔、EIPA、苄普地尔、KB-R7943、5-(N-甲基-N-异丁基)阿米洛利、5-(N,N-六亚甲基)阿米洛利和5-(N,N-二甲基)阿米洛利盐酸盐。

[0100] 在另外的实施方式中，所述药物组合物包含阿米洛利类似物或其药学上可接受的盐，其中所述阿米洛利类似物选自苯扎米尔的甲基化类似物、含有形成于胍基团上的环的阿米洛利类似物、含有酰胍基的阿米洛利类似物和含有形成于胍基团上的水增溶性基团的阿米洛利类似物，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

[0101] 在另外的实施方式中，所述药物组合物进一步包含一种或多种其它的ASIC抑制剂。在一个实施方式中，所述一种或多种其它的ASIC抑制剂包括PcTx1或PcTx1衍生物。

[0102] 本申请的另一个方面涉及一种用于减轻神经系统损伤的药物组合物。所述药物组合物包含有效量的阿米洛利类似物或其药学上可接受的盐；以及药学上可接受的载体。

[0103] 在一些实施方式中，将所述药物组合物配制为用于静脉、鞘内、侧脑室或肌肉注射。

[0104] 在一个实施方式中，所述阿米洛利类似物为苯扎米尔的甲基化类似物。在另一个实施方式中，所述阿米洛利类似物包含形成于胍基团上的环。在另一个实施方式中，所述阿

米洛利类似物包含酰胍基。在再一个实施方式中，所述阿米洛利类似物包含形成于胍基团上的水增溶性基团，其中所述水增溶性基团为N,N-二甲基氨基或糖基。

[0105] 在另外的实施方式中，所述药物组合物进一步包含一种或多种其它的ASIC抑制剂。在一个实施方式中，所述一种或多种其它的ASIC抑制剂包括PcTx1或PcTx1衍生物。

[0106] 如在本文中所用的，“药学上可接受的载体”包括任意和全部的溶剂、分散介质、包膜、抗菌剂和抗真菌剂、等渗和吸收延迟剂、甜味剂等。所述药学上可接受的载体可由广泛的材料来制备，所述材料包括但不仅限于：调味剂，甜味剂，以及其它的材料，如为了制备特定的治疗组合物而可能会需要的缓冲剂和吸收剂。这些具有药学活性物质的介质和药剂的使用是本领域公知的。除了任何常规介质或药剂与活性组分不相容之外，其在所述治疗组合物中的用途是可预期的。可选地，可将阿米洛利和/或阿米洛利类似物混合于包含不与所述阿米洛利和/或阿米洛利类似物相忌的补充活性组分的药物组合物中。

[0107] 在一些实施方式中，将所述药物组合物配制为用于静脉注射。在另外的实施方式中，所述药物组合物包含配制为用于静脉注射的阿米洛利和/或阿米洛利类似物。在另外的实施方式中，所述药物组合物包含配制为用于侧脑室注射的阿米洛利和/或阿米洛利类似物。在另外的实施方式中，所述药物组合物包含配制为用于鞘内注射的阿米洛利和/或阿米洛利类似物。在另外的实施方式中，所述药物组合物包含配制为用于肌肉注射的阿米洛利和/或阿米洛利类似物。适于注射用途的药物组合物包括无菌水溶液(水溶性的情况下)或分散液及供无菌注射用溶液或分散液的临时配制的无菌粉剂。对于静脉给药，适宜的载体包括生理盐水、抑菌水、Cremophor EL™(BASF，Parsippany，NJ)或磷酸盐缓冲盐水(PBS)。在所有的情况下，注射用组合物为无菌的，且为达到易于以注射液存在的程度的液体。所述注射用组合物在制造和储存的条件下必须是稳定的，且必须防微生物(如细菌和真菌)的污染行为而保留下来。所述载体可以是含有，例如，水、乙醇、多元醇(例如，甘油、丙二醇及液态聚乙二醇等)和它们的适宜的混合物的溶液或分散介质。例如，通过包膜(如卵磷脂)的使用，在分散液的情况下通过维持所需的粒度，以及通过表面活性剂的使用，可保持适当的流动性。可通过各种抗菌剂和抗真菌剂(例如，对羟基苯甲酸酯、氯丁醇、酚、抗坏血酸、硫柳汞等)来实现对微生物作用的防范。在许多情况下，优选在所述组合物中包含等渗剂，例如，糖类、多元醇(如甘露醇、山梨醇)、氯化钠。所述注射用组合物的持久吸收可通过在所述组合物中包含延迟吸收的药剂，例如，单硬脂酸铝和明胶而实现。

[0108] 无菌注射溶液可通过将所需量的阿米洛利和/或阿米洛利类似物混入适当的溶剂中，然后过滤灭菌来制备。通常地，分散液通过将活性化合物混入含有基本的分散介质和所需的以上所列举的那些其它成分的无菌载体来制备。在供无菌注射溶液的制备的无菌粉剂的情况下，优选的制备方法为真空干燥和冷冻干燥，其由之前的无菌过滤溶液生成活性组分加任意额外的所需组分的粉剂。

[0109] 在一些实施方式中，所述药物组合物以干燥的形式被提供，并被配制为片剂或胶囊的形式。可根据常规工序采用本领域公知的固态载体来配制片剂。本发明中使用的硬和软胶囊可由任何药学上可接受的材料，如明胶或纤维素衍生物来制备。

[0110] 在某些实施方式中，将所述药物组合物配制为即刻释放、缓释、延迟释放或它们的组合。缓释，又称持久释放、延时释放或长效释放、控释(CR)、改良释放(MR)或者持续释放(CR或Contin)，是用于医用片剂或胶囊以缓慢溶解并随着时间的推移释放所述活性组分的

机制。持久释放的片剂或胶囊的优点在于其通常可比同样药物的立刻释放的剂型吸收少，以及在于其保持了药物在血流中更稳定的水平，因而延长了药物活性的持续时间。

[0111] 在一个实施方式中，通过在不溶物(如丙烯酸类或甲壳素)的基质中包埋所述活性组分来将所述药物组合物配制为用于缓释。设计缓释形式以通过维持恒定的药物水平特定的时间段而在预定的速率下释放活性成分。这可通过多种剂型而实现，包括但不仅限于脂质体及药物-聚合物的结合物，如水凝胶。

[0112] 在另一个实施方式中，将所述药物组合物配制为用于延迟释放，从而使得所述活性组分在给药时并不立即释放。延迟释放载体的非限定性实例为在肠中而非胃中溶解的肠溶衣口服药物。

[0113] 在另外的实施方式中，将所述药物组合物配制为一部分所述活性组分即刻释放，剩余活性组分随后缓释。在一个实施方式中，所述药物组合物被配制为可被服用以使所述活性组分快速释放的粉剂。在另一个实施方式中，所述药物组合物被配制为液体、胶体、液态的悬浮液或乳剂的形式。所述液体、胶体、悬浮液或乳剂可以裸剂的形式或包含于胶囊中而被受治者服下。

[0114] 在再一个实施方式中，所述药物组合物可被提供为皮肤或透皮贴剂以控制和/或持久地局部施用所述活性组分。

[0115] 实施例

[0116] 以下实施例描述了本发明选择的方面和实施方式，尤其是描述了ASIC抑制作用的体外和体内效果的数据，以及示例性的用作抑制剂的胱氨酸结肽。这些实施例用于说明的目的，而不应当被理解为限定本发明的范围。

[0117] 实施例1:通过阻塞可渗透钙的酸敏感离子通道的局部缺血的神经保护

[0118] 参见图2-10，此实施例描述了显示ASIC1a在调解局部缺血性损伤中的作用以及ASIC1a抑制剂减轻局部缺血性损伤的能力的实验。Ca^{2+}毒性在局部缺血性脑损伤中扮演了关键性的角色。由于谷氨酸拮抗剂的大量人体试验显示在中风中不能有效的保护神经，在局部缺血的脑部中发生细胞的毒性Ca^{2+}负载的机理变得尚不明朗。酸中毒是局部缺血的常见特征，并在脑损伤中扮演了关键性的角色。此实施例证实:酸中毒激活了可导致与谷氨酸受体无关的、与Ca^{2+}有关的神经元损伤的可渗透Ca^{2+}的酸敏感离子通道(ASIC)。因此，缺乏内源性的ASIC的细胞可抵御酸的损害，而可渗透Ca^{2+}的ASIC1a的转染则可建立敏感性。在局灶性缺血中，侧脑室注射ASIC1a阻滞剂或敲除ASIC1a基因可保护大脑免遭局部缺血性损伤，并可比谷氨酸拮抗作用产生更大的效力。

[0119] 正常的大脑需要葡萄糖的完全氧化以满足其能量需求。在局部缺血期间，氧气耗尽迫使大脑切换到无氧糖酵解。作为糖酵解的副产物的乳酸与ATP水解产生的质子的积累会导致局部缺血的大脑中的pH的下降并加剧局部缺血性脑损伤。

[0120] 酸敏感离子通道(ASIC)为一类在哺乳动物中枢和周围神经系统的各处神经元表达的配体门控通道。迄今为止，已克隆了六种ASIC亚单位。这些亚单位中的四种形成由酸性pH激活的功能性的同源多聚(homomultimeric)通道，以传导钠选择性的、阿米洛利敏感的阳离子电流。所述ASIC亚单位中的两种(ASIC1a和ASIC2a亚单位)已被证明富含于大脑中。

[0121] 实验过程

[0122] 神经元的培养

[0123] 氟烷麻醉之后，从E16瑞士小鼠(Swiss mice)或P1ASIC1$^{+/+}$和ASIC1$^{-/-}$小鼠身上解剖出大脑皮层，并在37℃下用0.05％的胰蛋白酶-EDTA孵育10分钟。然后用火焰抛光的玻璃吸管将组织捣碎，以2×10^{5}细胞/孔或10^{6}细胞/盖玻片的密度放置于聚-L-鸟氨酸涂覆的24孔培养板或25×25mm玻璃盖玻片上。神经元用补充了10％马血清的MEM(用于E16的培养)或补充了B27的神经基质培养基(Neurobasal medium)(用于P1的培养)来培养，并在12天之后用作电生理学和毒性的研究。通过添加5-氟-2-脱氧尿苷和尿苷来抑制神经胶质的生长，制得了含90％神经元(由NeuN和GFAP染色法(数据未显示)所测定)的培养细胞。

[0124] 电生理学

[0125] ASIC的电流用全细胞膜片钳(whole-cell patch-clamp)和快速灌注技术(fast-perfusion technique)来记录。正常的细胞外溶液(ECF)包含(单位mM)：140NaCl、5.4KCl、25HEPES、20葡萄糖、1.3$CaCl_2$、1.0$MgCl_2$、0.0005TTX(pH 7.4)、320-335mOsm。为生成低pH溶液，加入了各种不同量的HCl。对于pH<6.0的溶液，采用MES代替HEPES以更可靠地缓冲pH。贴片电极包含(单位mM)：140CsF、2.0$MgCl_2$、1.0$CaCl_2$、10HEPES、11EGTA、4MgATP(pH 7.3)、300mOsm。无Na^+的溶液由10mM $CaCl_2$、25mM HEPES组成，并以等渗的NMDG或蔗糖来替代NaCl(Chu et al.,2002)。利用多管灌注系统(SF-77B，Warner Instrument公司)以供溶液的快速交换。

[0126] 细胞损伤试验——LDH测定

[0127] 将细胞用ECF洗涤三次，随机分为治疗组。在所有组中加入MK801(10μM)、CNQX(20μM)和尼莫地平(nimodipine)(5μM)以消除谷氨酸受体和电压门控Ca^{2+}通道的潜在的二次激活。之后是酸孵育，37℃下在神经基质培养基中洗涤和孵育神经元。利用LDH测定工具(Roche Molecular Biochemicals公司)在培养基中测定了LDH的释放。将介质(100μL)由培养孔转移至96孔培养板中，并与所述工具所提供的100μL的反应溶液混合。30分钟后，利用酶标仪(Spectra Max Plus，Molecular Devices公司)在492nm处测定了光密度。减去在620处的背景吸收。通过在各个实验的结尾用1％的Triton X-100的孵育15分钟，在各个孔中获得了最大释放的LDH。

[0128] Ca^{2+}成像

[0129] 将在25×25mm玻璃盖玻片上生长的皮层神经元用ECF洗涤三次，并在22℃下用5μM的Fura-2乙酰氧基甲基酯孵育40分钟，洗涤三次，再在常规ECF中孵育30分钟。在倒置显微镜(Nikon TE300)上将盖玻片转移至灌注室中。用氙灯照亮细胞，并用40x UV的萤石油浸物镜观察，并利用冷却的CCD摄影机(Sensys KAF 1401，Photometrics公司)获得了影像。在Axon成像工作台软件(Axon Imaging Workbench software)(Axon Instruments公司)控制的PC中获得并分析了数字图像。通过所述软件控制快门和滤光轮(Lambda10-2)以使得细胞在340或380nm激发波长下定时发光。在510nm的辐射波长处检测到了Fura-2荧光反应。凭借视野内的细胞的限定区域中的平均像素比值分析了比率图像(340/380)。所述值被输出至SigmaPlot以供进一步的分析。

[0130] 荧光素二乙酸酯染色与碘化丙啶吸收

[0131] 细胞在含有荧光素二乙酸酯(FDA)(5μM)和碘化丙啶(PI)(2μM)的ECF中孵育30分钟，之后用不含染料的ECF洗涤。在配备有对于PI在580/630nm处激发/辐射和对于FDA在500/550nm处激发/辐射的外荧光的显微镜(Zeiss)上观察和计数存活(FDA阳性)和死亡(PI

阳性)的细胞。利用配备有BQ 8000sVGA图像采集卡的光电DEI-730摄像机收集图像,并用计算机软件(Bioquant,TN公司)来分析。

[0132] COS-7细胞的转染

[0133] 在含10％HS和1％PenStrep(GIBCO)的MEM中培养了COS-7细胞。在～50％的融合下,利用FuGENE6转染剂(Roche Molecular Biochemicals公司),采用在pc^{DNA3}载体中的用于ASIC与GFP的cDNA共转染了细胞。用于ASIC的DNA(0.75μg)和0.25μg的用于GFP的DNA被用于每个35mm培养皿中。选取GFP阳性的细胞以在转染后用膜片钳记录48小时。为稳定ASIC1a的转染,在所述转染一周后将500μg/mL的G418加入到培养基中。在G418的存在下,幸存的耐G418的细胞被进一步地培养并传递>5代(passage)。然后细胞用膜片钳和免疫荧光染色来检查,用于ASIC1a的表达。

[0134] 氧糖剥夺(Oxygen-Glucose Deprivation)

[0135] 35℃下在含85％N_2、10％H_2和5％CO_2的气体的厌氧室(Model 1025,Forma Scientific公司)中,在pH 7.4或6.0下神经元被不含葡萄糖的ECF洗涤三次及孵育。1小时后通过在常规的细胞培养孵育器中用神经基质培养基替换所述不含葡萄糖的ECF并孵育培养物而终止氧糖剥夺(OGD)。随着HEPES缓冲的ECF的使用,1小时的OGD使pH由7.38稍稍降低至7.28(n＝3)和由6.0稍稍降低至5.96(n＝4)。

[0136] 局灶性缺血

[0137] 通过使利用1.5％异氟烷、70％N_2O和28.5％O_2而麻醉的插管和通气的雄性大鼠(SD,250-300g)和小鼠(具有同类系C57B16的背景,～25g)的大脑中动脉的线栓(MCAO)而引发了短暂的局灶性缺血。用恒温控制的加热板和发热灯将直肠和颞肌的温度维持在37℃±0.5℃。通过经颅激光多普勒来监测脑部血流。剔除血流未降低20％以下的动物。

[0138] 局部缺血24小时后用水合氯醛杀死动物。快速取出脑部,间隔1mm(小鼠)或2mm(大鼠)冠状切片,并通过浸入活体染料(2％)2,3,5-三苯基四氮唑盐酸盐(TTC)中来染色。通过从未局部缺血的半球的面积中减去局部缺血的半球中被TTC染色的正常区域来计算梗死面积。通过加和所有切片的梗死面积并乘以切片厚度来计算梗死体积。采用具有在后部至前囟0.8mm、侧边至中线1.5mm以及腹侧至硬脑膜3.8mm的立体定位下插入的导管的微量注射泵,通过立体定位技术进行了大鼠的脑室注射。所有的操作和分析均通过不知所属治疗组的个体来进行。

[0139] 结果

[0140] (a)酸中毒激活小鼠的皮层神经元的ASIC

[0141] 图3和4显示了关于培养小鼠的皮层神经元的ASIC的电生理学和药理学的示例性数据。图3A和3B为图示了通过pH由7.4降至所示的pH值而激活的ASIC电流的pH相关性的图表。剂量响应曲线符合具有6.18±0.06的平均$pH_{0.5}$的Hill公式(n＝10)。图3C和3D为图示了ASIC的电流-电压关系的图表(n＝5)。使在各个电压下的ASIC电流的幅度向在-60mV下记录的标准化。图4A和4B为图示了阿米洛利所造成的ASIC电流的剂量依赖性阻滞的图表,IC_{50}＝16.4±4.1μM,N＝8。图4C和4D为图示了PcTX毒素所造成的ASIC电流的阻滞的图表,**p<0.01。

[0142] 记录了培养小鼠的皮层神经元的ASIC电流(参见图3)。-60mV的维持电势下,在大多数的神经元中,细胞外的pH(pH_e)急速下降至低于7.0诱发了具有少量稳态成分的较大的

瞬态内向电流(图3)。内向电流的幅度随着pH_e的下降而以S形上升,生成了6.18±0.06的$pH_{0.5}$(n=10,图3B)。获得了线性I-V关系和接近于Na^+平衡电势的逆转(n=6,图3C和3D)。这些数据证实:降低pH_e可激活小鼠的皮层神经元中常见的ASIC。

[0143] 测试了阿米洛利,ASIC的非特异性阻滞剂,对酸激活电流的影响(参见图4)。如图4所示,阿米洛利剂量依赖性地阻滞了皮层神经元中的ASIC电流,IC_{50}为16.4±4.1μM(n=8,图4A和4B)。图4C和4D中显示了PcTX毒素对皮层神经元中的酸激活电流的影响。在100ng/mL下,PcTX毒素可逆地阻滞了ASIC电流的47%±7%的峰值幅度(n=15,图4C和4D),表明同源的ASIC1a对总的酸激活电流的显著贡献。在大多数的皮层神经元中,提高PcTX的浓度并不会导致ASIC电流的幅度的进一步的下降(n=8,数据未显示),表明在这些神经元中共存有PcTX不敏感的ASIC(例如,异源的ASIC1a/2a)。

[0144] (b)通过模拟的局部缺血增强了ASIC响应

[0145] 图5显示了表明模拟的局部缺血可提高ASIC的活性的示例性数据。图5A为一系列显示了1小时的OGD之后ASIC电流的幅度的上升和去敏感作用的下降的示例性曲线。图5B为阐释了在OGD神经元中ASIC电流幅度的上升的概括性数据的图表,N=40和44,*p<0.05。图5C为一系列显示了在OGD神经元中的下降的ASIC电流去敏感作用的示例性曲线和概括性数据,N=6,**p<0.01。图5D为一对显示了1小时的OGD之后,控制条件下在$ASIC1^{-/-}$神经元中在pH6.0缺乏酸激活电流的示例性曲线(n=12和13)。

[0146] 由于酸中毒可能是脑局部缺血的首要特征,决定测试ASIC是否可在局部缺血条件下被激活以及局部缺血是否可改变这些通道的特性,参见图5。记录1小时的氧糖剥夺(OGD)后神经元中的ASIC电流。简而言之,将一组培养物用不含葡萄糖的细胞外液(ECF)洗涤三次并接受OGD,同时使对照培养物接受含葡萄糖的ECF的洗涤并在常规的细胞培养孵育器中孵育。1小时后通过在所述常规的孵育器中用神经基质培养基替换不含葡萄糖的ECF并孵育培养物而终止OGD。然后当神经元不存在形态学上的变化时,在所述OGD之后1小时记录ASIC电流。OGD的治疗导致了ASIC电流的幅度的平缓上升(对照组中1520±138pA,N=44;1小时OGD后的神经元中1886±185pA,N=40,p<0.05,图5A和5B)。更重要的是,电流衰减的时间常数的增大表明,OGD导致了ASIC去敏感作用的突然降低(对照组中814.7±58.9ms,N=6;OGD后的神经元中1928.9±315.7ms,N=6,p<0.01,图5A和5C)。类似于之前在海马神经元中的研究(Wemmie et al.,2002),在来自$ASIC1^{-/-}$小鼠的培养皮层神经元中,pH由7.4降低至6.0并不会激活任何的内向电流(n=52)。在这些神经元中,1小时的OGD并不会激活或增强酸引发的响应(图5D,n=12和13)。

[0147] (c)酸中毒引发经由ASIC1a的与谷氨酸无关的Ca^{2+}的进入

[0148] 图6和7显示了表明皮层神经元中的ASIC可以是可渗透的Ca^{2+},且Ca^{2+}的渗透性可取决于ASIC1a的示例性数据。图6A显示了用含10mM Ca^{2+}的无Na^+的ECF作为仅有的电荷载体获得的示例性曲线。在pH6.0下记录了内向电流。校正液体接界电势(n=5)之后的平均逆转电势为~-17mV。图6B显示了阐释由阿米洛利和PcTX毒素造成的Ca^{2+}介导的电流的阻滞的代表性曲线和概括性数据。Ca^{2+}介导的电流的峰值幅度通过100μM阿米洛利(n=6,p<0.01)降至对照组值的26%±2%以及通过100ng/mL PcTX毒素(n=5,p<0.01)降至22%±0.9%。图7A显示了表明由pH降至6.0导致的$[Ca^{2+}]_i$的上升的作为pH的函数的示例性的340/380nm比率。将神经元浸入具有用于电压门控Ca^{2+}通道的阻滞剂(5μM尼莫地平和1μMω-芋螺毒素

MVIIC)和谷氨酸受体(10μM MK801和20μM CNQX)的含1.3mM $CaCl_2$的常规ECF中。图7A的小图显示了示例性的100μM阿米洛利对$[Ca^{2+}]_i$的酸诱导上升的抑制。图7B显示了阐释相比于pH6.0组,阿米洛利和PcTX毒素对$[Ca^{2+}]_i$的酸诱导上升的抑制的示例性的概括性数据,N=6-8,**p<0.01。图7C显示了NMDA出现/缺席时作为pH的函数的示例性的340/380nm比率,证实$ASIC1^{-/-}$神经元中缺乏$[Ca^{2+}]_i$的酸诱导上升;神经元具有对NMDA的正常响应(n=8)。图7D显示了阐释在$ASIC1^{-/-}$神经元中在pH6.0时缺乏酸激活电流的示例性曲线。

[0149] 利用标准离子置换方案(Jia et al.,Neuron,1996,17:945-956)和Fura-2荧光反应的Ca^{2+}成像技术(Chu et al.,2002,J.Neurophysiol.87:2555-2561)确定了皮层神经元中的ASIC的Ca^{2+}渗透性。利用含有10mM Ca^{2+}的浴溶液(不含Na^+和不含K^+)作为仅有的电荷载体并在-60mV的维持电势下,我们记录下18个神经元中的15个的内向电流大于50pA,显示了大多数皮层神经元中的ASIC的显著的Ca^{2+}渗透性(图6A)。与同源ASIC1a通道的激活相一致,10mM Ca^{2+}所承载的电流被非特异性ASIC阻滞剂阿米洛利和ASIC1a特异性阻滞剂PcTX毒素极大地阻滞(图6B)。Ca^{2+}介导的电流的峰值幅度通过100μM阿米洛利(n=6,p<0.01)降至对照的26%±2%以及通过100ng/mL PcTX毒素(n=5,p<0.01)降至22%±0.9%。在其它主要Ca^{2+}进入途径的阻滞剂(针对谷氨酸受体的MK801 10μM和CNQX 20μM;针对电压门控Ca^{2+}通道的尼莫地平5μM和ω-芋螺毒素MVIIC 1μM)的存在下,Ca^{2+}成像证实:20个神经元中的18个对pH下降作出了响应,其具有可检测到的细胞内Ca^{2+}($[Ca^{2+}]_i$)的浓度的升高(图7A)。一般而言,$[Ca^{2+}]_i$残余会在低pH溶液的长期灌注期间升高。在一些细胞中,$[Ca^{2+}]_i$升高的持续时间甚至长于酸灌注的持续时间(图7A)。长期持续的Ca^{2+}响应表明在无损神经元中的ASIC响应可能会比在全细胞记录中更少地被去敏感,或表明经由ASIC的Ca^{2+}的进入可引发随后的自细胞内存储的Ca^{2+}释放。采用1μM毒胡萝卜内酯(thapsigargin)的神经元预孵育部分地抑制了Ca^{2+}升高的持久成分,表明自细胞内存储的Ca^{2+}释放也可促进酸诱导细胞内Ca^{2+}的累积(n=6,数据未显示)。类似于Ca^{2+}离子承载的电流(图6B),$[Ca^{2+}]_i$的峰值和持续升高被阿米洛利和PcTX毒素极大地抑制(图7A和7B,n=6-8),这与酸诱导$[Ca^{2+}]_i$的升高中同源ASIC1a的参与相一致。ASIC1基因的敲除在所有神经元中消除了酸诱导$[Ca^{2+}]_i$的升高而不会影响NMDA受体介导的的Ca^{2+}响应(图7C,n=8)。膜片钳的记录证实了52个$ASIC1^{-/-}$神经元中的52个在pH6.0处缺乏酸激活电流,这与ASIC1a亚单位的缺席相一致。然而,将pH降至5.0或4.0,在52个$ASIC1^{-/-}$神经元中的24个中激活了可检测到的电流,表明这些神经元中ASIC1a亚单位的出现(图7D)。进一步的电生理学研究表明$ASIC1^{-/-}$神经元具有对各种电压门控通道和NMDA、GABA受体门控通道的正常响应(数据未显示)。

[0150] (d)ASIC的阻滞保护了酸中毒引发的、与谷氨酸无关的神经元损伤

[0151] 图8显示了表明酸孵育可引发与谷氨酸受体无关的受ASIC的阻滞所保护的神经元损伤的示例性数据。图8A和8B显示了相比于相同时间点的pH7.4组(通过幸存神经元的细胞体染色和死亡神经元的核的碘化丙啶(PI)染色,还分析了用荧光素二乙酸酯(FDA)的酸诱导神经元损伤),通过皮层神经元在pH7.4(实心柱)或6.0(空心柱)的ECF中孵育1小时(图8A)或24小时(图8B)所引发的与时间相关的LDH释放的示例性数据的图表,N=20-25孔,*p<0.05,以及**p<0.01。图8C显示了阐释由100μM阿米洛利或100ng/mL PcTX毒素所造成的酸诱导LDH释放的抑制的图表(n=20-27,*p<0.05,以及**p<0.01)。对于所有的实验,在ECF中均存在MK801、CNQX和尼莫地平(图8A-C)。

[0152] 参见图8,研究了在pH7.4或6.0的含MK801、CNQX和尼莫地平的ECF中孵育的24孔培养板上生长的神经元的酸诱导损伤。通过测定各个时间点的乳酸脱氢酶(lactate dehydrogenase,LDH)的释放(Koh and Choi,J.Neurosci.,1987,20:83-90)(图8A和8B)及通过幸存/死亡细胞的荧光染色测定了细胞损伤。相比于pH7.4下处理的神经元,1小时的酸孵育(pH6.0)引发了LDH释放的时间相关性增长(图8A)。24小时后,引发了最大LDH释放的45.7%±5.4%(n=25孔)。pH6.0下的持续处理引发了更大的细胞损伤(图8B,n=20)。与LDH测定相一致,采用荧光素二乙酸酯和碘化丙啶的幸存/死亡染色显示出类似的由1小时酸处理造成的细胞死亡的增长(数据未显示)。采用pH6.5的ECF的1小时孵育同样引发了显著但比采用pH6.0的ECF更少的LDH释放(n=8孔,数据未显示)。

[0153] 测试了阿米洛利和PcTX毒素对酸诱导LDH释放的影响,以确定ASIC的激活是否参与了酸诱导的、与谷氨酸受体无关的神经元损伤。在1小时的酸孵育之前的10分钟或期间加入100μM阿米洛利或100ng/mL PcTX毒素显著降低了LDH释放(图8C)。经过24小时,LDH释放由45.3%±3.8%被阿米洛利降至31.1%±2.5%以及被PcTX毒素降至27.9%±2.6%(n=20-27,$p<0.01$)。尽管仅使用阿米洛利的持久孵育(例如,5小时)增加了LDH释放,但在pH7.4的ECF中添加阿米洛利或PcTX毒素后1小时并不会影响基线LDH释放(n=8,数据未显示)。

[0154] (e)同源ASIC1a的激活是造成酸中毒引发的损伤的原因

[0155] 图9为一系列呈现出表明ASIC1a可能参与了体外的酸诱导损伤的示例性数据的图表。图9A显示了阐释通过降低$[Ca^{2+}]_e$来抑制酸诱导LDH释放的示例性数据(n=11-12,**$p<0.01$,相比于pH 6.0,1.3Ca^{2+})。图9B显示了阐释酸孵育引发了在ASIC1a转染的而非未转染的COS-7细胞中的LDH释放的增长的示例性数据(n=8-20)。阿米洛利(100μM)抑制了ASIC1a转染细胞中的酸诱导LDH释放(对于7.4比6.0和6.0比6.0+阿米洛利,*$p<0.05$)。图9C显示了阐释$ASIC1^{-/-}$神经元中酸诱导损伤的缺乏及凭借阿米洛利和PcTX毒素的保护的示例性数据(各组中n=8,$p>0.05$)。图9D显示了阐释在OGD下培养的皮层神经元中LDH释放的酸诱导增长的示例性数据(n=5)。通过结合1小时OGD/酸中毒而引发的LDH释放并不会被水溶性维生素E(trolox)和左旋硝基精氨酸甲酯(L-NAME)所抑制(n=8-11)。在$ASIC1^{-/-}$神经元中OGD并不会增强酸诱导的LDH释放(对于pH 7.4比pH 6.0,**$p<0.01$,以及对于pH 6.0比6.0+PcTX毒素,*$p<0.05$)。对于所有的实验,在ECF中均存在MK801、CNQX和尼莫地平(图9A-D)。

[0156] 在常规或减少的$[Ca^{2+}]_e$的存在下用pH 6.0的ECF处理神经元以确定Ca^{2+}的进入是否在酸诱导损伤中起了作用(参见图9)。如同用PcTX毒素阻滞ASIC1a,Ca^{2+}由1.3降至0.2mM抑制了酸诱导的LDH释放(由40.0%±4.1%降至21.9%±2.5%)(n=11-12,$p<0.01$;图9A)。未测试不含Ca^{2+}的溶液,因为$[Ca^{2+}]_e$的完全移除可能会激发大的经由Ca^{2+}敏感阳离子通道的内向电流,这可在其它方面使得数据理解变得复杂。通过阿米洛利和PcTX(非特异性和特异性ASIC1a阻滞剂)或通过降低$[Ca^{2+}]_e$而对酸损伤的抑制表明:可渗透Ca^{2+}的ASIC1a的激活可能参与了酸诱导神经元损伤。

[0157] 研究了未转染和ASIC1a转染的COS-7细胞的酸损伤,以提供ASIC1a的激活参与了酸损伤的另外的证据。COS-7为通常用于ASIC的表达的细胞系,这是因为其缺乏内源性的通道。融合后(铺板36-48小时后),用pH 7.4或6.0的ECF处理细胞1小时。酸孵育后24小时测定LDH释放。相比于pH7.4处理的细胞,用pH 6.0的ECF处理未转染的COS-7细胞并不会造成LDH释放的增长(对于pH 7.4,10.3%±0.8%,以及对于pH 6.0,9.4%±0.7%,N=19和20孔;p

>0.05,图9B)。然而,在稳定转染ASIC1a的COS-7细胞中,pH 6.0下的1小时孵育使LDH释放由15.5%±2.4%显著增长至24.0%±2.9%(n=8孔,p<0.05)。在这些细胞中,阿米洛利(100μM)的添加抑制了酸诱导的LDH释放(图9B)。

[0158] 同样研究了单独用编码GFP的cDNA或GFP加ASIC1a的cDNA瞬时转染的CHO细胞的酸损伤。所述转染之后(24-36小时),用酸性溶液(pH 6.0)孵育细胞1小时,并在酸孵育的24小时之后测定细胞损伤。一小时的酸孵育在GFP/ASIC1a组中而非用单独的GFP转染的组中极大地减少了幸存的GFP阳性细胞(数据未显示)。

[0159] 进行了针对来自$ASIC^{+/+}$和$ASIC1^{-/-}$小鼠的培养皮层神经元的细胞毒性实验,以进一步证实ASIC1a在酸中毒引发的神经损伤中的参与。再一次地,$ASIC^{+/+}$神经元在6.0时的1小时酸孵育引发了被阿米洛利和PcTX毒素所减少的可观的LDH释放(n=8-12)。然而,$ASIC^{+/+}$神经元的一小时酸处理并不会在24小时后造成LDH释放的显著增长(对于pH 7.4,13.8%±0.9%,以及对于pH 6.0,14.2%±1.3%,N=8,p>0.05),这显示出这些神经元对酸损伤的耐力(图9C)。另外,ASIC1基因的敲除同样消除了阿米洛利和PcTX毒素对酸诱导的LDH释放的影响(图9C,各自n=8),进一步表明阿米洛利和PcTX毒素对皮层神经元的酸诱导损伤的抑制(图8C)是由于ASIC1亚单位的阻滞。与酸孵育相反,用1mM NMDA+10μM甘氨酸对$ASIC1^{-/-}$神经元的1小时处理(在不含Mg^{2+}的[pH 7.4]ECF中)在24小时后引发了最大LDH释放的84.8%±1.4%(n=4,图9C),显示出对其它细胞损伤过程的常规响应。

[0160] (f)模拟的局部缺血通过ASIC加剧了酸中毒导致的、与谷氨酸无关的神经元损伤

[0161] 由于ASIC电流的大小可被脑缺血细胞溶胀的细胞和神经化学成分,花生四烯酸和乳酸盐所增大,且更重要的是,ASIC电流的去敏感作用可被模拟的局部缺血所显著降低(参见图5A和5C),则可预见在局部缺血状况中的ASIC的激活会产生更严重的神经元损伤。为了检验这个假设,使神经元在氧糖剥夺(OGD)条件下接受1小时的酸处理。在所有的溶液中加入MK801、CNQX和尼莫地平以抑制电压门控Ca^{2+}通道和与OGD有关的谷氨酸受体介导的细胞损伤。在OGD条件下采用pH 7.4的ECF的一小时孵育在24小时后仅引发了最大LDH释放的27.1%±3.5%(n=5,图9D)。这个发现与带有谷氨酸受体和电压门控Ca^{2+}通道的阻滞的1小时的OGD不会引发实质的细胞损伤的在先报导(Aarts et al.,2003)相符。然而,1小时的OGD,结合酸中毒(pH 6.0),引发了最大LDH释放的73.9%±4.3%(n=5,图9D,p<0.01),明显大于没有OGD时酸引发的LDH释放(参见图8A,p<0.05)。ASIC1a阻滞剂PcTX毒素(100ng/mL)的添加使酸/OGD引发的LDH释放显著降至44.3%±5.3%(n=5,p<0.05,图9D)。

[0162] 用来自$ASIC1^{-/-}$小鼠的培养神经元进行了同样的实验。然而,不同于含有ASICI的神经元,结合OGD和酸的1小时处理在$ASIC1^{-/-}$神经元中仅稍稍提高了LDH释放(由26.1%±2.7%升至30.4%±3.5%,N=10-12,图9D)。这个发现表明:OGD造成的酸引发的损伤的加剧很可能是由于OGD加剧了ASIC1介导的中毒。

[0163] 已证实由反应性氧/氮物质激活的可渗透Ca^{2+}的非选择性阳离子传导的激活导致了与谷氨酸受体无关的神经元损伤(Aarts et al,Cell,2003,115:863-877)。操作者可通过直接清理自由基(例如,水溶性维生素E)或减少自由基的产生(例如,左旋硝基精氨酸甲酯)来显著降低长期OGD引发的细胞损伤。为了确定短期OGD与酸中毒的结合所引发的神经元损伤是否涉及类似的机制,检验了水溶性维生素E和左旋硝基精氨酸甲酯对OGD/酸诱导的LDH释放的效果。如图9D所示,无论水溶性维生素E(500μM)还是左旋硝基精氨酸甲酯(300

μM),均不具有对结合的1小时OGD/酸中毒引发的神经元损伤的明显效果(n=8-11)。另外的实验还证实:ASIC阻滞剂阿米洛利和PcTX毒素对TRPM7通道的传导无效(Aarts et al.supra)。综上,这些发现有力地表明:在我们的研究中,ASIC而非TRPM7通道的激活很可能是造成结合的1小时OGD/酸中毒引发的神经元损伤的原因。

[0164] (g)体内局部缺血性脑损伤中ASIC1a的激活

[0165] 图10显示了阐释体内脑局部缺血中,凭借ASICI阻滞剂和ASICI基因敲除的神经保护的数据。图10A显示了得自TTC染色的大脑切片的示例性数据的图表,其中显示了来自注射了aCSF(n=7)、阿米洛利(n=11)或PcTX毒素(n=5)的大鼠的大脑中的染色体积("梗死体积"),与aCSF注射组相比,*p<0.05和**p<0.01。图10B显示了阐释来自$ASIC1^{-/-}$小鼠的大脑中的梗死体积的减少(各组n=6)的示例性数据的图表,与+/+组相比,*p<0.05和**p<0.01。图10C显示了阐述来自腹腔注射10mg/kg美金刚(memantine,Mem)或者腹腔注射美金刚并伴随着侧脑室注射PcTX毒素(500ng/mL)的小鼠的大脑中的梗死体积的减少的示例性数据的图表,与aCSF注射相比并在美金刚与美金刚+PcTX毒素之间,**p<0.01(各组n=5)。图10D显示了阐述来自腹腔注射美金刚的$ASIC1^{+/+}$(wt)或$ASIC1^{-/-}$小鼠的大脑中的梗死体积的减少的示例性数据的图表(各组n=5),*p<0.05和**p<0.01。

[0166] 测试了短暂局灶性缺血的大鼠模型中阿米洛利和PcTX毒素的保护效果(Longa et al.,Stroke,1989,20:84-91),以确定ASIC1a的激活是否参与了体内局部缺血性脑损伤。通过短暂地阻塞大脑中动脉(MCAO)而引发了局部缺血(100分钟)。在所述局部缺血之前的30分钟和之后侧脑室注射总共6μL的单独的人造CSF(aCSF)、含阿米洛利(1mM)的aCSF、或者PcTX毒素(500ng/mL)。用于4周龄大鼠的脑室和脊髓液的体积估计为~60μL。假定注入的阿米洛利和PcTX均匀地分散于CSF中,则预计阿米洛利的浓度为~100μM及PcTX的浓度为~50ng/mL,此为被发现在细胞培养实验中有效的浓度。局部缺血后24小时通过TTC染色确定了梗死体积(Bederson et al.,Stroke,1986,17:1304-1308)。局部缺血(100分钟)在注射aCSF的大鼠中产生了329.5±25.6mm^3的梗死体积(n=7),但在注射阿米洛利的大鼠中仅为229.7±41.1mm^3(n=11,p<0.05)以及在注射PcTX毒素的大鼠中仅为130.4±55.0mm^3(降低了~60%)(n=5,p<0.01)(图10A)。

[0167] $ASIC1^{-/-}$小鼠被用于进一步证实ASIC1a在体内局部缺血性脑损伤中的参与。如之前所描述的(Stenzel-Poore et al.,Lancet,2003,362:1028-1037),使雄性$ASIC1^{+/+}$、$ASIC1^{+/-}$和$ASIC1^{-/-}$小鼠(~25g,具有同类系C57B16背景)接受60分钟的MCAO。与通过ASIC1a的药物学阻滞的保护(如上所述)相一致,与+/+小鼠(84.6±10.6mm^3,N=6,p<0.01)相比,-/-小鼠显示出明显较小(降低了~61%)的梗死体积(32.9±4.7mm^3,N=6)。+/-小鼠也显示出减小的梗死体积(56.9±6.7mm^3,N=6,p<0.05)(图10B)。

[0168] 为了确定在谷氨酸受体阻滞的背景下,ASIC1a通道的阻滞或ASIC1基因的敲除是否会在体内提供额外的保护,则在60分钟的MCAO之后立即向C57B16小鼠腹腔(i.p.)注射美金刚(10mg/kg)并伴随着在局部缺血之前的15分钟或之后侧脑室(i.c.v.)注射总体积0.4μL的单独的aCSF或含PcTX毒素(500ng/mL)的aCSF。在i.p.注射生理盐水并i.c.v.注射aCSF的对照小鼠中,60分钟的MCAO造成了123.6±5.3mm^3的梗死体积(n=5,图10C)。在腹腔注射美金刚且侧脑室注射aCSF的小鼠中,相同持续时间的局部缺血造成了73.8±6.9mm^3的梗死体积(n=5,p<0.01)。然而,在注射美金刚和PcTX毒素的小鼠中,仅造成了47.0±1.1mm^3的

梗死体积(相比于对照组和美金刚组,n＝5,p<0.01,图10C)。这些数据表明同源ASIC1a的阻滞可在NMDA受体阻滞的背景下,在体内局部缺血中提供额外的保护。额外的保护还可在用药物NMDA阻滞处理的$ASIC1^{-/-}$小鼠中观察到(图10D)。在i.p.注射生理盐水或10mg/kg美金刚的$ASIC1^{+/+}$小鼠中,60分钟的MCAO分别造成了101.4±9.4mm^3或61.6±12.7mm^3的梗死体积(各组中n＝5,图10D)。然而,在注射美金刚的$ASIC1^{-/-}$小鼠中,相同持续时间的局部缺血造成了27.7±1.6mm^3的梗死体积(n＝5),明显小于注射美金刚的$ASIC1^{+/+}$小鼠的梗死体积(p<0.05)。

[0169] 总之,这些数据证实:可渗透Ca^{2+}的ASIC1a的激活是新颖的、与谷氨酸无关的基于局部缺血性脑损伤的生物机制。

[0170] 实施例2:PcTX神经保护的时间窗

[0171] 参见图11,本实施例描述了测定啮齿动物中风发作后PcTX毒素在不同时间下的神经保护效果的示例性的实验。简而言之,通过阻塞大脑中动脉(MCAO)而引发了啮齿动物的脑局部缺血(中风)。在引发后的指定时间下,向各个啮齿动物的侧脑室注入人造脑脊液(aCSF)、PcTX毒素(0.5μL,500ng/mL总蛋白)或灭活(煮沸)毒素。如图11所示,PcTX毒素的给药在中风发作后一小时和三小时均使中风体积减少了60％。另外,若在MCAO开始后五小时停止治疗,仍可维持可观的中风体积的减少。因此,由ASIC的抑制所致的神经保护可具有中风发作后延长了的治疗时间窗,使得中风受治者从中风开始数小时后的治疗中获益。ASIC阻滞在中风神经保护方面的这个效果比采用谷氨酸拮抗剂的NMDA受体的钙通道阻滞(实验性中风治疗的主要目标)的效果更强。迄今为止,没有谷氨酸拮抗剂具有如此处ASIC1a选择性抑制所示的这种良好的曲线。

[0172] 实施例3:示例性的胱氨酸结肽

[0173] 此实施例描述了示例性的胱氨酸结肽,包括全长PcTX和缺失PcTX衍生物,其可在培养细胞中被筛选,在局部缺血的动物(例如,啮齿动物,如小鼠或大鼠)中测得,和/或被施用至局部缺血的人类受治者。

[0174] 图12显示了以50示出的示例性胱氨酸结肽PcTx1的以单字母编码的主要氨基酸序列(SEQ ID NO:1),其具有所示的与氨基酸位置1-40有关的多种示例性肽特征。肽50可包含六个半胱氨酸残基,其形成胱氨酸键52、54、56以生成胱氨酸结基序58。所述肽还可包括一个或多个β折叠区域60和带正电的区域62。N端区域64和C端区域66可位于所述胱氨酸结基序的两侧。

[0175] 图13显示了图12的PcTx1肽50与该肽的多种示例性缺失衍生物相对齐的对比。这些衍生物可包括:N端缺失70(SEQ ID NO:2)、部分C端缺失72(SEQ ID NO:3)、全C端缺失74(SEQ ID NO:4)和N/C端缺失76(SEQ ID NO:5)。其它的PcTx1衍生物可包括,例如,在维持与原始PcTx1序列的至少约25％或约50％的序列相似性或一致性的同时,一个或多个氨基酸的缺失、嵌入或替换。

[0176] 可检测各个PcTx1衍生物的选择性抑制ASIC蛋白的能力和/或针对局部缺血的效果(如果有的话)。可采用任何适宜的检测系统以进行包括本发明别处所述的任何的基于细胞的测定系统和/或动物模型系统的此检测。同样或另选地,可在局部缺血的人类受治者中检测所述PcTx1衍生物。

[0177] 实施例4:PcTX毒素对ASIC1a的选择性

[0178] 此实施例描述了相对于培养细胞中表达的其它ASIC蛋白或ASIC蛋白的组合，测定PcTX毒素(及由此获得的PcTx1毒素)对单独的ASIC1a的选择性的实验。用PcTX毒素(25ng/mL，针对表达ASIC1a的细胞；以及500ng/mL，针对表达ASIC2a、ASIC3或ASIC1a+2a的细胞)处理表达指定的ASIC蛋白的COS-7细胞。在通道半最大激活的pH(pH 0.5)下测定通道电流。如图14所示，PcTX毒素在25ng/mL的蛋白质浓度下极大地阻滞了由ASIC1a同源通道介导的电流，并在500ng/mL下对由同源的ASIC2a、ASIC3或异源的ASIC1a/ASIC2a介导的电流没有影响(n＝3-6)。在500ng/mL下，PcTX毒素也不会影响由其它配体门控通道(例如，NMDA和GABA受体门控通道)或电压门控通道(例如，Na^+、Ca^{2+}和K^+通道)介导的电流(n＝4-5)。这些实验表明：PcTX毒素及由此获得的PcTx1肽为针对同源的ASIC1a的特定的阻滞剂。利用此基于细胞的测定系统，可测定多种合成肽或其它的候选抑制剂的ASIC抑制作用的效力和选择性(例如，参见实施例3)。

[0179] 实施例5：PcTX毒素的鼻腔给药的神经保护

[0180] 此实施例描述了说明经鼻腔施用PcTX毒素在中风的动物模型系统中减轻局部缺血引发的损伤的效力的示例性数据。通过阻塞大脑中动脉在雄性小鼠中引发了脑局部缺血。在阻塞开始后一小时，将动物作为对照处理或用PcTX毒素处理(鼻腔导入50μL 5ng/mL(总蛋白)的PcTx)。如图15所示，相对于对照处理，PcTX毒素的鼻腔给药导致如梗死体积所定义的局部缺血引发的损伤(局部缺血性损害)降低了55％。鼻腔给药可凭借基本上沉积于鼻腔通道而非吸入肺中的喷剂和/或可凭借至少部分吸入肺中的气雾剂而实现。在一些实施例中，鼻腔给药相比于其它的给药路径可具有许多的优点，如更有效地传送至大脑和/或适于局部缺血受治者的自主给药。

[0181] 实施例6：阿米洛利和阿米洛利类似物对ASIC1a通道的抑制作用

[0182] 如图16所示，阿米洛利和阿米洛利类似物苯扎米尔、非那米尔和EIPA以剂量依赖的方式阻滞了ASIC1a电流。类似地，阿米洛利和阿米洛利类似物苯扎米尔和EIPA以剂量依赖的方式阻滞了ASIC2a电流(图17)。表1总结了阿米洛利和阿米洛利类似物对ASIC1a通道的抑制作用。阿米洛利为此通道的有效抑制剂，具有7.7μM的IC50。

[0183] 表1阿米洛利和阿米洛利类似物对ASIC1a通道的抑制作用

[0184]

敲除1a	39.1 ± 3.8 (n=4)	
敲除2a	5.14 ± 0.79 (n=5)	
神经元(-60 mV)	43.3 ± 1.43 (n=6)	EIPA
神经元(-20 mV)	32.2 ± 6.3 (n=3)	
CHO 1a	111 ± 30 (n=5)	
CHO 2a	31 (n=1)	
敲除1a	35.9 ± 2.1 (n=5)	
敲除2a	20.1 ± 2.2 (n=2)	
神经元(-60 mV)	82.9 ± 5.2 (n=8)	苄普地尔
神经元(-60 mV)	100 ± 11 (n=10)	KB-R7943
神经元(-60 mV)	24.3 ± 17.2 (n=2)	5-(N-甲基-N-异丁基)阿米洛利
神经元(-60 mV)	15.0 ± 11.7 (n=3)	5-(N,N-六亚甲基)阿米洛利
神经元(-60 mV)	14.8 ± 7.1 (n=2)	5-(N,N-二甲基)阿米洛利盐酸盐

[0185] 实施例7:阿米洛利和阿米洛利类似物的侧脑室注射降低了小鼠的梗死体积

[0186] 如上所述,使小鼠接受60分钟的阻塞大脑中动脉(MCAO)。MCAO后一小时通过侧脑室注射施用阿米洛利或阿米洛利类似物苯扎米尔、苄普地尔(bepridel)、EIPA或KB-R7943。局部缺血引发一天后评价动物。如图18所示,阿米洛利或阿米洛利类似物苯扎米尔、苄普地尔、EIPA或KB-R7943的侧脑室注射有效减少了梗死体积。

[0187] 实施例8:阿米洛利的静脉注射降低了小鼠的梗死体积

[0188] 如上所述,使小鼠接受60分钟的阻塞大脑中动脉(MCAO)。MCAO后1、3或5小时通过静脉注射施用阿米洛利。局部缺血引发一天后评价动物。如图19所示,阿米洛利的静脉注射有效减少了梗死体积。阿米洛利的有效的CNS渗透可通过脑局部缺血/再灌注后危及了血脑

障壁的事实来解释。图20显示了阿米洛利的静脉注射具有5h的长效治疗窗。

[0189] 实施例9:疏水性的阿米洛利类似物对各种通道的结构活性关系

[0190] 如表1所示,用烷基取代阿米洛利的C-5氨基导致了对ASIC1a通道的效力的减小。该取代提高了对ASIC3通道的效力(Kuduk et al.,Bioorg.Med.Chem.Lett.,2009,19:2514-2518)。当将疏水性基团取代到结构的胍基部分上时,会获得相反的结果。实际上,苄基取代的胍基类似物(苯扎米尔)为测得的最强效的ASIC1a阻滞化合物(IC50=4.9μM))。总之,这些结果显示:阿米洛利为ASIC1a通道的有效抑制剂,具有7.7μM的IC_{50}。它们还提供了结构活性关系(参见图21)以用于设计可抑制ASIC1a通道的阿米洛利类似物。因此,在一些实施方式中,通过在阿米洛利结构的胍部分引入变化来产生阿米洛利类似物。由于阿米洛利仅是Na^+/Ca^{2+}离子交换器的非常弱的抑制剂(IC_{50}=1.1mM)。所述阿米洛利类似物也很可能是Na^+/Ca^{2+}离子交换器的非常弱的抑制剂。在一些实施方式中,所述阿米洛利类似物被设计为具有比ASIC3通道更高的针对ASIC1a的选择性。在另外的实施方式中,在阿米洛利结构中引入了环结构(如环胍基团)以提高ASIC1a电流的抑制效力。阿米洛利的一个或多个N-H基团也很可能会形成与3-氨基的内部的或与离子通道的H键。

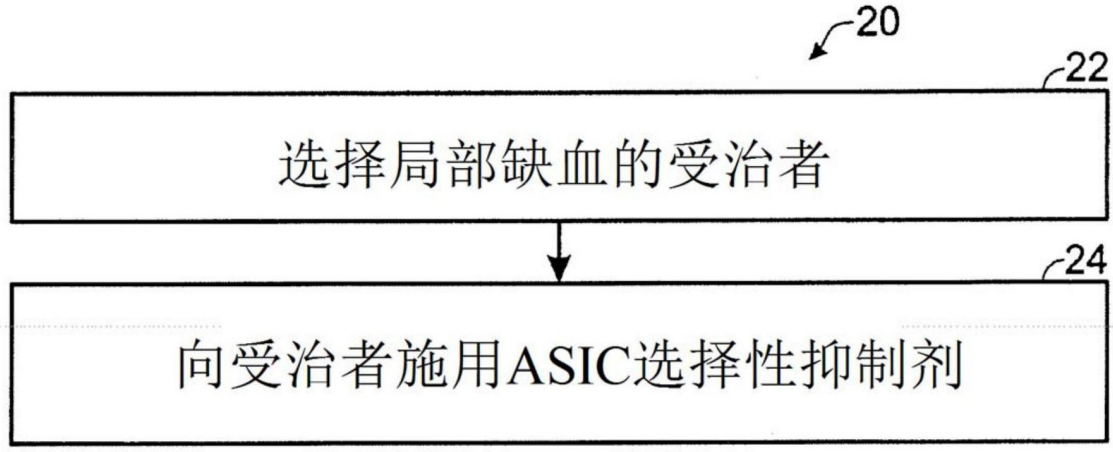
[0191] 小鼠的体内结果显示:在32.5μM的血浆浓度(50μL×1mM的静脉剂量)和12.5μM的总的脑浓度(1μL×500μM的侧脑室剂量)下可实现功效。由此可估计:为实现适于人类中风的急性治疗的有效浓度,仅需要在效力上增长10倍。因此,筛选新颖的类似物以使ASIC1a IC50的效力由对于阿米洛利和苯扎米尔来说的4至8μM提高至<1μM。

[0192] 在一些实施方式中,所述阿米洛利类似物包括苯扎米尔的甲基化类似物(图21的式1-5)和苯扎米尔的脒基类似物(图21的式6)。在另外的实施方式中,所述阿米洛利类似物包含形成于胍基团上的环。在另外的实施方式中,所述阿米洛利类似物包含用于提高ASIC1a电流的抑制效力的酰胍基。

[0193] 阿米洛利以1mM是可溶于水的,且在50μL每针的剂量下在治疗小鼠模型的局部缺血中是有效的。在65kg人类上的相同剂量(以mg/kg为基准)可能会接近40mg,且需要超过160mL的注射体积。类似地,已有报导苯扎米尔具有在0.9%生理盐水中的0.4mg/mL(1.7mM)的溶解度,这允许在10mL注射液中仅5mg苯扎米尔二盐酸盐的给药。因此,需要具有更高的水溶性的阿米洛利类似物。在一些实施方式中,所述阿米洛利类似物在胍基上包含水增溶性基团,如N,N-二甲基氨基或糖基,以改善水溶性。在一些实施方式中,所述阿米洛利类似物具有5mM、10mM、20mM、30mM、40mM、50mM、60mM、70mM、80mM、90mM、100mM或更高的水溶性。在另外的实施方式中,所述阿米洛利类似物具有允许在单次10mL注射液中将10mg、25mg、50mg、100mg、150mg、200mg、250mg、300mg、400mg或500mg的剂量通过静脉施用至人体的溶解度。在更另外的实施方式中,所述阿米洛利类似物具有允许在单次2mL注射液中将10mg、25mg、50mg、100mg、150mg、200mg、250mg、300mg、400mg或500mg的剂量通过侧脑室施用至人体的溶解度。

[0194] 以上所述的本公开可包括一个或多个具有独立用途的不同的发明。各个发明已以其优选的方式被公开。这些优选的方式,包括本文中公开和阐释的具体实施方式,不应以限制性的观念去理解,因为还可以有许多的变体。本发明的主题包括本文所公开的各种元素、特征、功能和/或性能的全部的新颖的和非显而易见的组合和次组合。以下的权利要求特别指出了认为是新颖性和非显而易见性的组合和次组合。在元素、特征、功能和/或性能的其

它组合和次组合中体现的发明可在要求本申请或相关申请的优先权的申请中声明。这些权利要求，无论是否指向不同的发明或相同的发明，以及无论是否比原始权利要求的保护范围更宽、更窄、等同或不同，均应被视为包含于本公开的发明的主题之内。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选择局部缺血的受治者
24
向受治者施用ASIC选择性抑制剂

图1

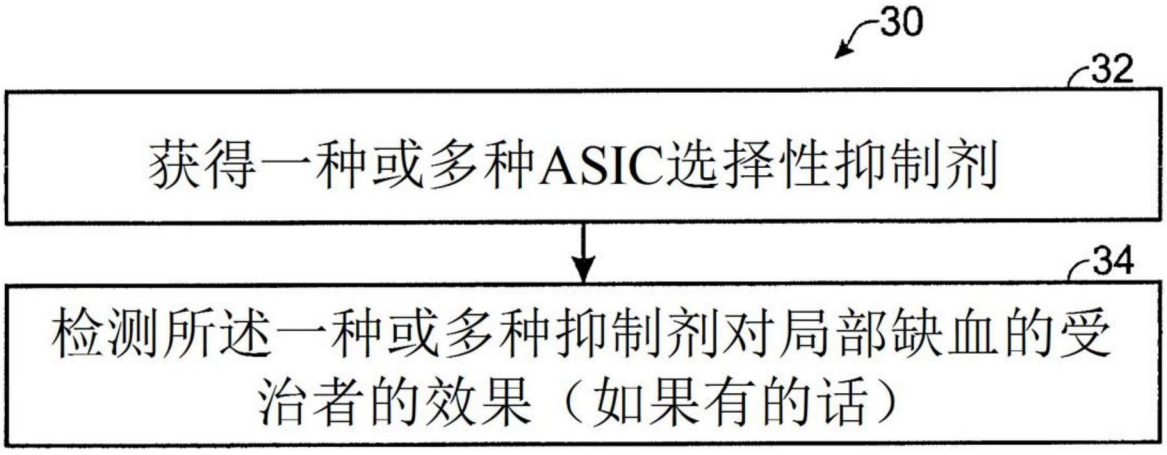

30
32
获得一种或多种ASIC选择性抑制剂
34
检测所述一种或多种抑制剂对局部缺血的受治者的效果（如果有的话）

图2

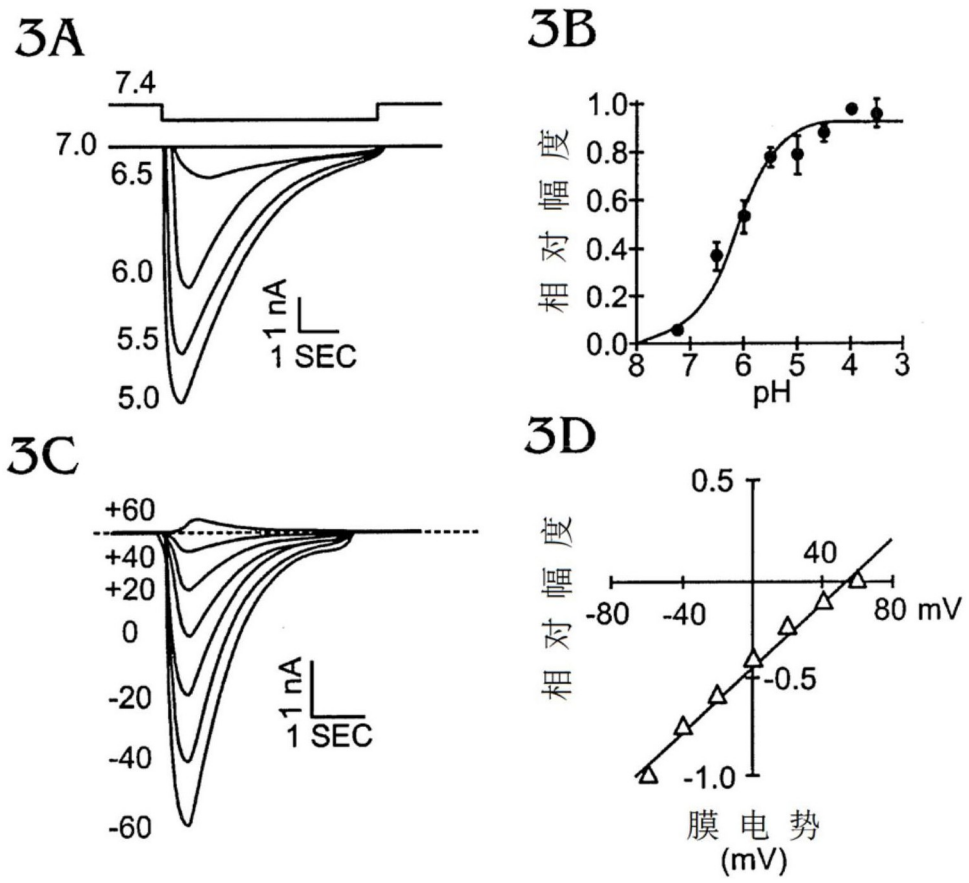

3A
7.4
7.0
6.5
6.0
5.5
5.0
1 nA
1 SEC
3B
相对幅度
1.0
0.8
0.6
0.4
0.2
0.0
8 7 6 5 4 3
pH
3C
+60
+40
+20
0
-20
-40
-60
1 nA
1 SEC
3D
相对幅度
0.5
40
-80 -40
80 mV
-0.5
-1.0
膜电势
(mV)

图3

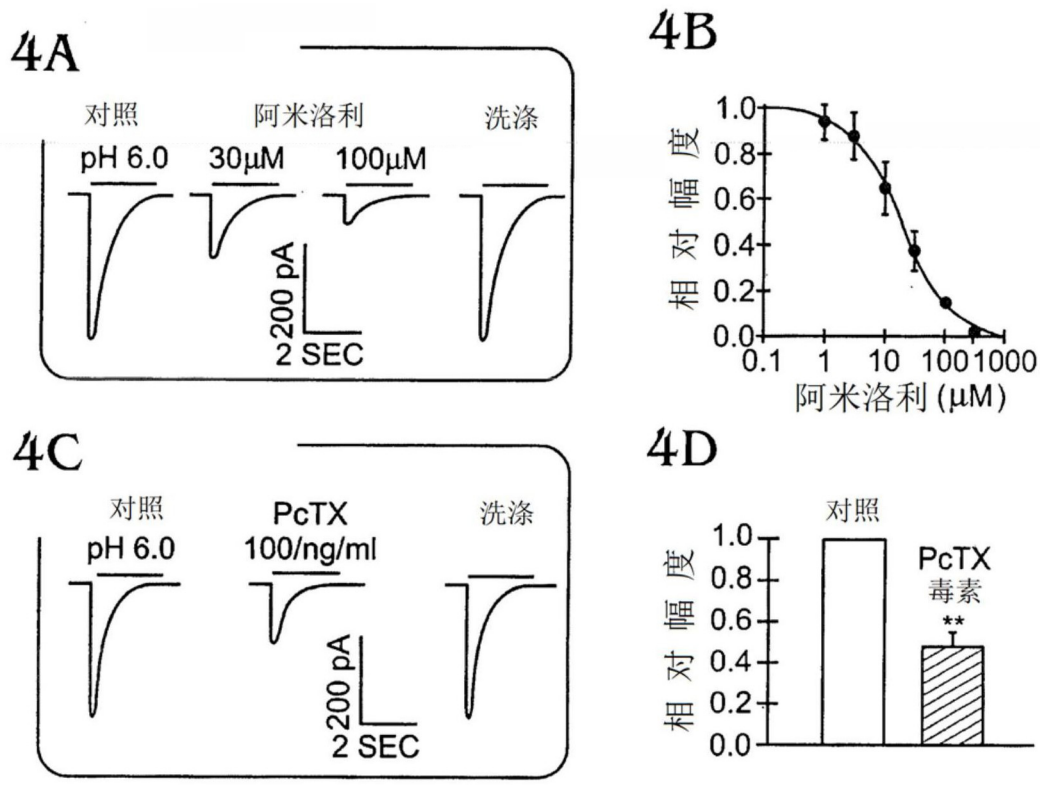

4A
对照
阿米洛利
洗涤
pH 6.0
30μM
100μM
200 pA
2 SEC
4B
相对幅度
1.0
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
1000
阿米洛利 (μM)
4C
对照
PcTX
100/ng/ml
洗涤
pH 6.0
200 pA
2 SEC
4D
对照
PcTX
毒素
**
相对幅度
1.0
0.8
0.6
0.4
0.2
0.0

图4

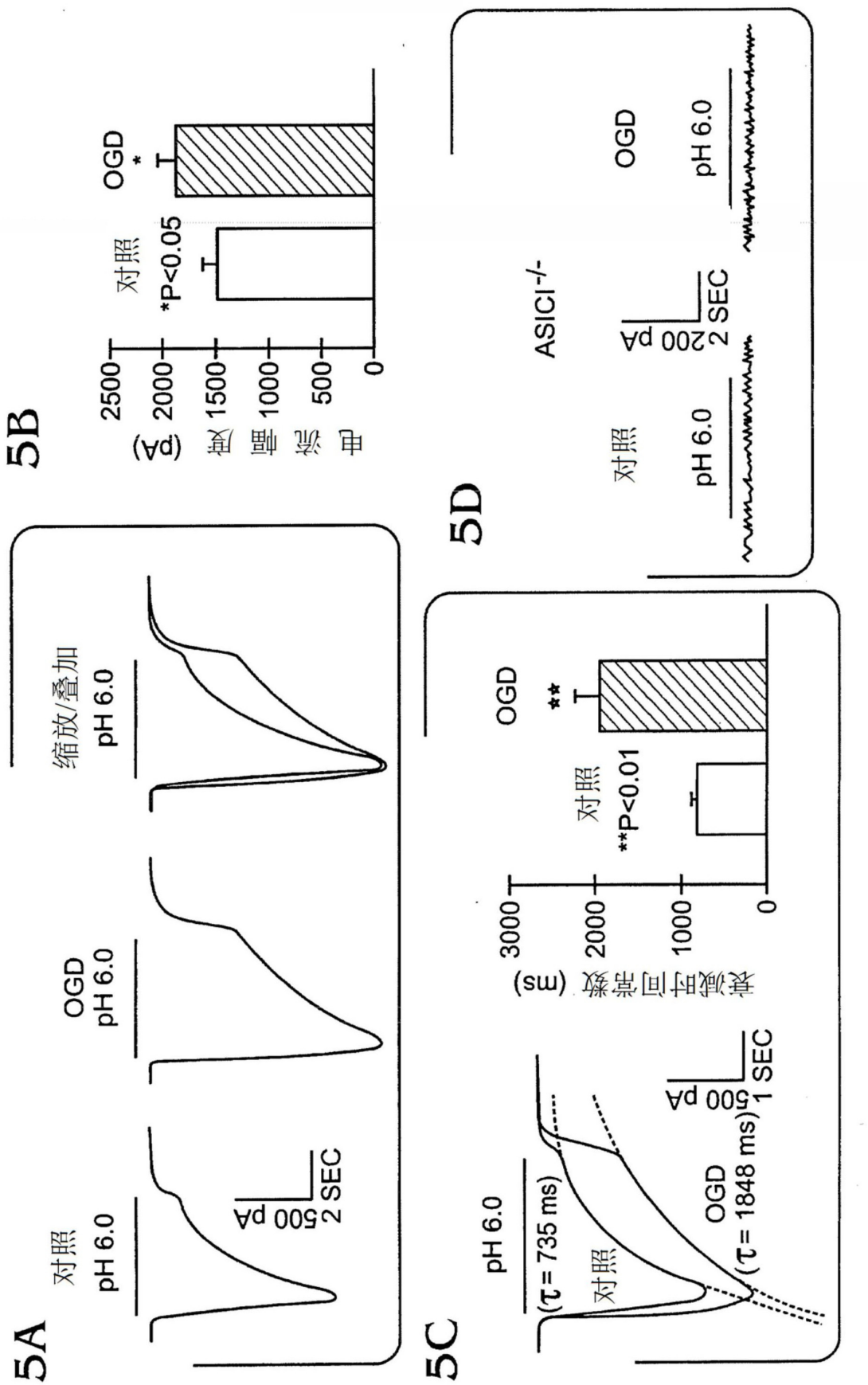

5A
对照
pH 6.0
OGD
pH 6.0
缩放/叠加
pH 6.0
500 pA
2 SEC
5B
电流幅度 (pA)
2500
2000
1500
1000
500
0
对照
*P<0.05
OGD
*
5C
pH 6.0
(τ= 735 ms)
对照
OGD
(τ= 1848 ms)
500 pA
1 SEC
衰减时间常数 (ms)
3000
2000
1000
0
对照
**P<0.01
OGD
**
5D
ASIC1-/-
对照
pH 6.0
200 pA
2 SEC
OGD
pH 6.0

图5

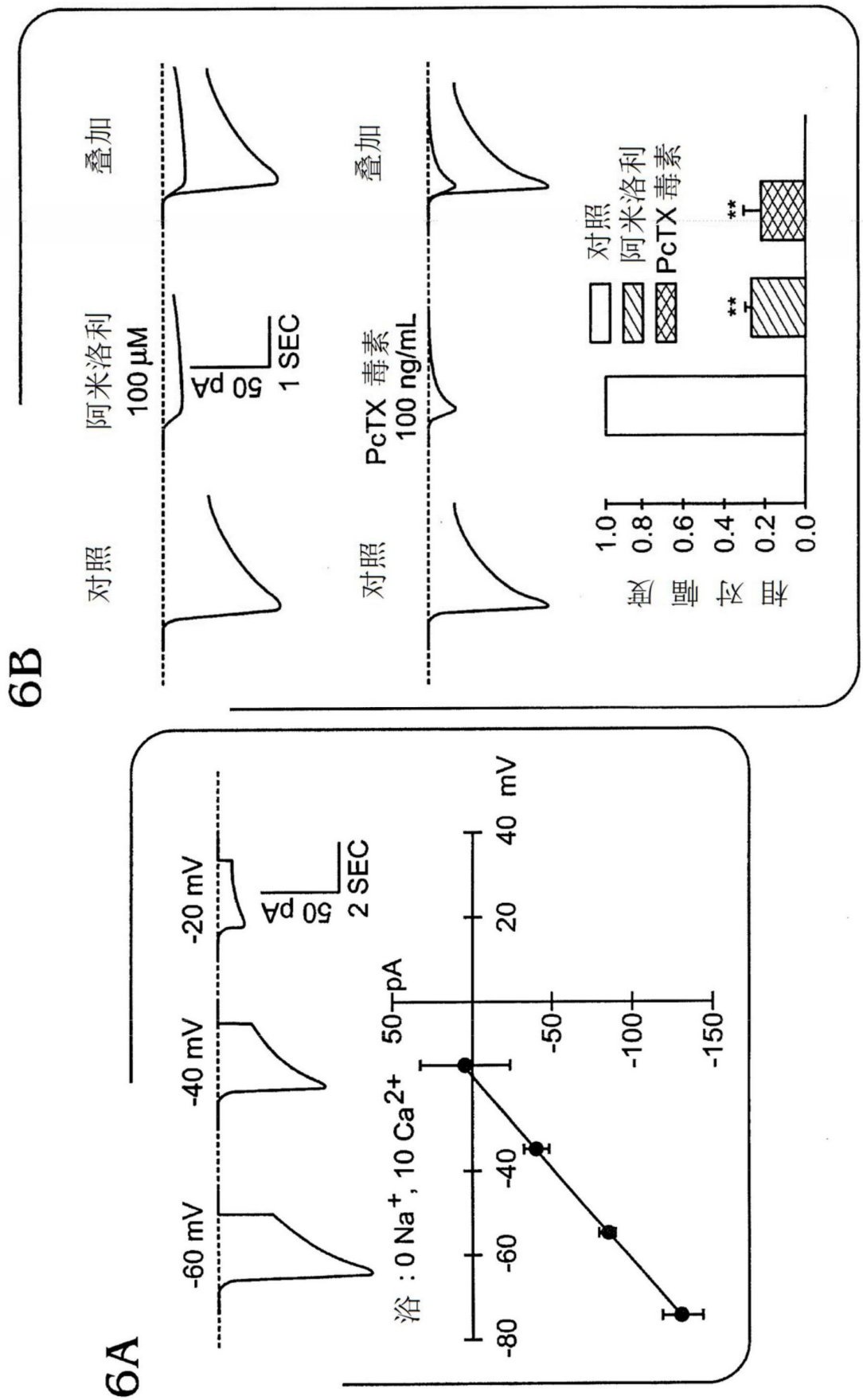

6A
-60 mV
-40 mV
-20 mV
50 pA
2 SEC
浴 : 0 Na+, 10 Ca2+
50 pA
-80
-60
-40
-50
-100
-150
20
40 mV
6B
对照
阿米洛利
100 μM
叠加
50 pA
1 SEC
对照
PcTX 毒素
100 ng/mL
叠加
对照
阿米洛利
PcTX 毒素
**
**
相对幅度
1.0
0.8
0.6
0.4
0.2
0.0

图6

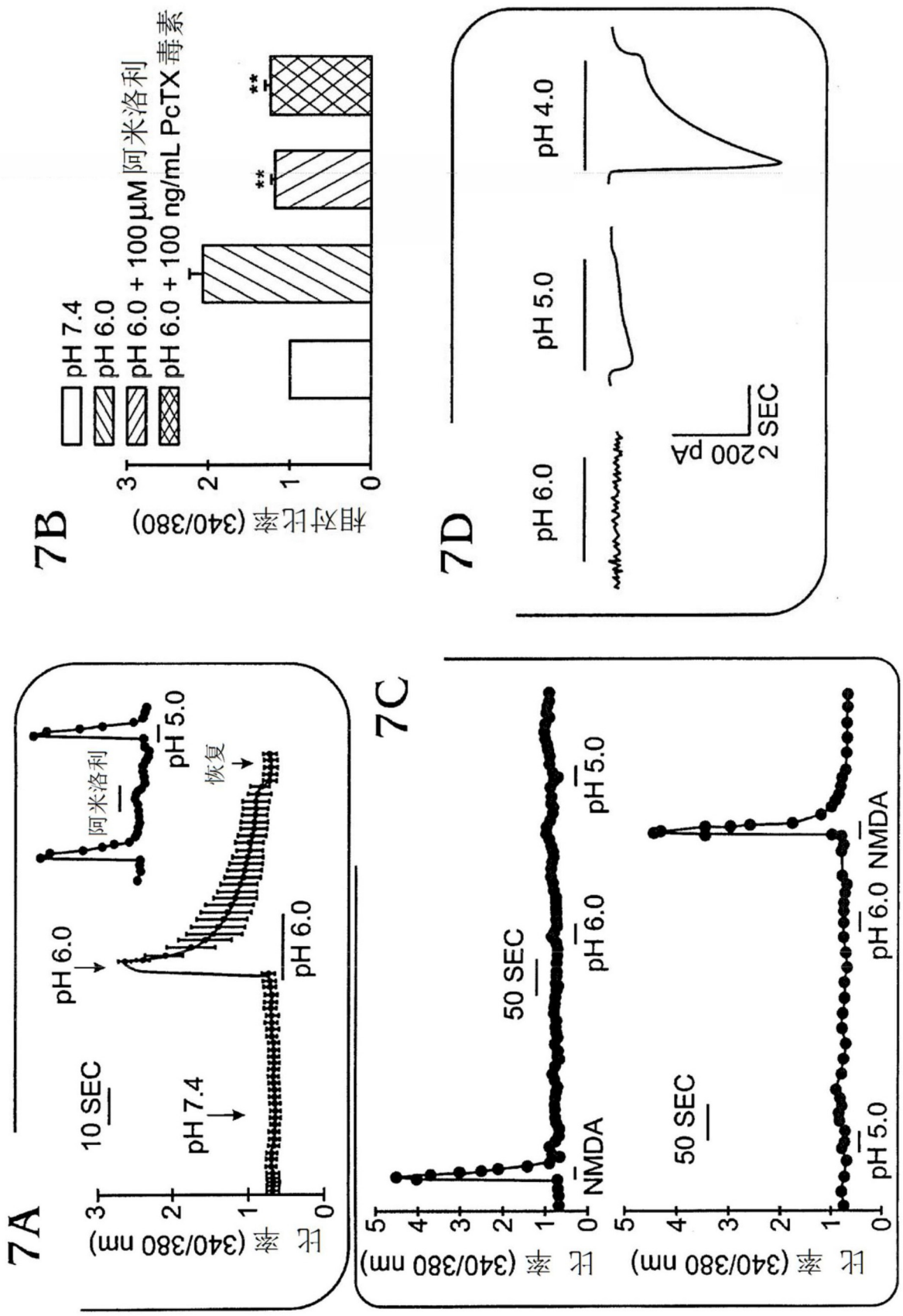

7A
比 率 (340/380 nm)
10 SEC
pH 7.4
pH 6.0
阿米洛利
pH 5.0
恢复
pH 6.0
7B
相对比率 (340/380)
pH 7.4
pH 6.0
pH 6.0 + 100 μM 阿米洛利
pH 6.0 + 100 ng/mL PcTX 毒素
**
**
7C
比 率 (340/380 nm)
50 SEC
NMDA
pH 6.0
pH 5.0
50 SEC
pH 5.0
pH 6.0 NMDA
7D
pH 6.0
pH 5.0
pH 4.0
200 pA
2 SEC

图7

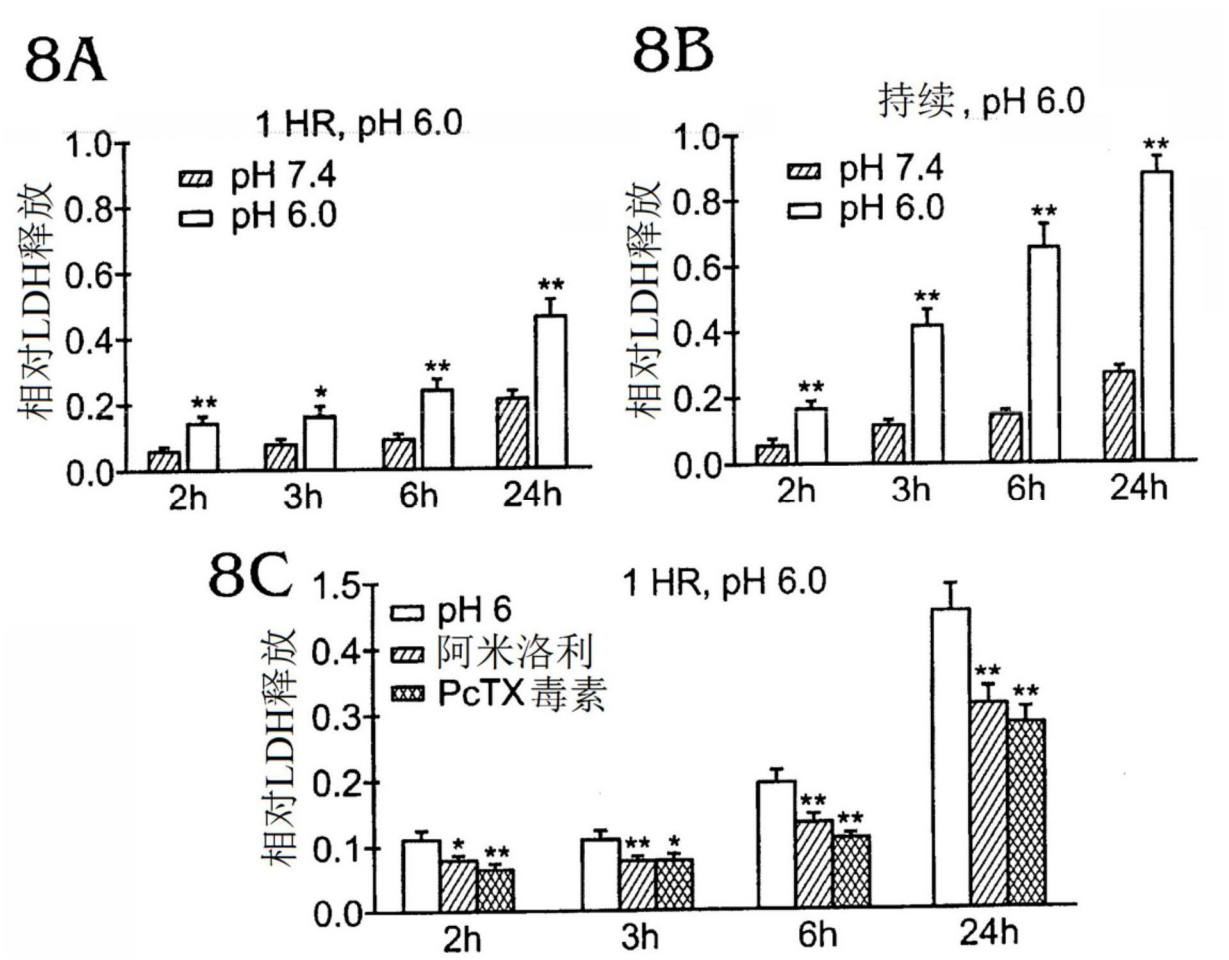

8A
1 HR, pH 6.0
pH 7.4
pH 6.0
相对LDH释放
1.0
0.8
0.6
0.4
0.2
0.0
2h
3h
6h
24h
8B
持续, pH 6.0
pH 7.4
pH 6.0
相对LDH释放
2h
3h
6h
24h
8C
1 HR, pH 6.0
pH 6
阿米洛利
PcTX毒素
相对LDH释放
1.5
0.4
0.3
0.2
0.1
0.0
2h
3h
6h
24h

图8

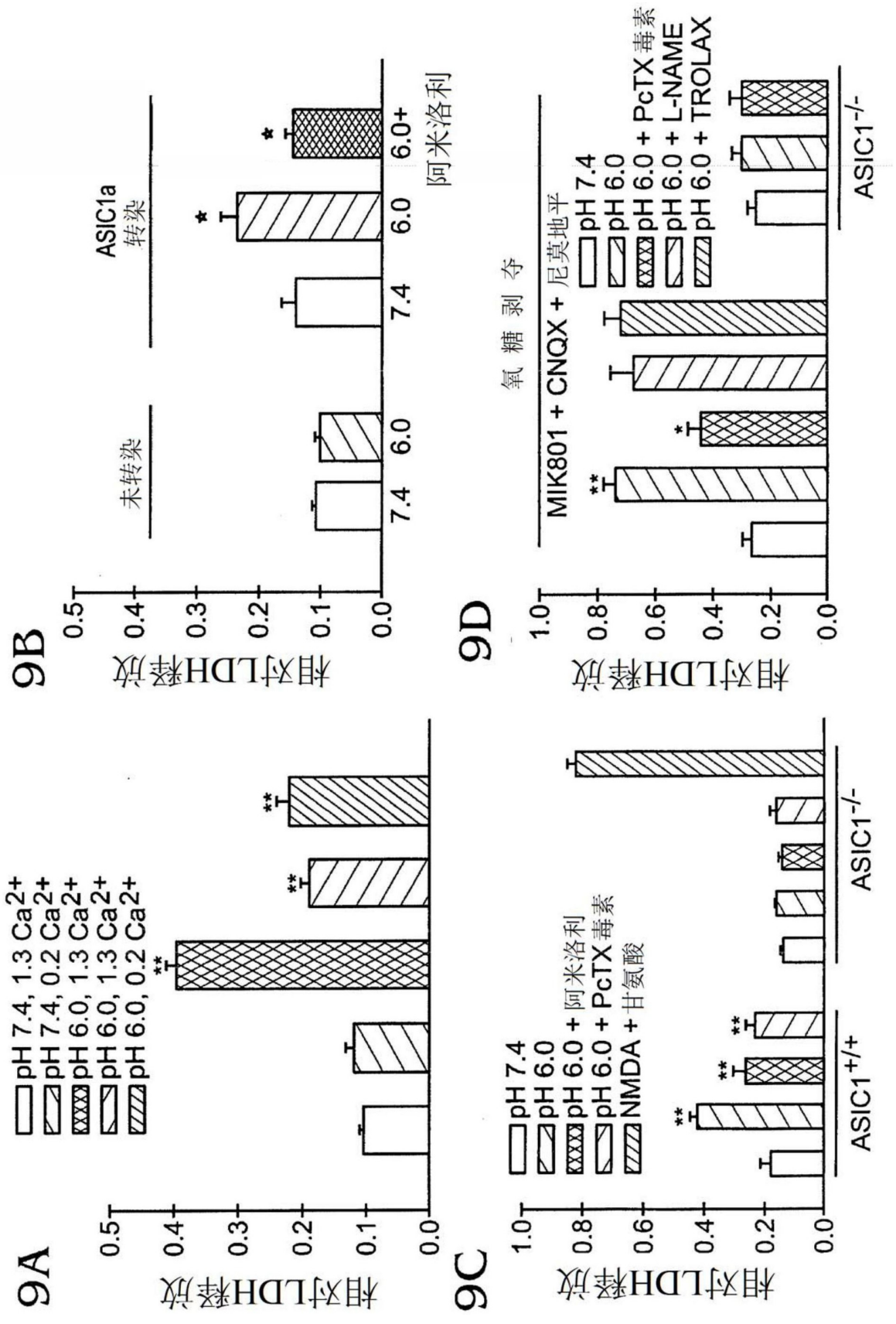

9A
相对LDH释放
pH 7.4, 1.3 Ca2+
pH 7.4, 0.2 Ca2+
pH 6.0, 1.3 Ca2+
pH 6.0, 1.3 Ca2+
pH 6.0, 0.2 Ca2+
9B
相对LDH释放
未转染
ASIC1a 转染
7.4 6.0 7.4 6.0 6.0+
阿米洛利
9C
相对LDH释放
pH 7.4
pH 6.0
pH 6.0 + 阿米洛利
pH 6.0 + PcTX 毒素
NMDA + 甘氨酸
ASIC1+/+
ASIC1-/-
9D
相对LDH释放
氧 糖 剥 夺
MIK801 + CNQX + 尼莫地平
pH 7.4
pH 6.0
pH 6.0 + PcTX 毒素
pH 6.0 + L-NAME
pH 6.0 + TROLAX
ASIC1-/-

图9

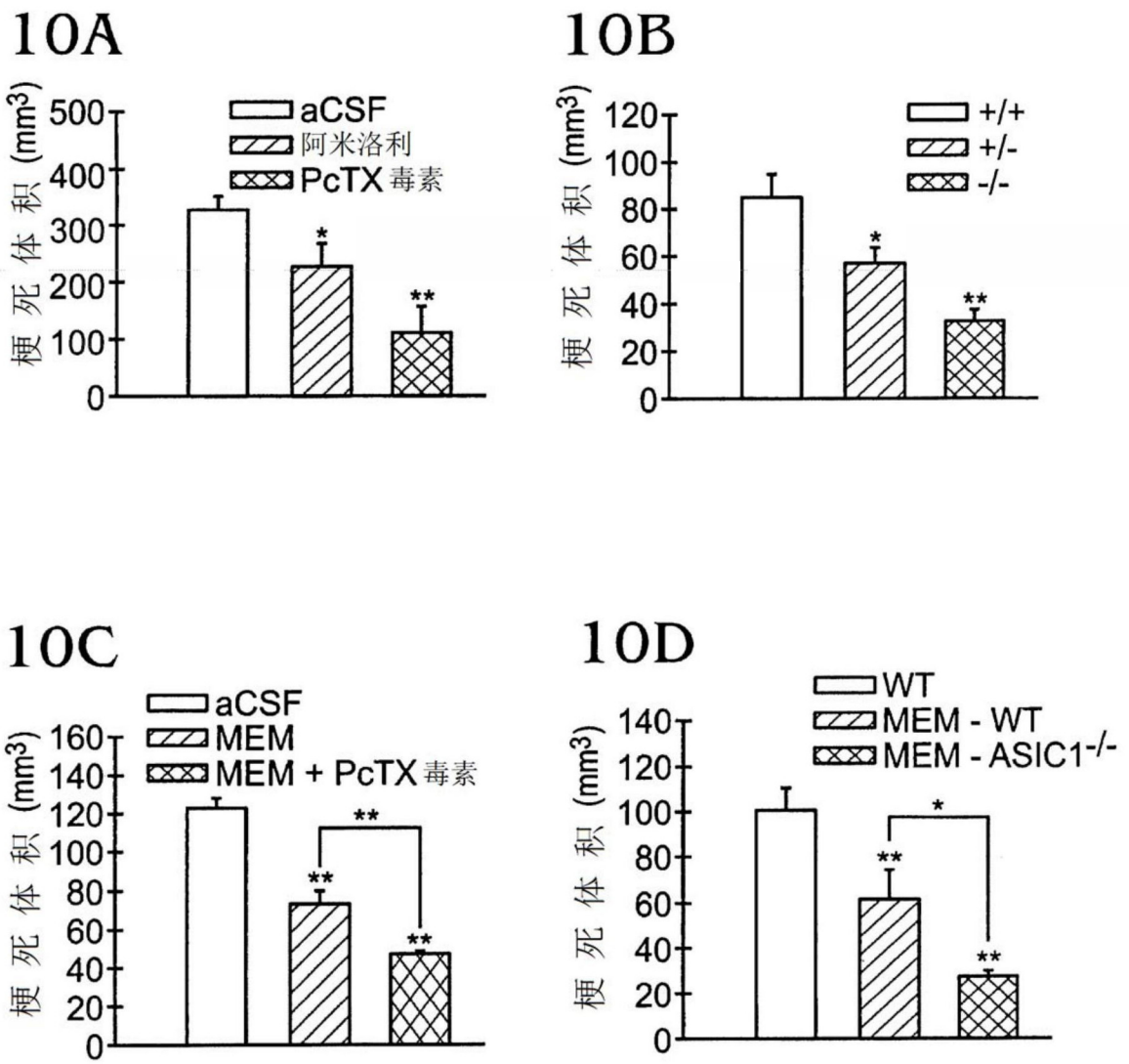

10A
梗死体积 (mm3)
500
400
300
200
100
0
aCSF
阿米洛利
PcTX 毒素
*
**
10B
梗死体积 (mm3)
120
100
80
60
40
20
0
+/+
+/-
-/-
*
**
10C
梗死体积 (mm3)
160
140
120
100
80
60
40
20
0
aCSF
MEM
MEM + PcTX 毒素
**
**
**
10D
梗死体积 (mm3)
140
120
100
80
60
40
20
0
WT
MEM - WT
MEM - ASIC1-/-
*
**
**

图10

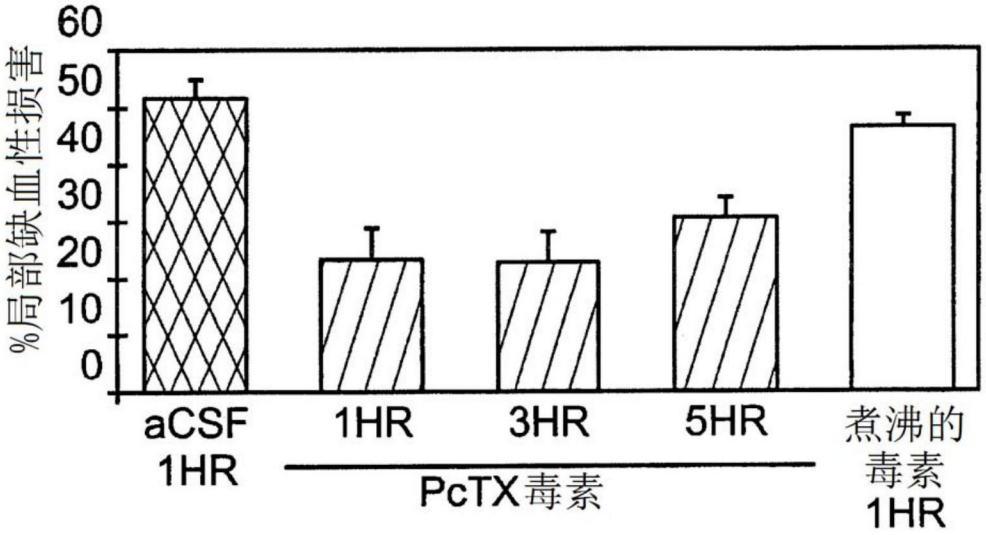

60
50
40
30
20
10
0
%局部缺血性损害
aCSF
1HR
1HR
3HR
5HR
PcTX毒素
煮沸的
毒素
1HR

图11

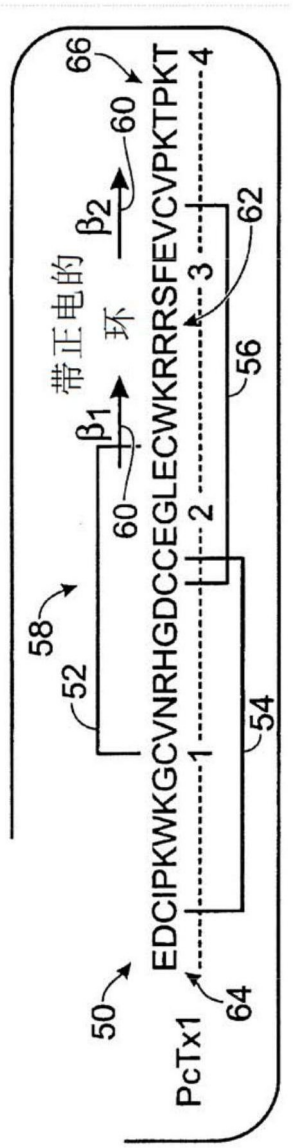

50
PcTx1
64
EDCIPKWKGCVNRHGDCCEGLECWKRRRSFEVCVPKTPKT
1
2
3
4
52
54
56
58
60
62
66
β1
β2
带正电的
环

图12

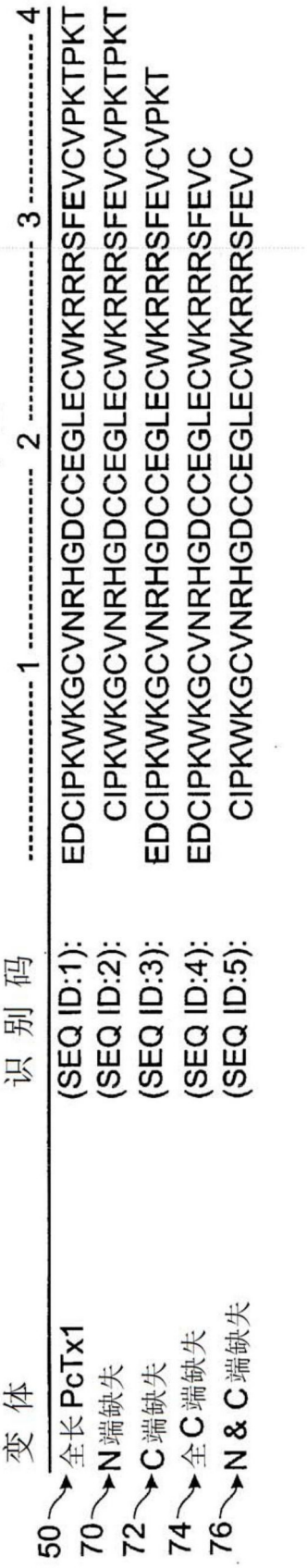

位置
1
2
3
4
识 别 码
变 体
50 全长 PcTx1 (SEQ ID:1): EDCIPKWKGCVNRHGDCCEGLECWKRRRSFEVCVPKTPKT
70 N 端缺失 (SEQ ID:2): CIPKWKGCVNRHGDCCEGLECWKRRRSFEVCVPKTPKT
72 C 端缺失 (SEQ ID:3): EDCIPKWKGCVNRHGDCCEGLECWKRRRSFEVCVPKT
74 全 C 端缺失 (SEQ ID:4): EDCIPKWKGCVNRHGDCCEGLECWKRRRSFEVC
76 N & C 端缺失 (SEQ ID:5): CIPKWKGCVNRHGDCCEGLECWKRRRSFEVC

图13

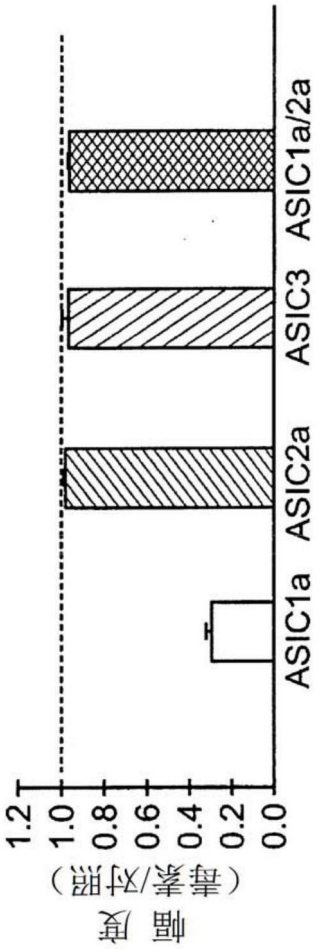

幅 度
(毒素/对照)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ASIC1a
ASIC2a
ASIC3
ASIC1a/2a

图14

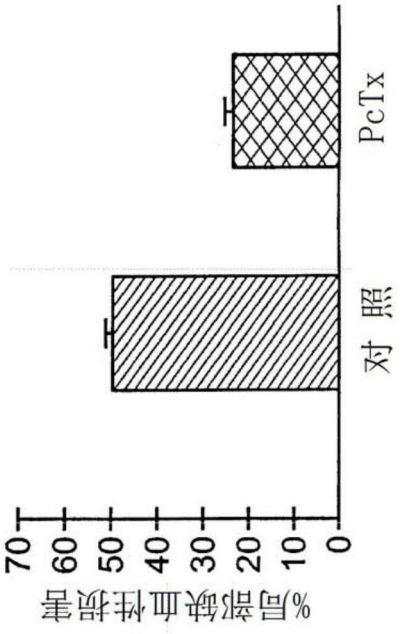


图15

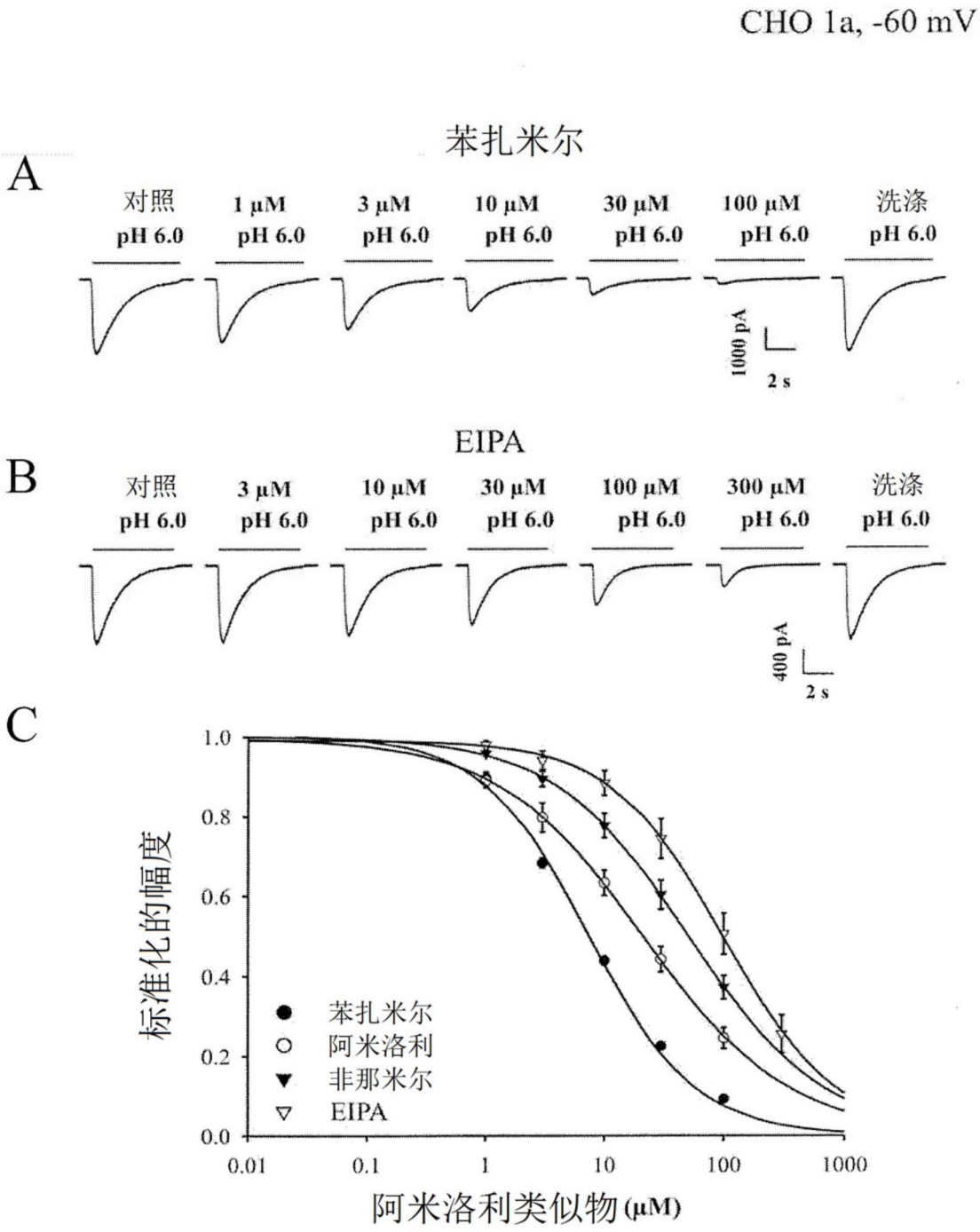

CHO 1a, -60 mV
A
苯扎米尔
对照
pH 6.0
1 μM
pH 6.0
3 μM
pH 6.0
10 μM
pH 6.0
30 μM
pH 6.0
100 μM
pH 6.0
洗涤
pH 6.0
1000 pA
2 s
B
EIPA
对照
pH 6.0
3 μM
pH 6.0
10 μM
pH 6.0
30 μM
pH 6.0
100 μM
pH 6.0
300 μM
pH 6.0
洗涤
pH 6.0
400 pA
2 s
C
标准化的幅度
1.0
0.8
0.6
0.4
0.2
0.0
苯扎米尔
阿米洛利
非那米尔
EIPA
0.01
0.1
1
10
100
1000
阿米洛利类似物 (μM)

图16

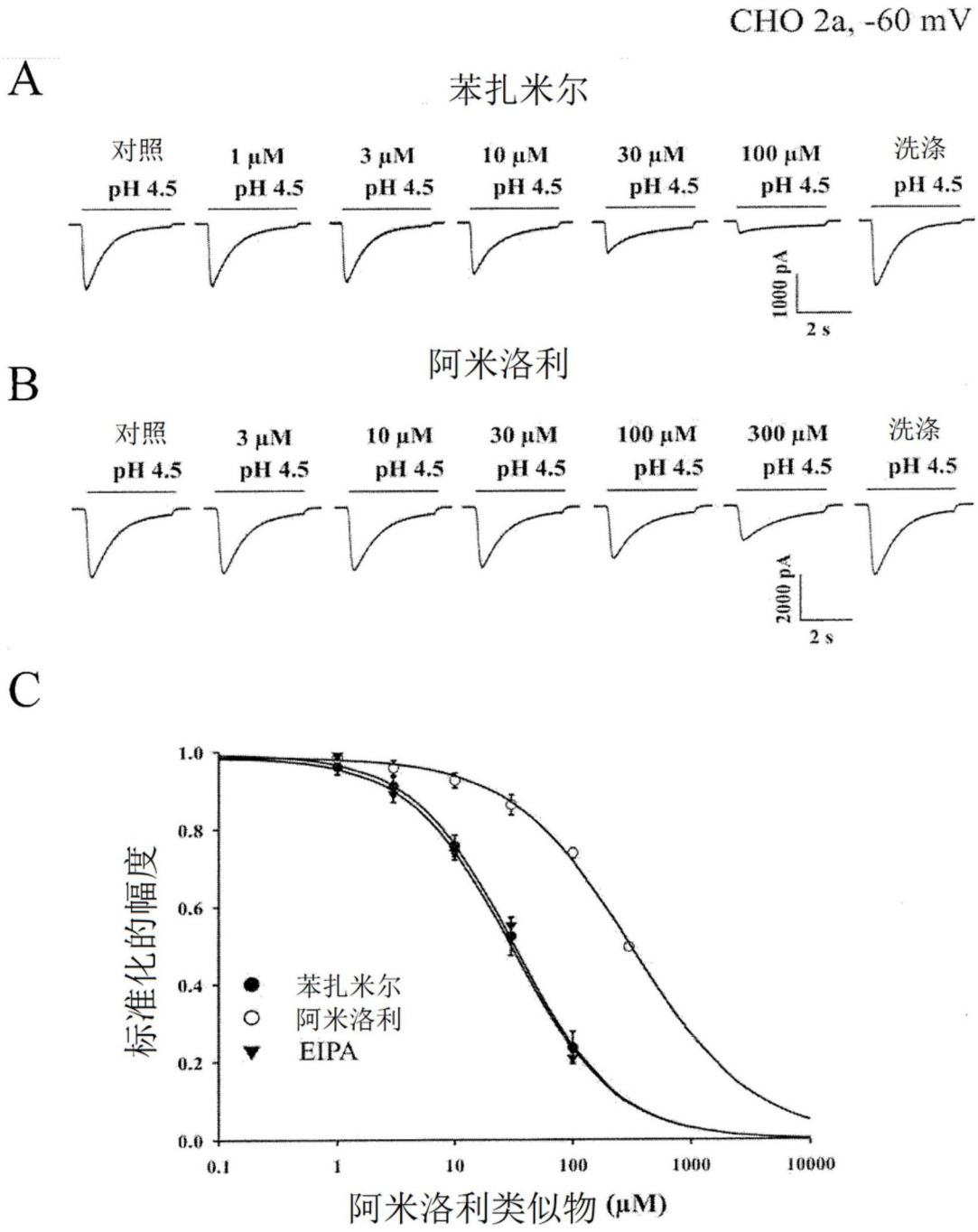

CHO 2a, -60 mV
A
苯扎米尔
对照
pH 4.5
1 μM
pH 4.5
3 μM
pH 4.5
10 μM
pH 4.5
30 μM
pH 4.5
100 μM
pH 4.5
洗涤
pH 4.5
1000 pA
2 s
B
阿米洛利
对照
pH 4.5
3 μM
pH 4.5
10 μM
pH 4.5
30 μM
pH 4.5
100 μM
pH 4.5
300 μM
pH 4.5
洗涤
pH 4.5
2000 pA
2 s
C
标准化的幅度
1.0
0.8
0.6
0.4
0.2
0.0
苯扎米尔
阿米洛利
EIPA
0.1
1
10
100
1000
10000
阿米洛利类似物 (μM)

图17

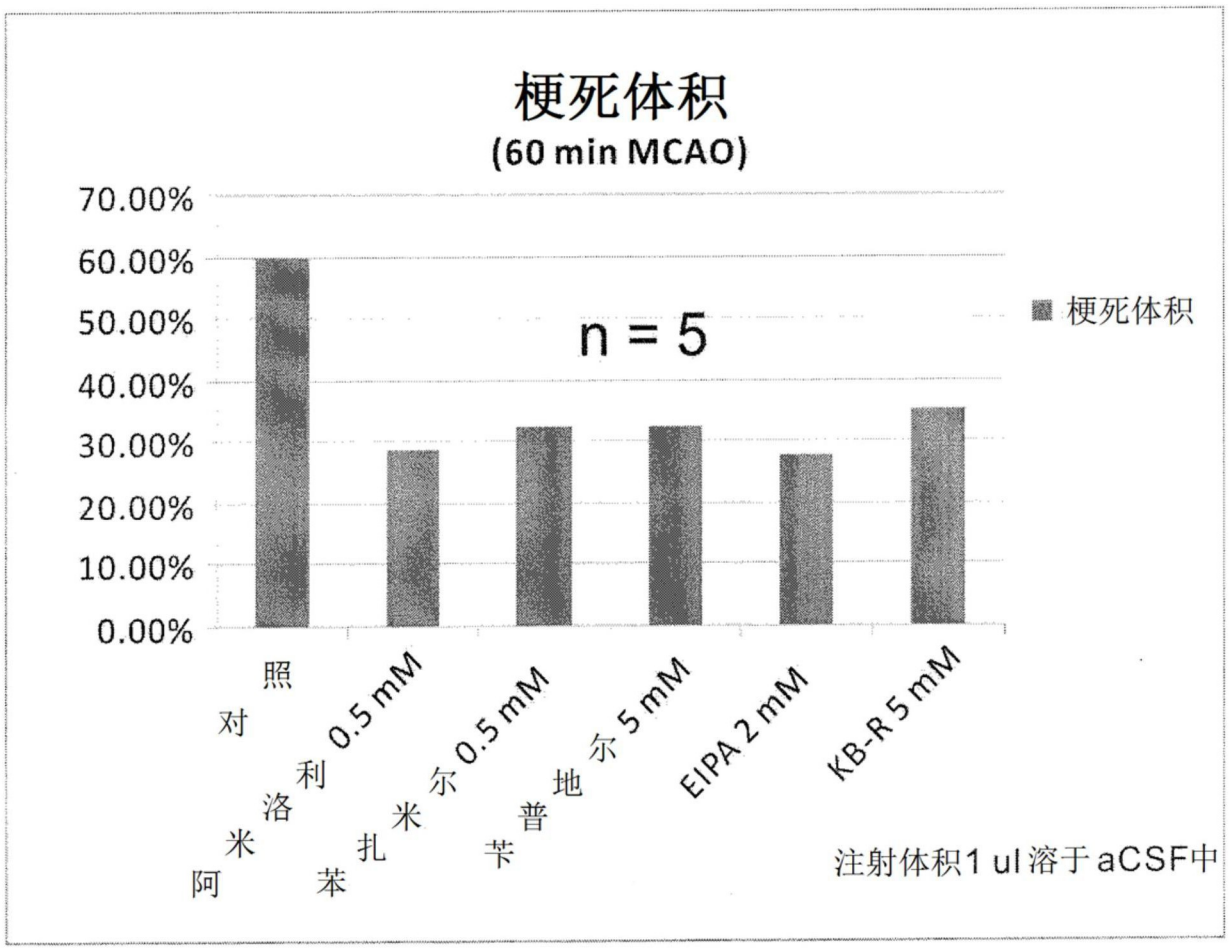

梗死体积
(60 min MCAO)
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
n = 5
梗死体积
照对
阿米洛利 0.5 mM
苯扎米尔 0.5 mM
普地尔 5 mM
EIPA 2 mM
KB-R 5 mM
注射体积1 ul 溶于 aCSF中

图18

生理盐水 50 ul 阿米洛利 1 mM x 50 ul

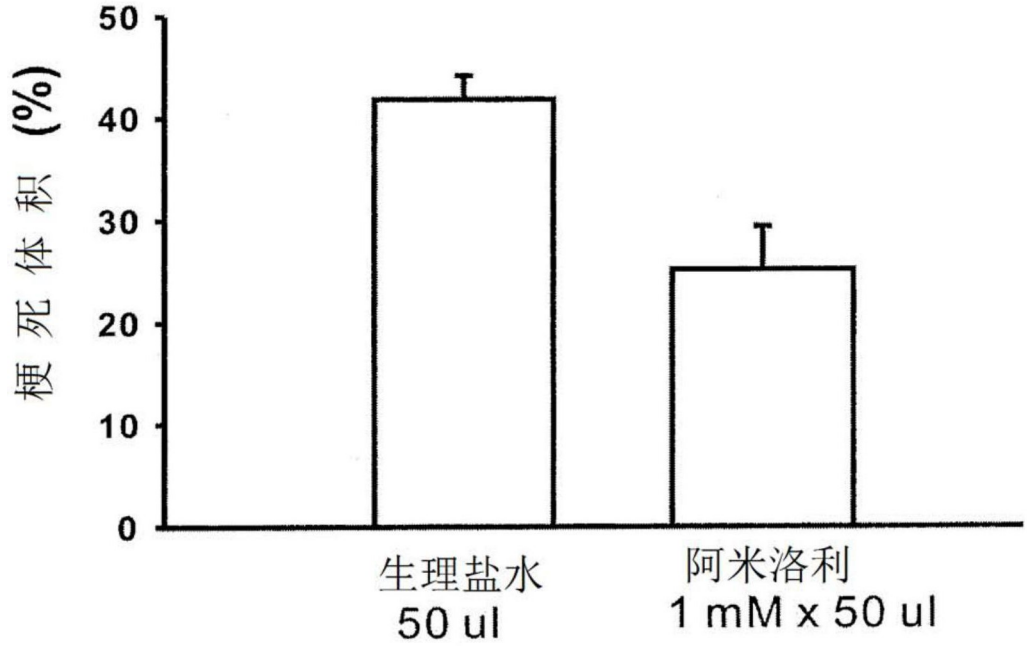
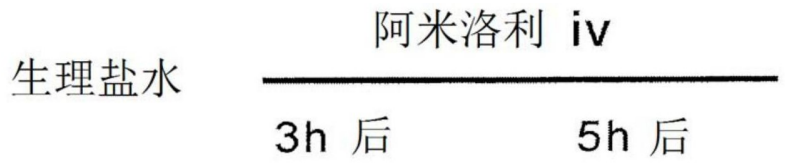


图19


阿米洛利 iv
生理盐水
3h 后
5h 后

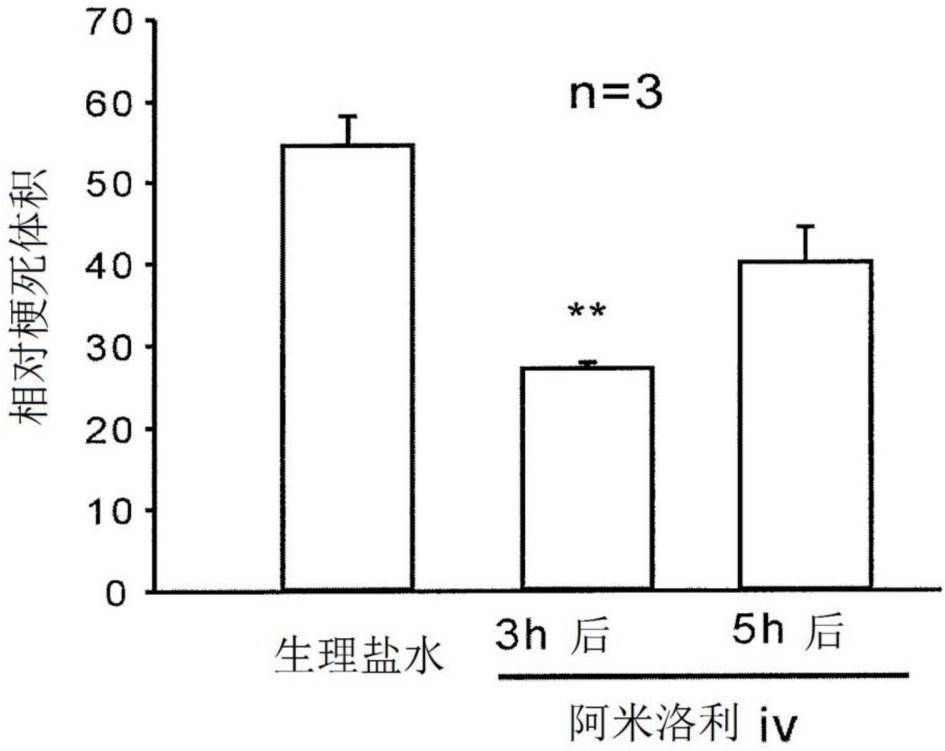

70
60
50
40
30
20
10
0
相对梗死体积
n=3
**
生理盐水
3h 后
5h 后
阿米洛利 iv

图20

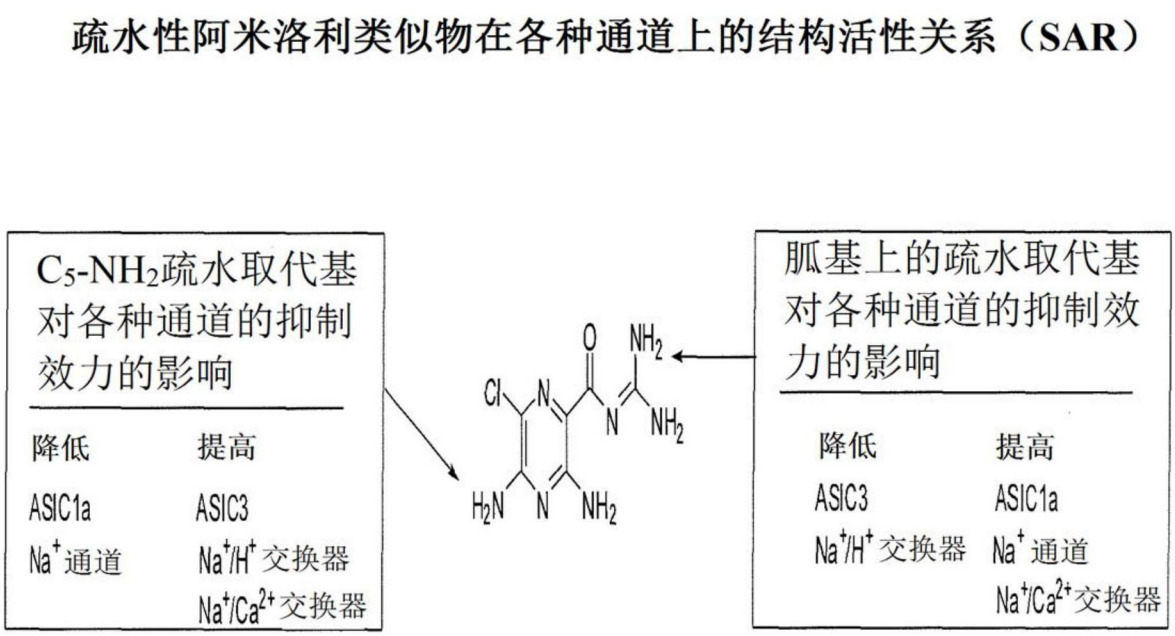

疏水性阿米洛利类似物在各种通道上的结构活性关系（SAR）
C5-NH2疏水取代基对各种通道的抑制效力的影响
降低
提高
ASIC1a
ASIC3
Na+通道
Na+/H+交换器
Na+/Ca2+交换器
胍基上的疏水取代基对各种通道的抑制效力的影响
降低
提高
ASIC3
ASIC1a
Na+/H+交换器
Na+通道
Na+/Ca2+交换器
Cl
O
NH2
N
H2N
NH2

图21