



(43) International Publication Date
15 January 2015 (15.01.2015)

(10) International Publication Number
WO 2015/006324 A2

- (51) International Patent Classification:
A61K 38/17 (2006.01)
- (21) International Application Number:
PCT/US2014/045748
- (22) International Filing Date:
8 July 2014 (08.07.2014)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
61/843,702 8 July 2013 (08.07.2013) US
61/902,935 12 November 2013 (12.11.2013) US
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- (81) Designated States (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,

BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,
ZW.

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

Published:

- *without international search report and to be republished
upon receipt of that report (Rule 48.2(g))*
- *with sequence listing part of description (Rule 5.2(a))*

(54) Title: COMPOSITIONS AND METHODS FOR TREATING NEUROPSYCHIATRIC DISORDERS USING AN ENDOTH-
ELIN-B RECEPTOR AGONIST

(57) Abstract: The present invention relates to compositions and methods for treating neuropsychiatric disorders in vertebrates and humans. More specifically, the present invention provides for use of an endothelin-B receptor agonist as a neuroprotective and a neuroregenerative agent.



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COMPOSITIONS AND METHODS FOR TREATING NEUROPSYCHIATRIC DISORDERS USING AN ENDOTHELIN-B RECEPTOR AGONIST

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit under 35 U.S.C. § 119(e) of U.S. Provisional Patent Application Number 61/843,702, filed July 8, 2013, and U.S. Provisional Patent Application Number 61/902,935, filed November 12, 2013, the disclosures of which are incorporated herein by reference in their entirety.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0002] This application contains, as a separate part of disclosure, a Sequence Listing in computer-readable form (filename: 48812_SeqListing.txt; created July 8, 2014, 659 bytes - ASCII text file) which is incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present invention relates to compositions and methods for treating neuropsychiatric disorders in vertebrates and humans. More specifically, the present invention provides for use of an endothelin-B receptor agonist as a neuroprotective and a neuroregenerative agent.

BACKGROUND OF THE INVENTION

[0004] Endothelin (ET) is an endogenous peptide which has been implicated in numerous physiological and pathological phenomena within the body. Acting upon two distinct receptors, ET_A and ET_B, ET influences a range of processes from regulation of blood pressure to neurotransmitters and hormones (Kojima et al., 1992; Levin, 1995; Schiffrin et al., 1997; Schneider et al., 2007). Although most widely studied for their actions on cardiovascular system, ET receptors are widespread throughout the body, including the brain. ET_B receptors, specifically, are located in abundance on neurons and glial cells, as well as endothelial lining of the cerebral vasculature (Schinelli, 2006). The exact function of these receptors within the brain, particularly during its development, is not well understood.

Development of the central nervous system

[0005] A deficiency in ET_B receptors at birth has been shown to result in a decrease in neuronal progenitor cells and an increase in apoptosis within the postnatal dentate gyrus and

cerebellum of rats (Ehrenreich et al., 2000; Vidovic et al., 2008). Additionally, ET_B knockout model in rats leads to congenital aganglionosis within the gut and associated CNS disturbances (Dembowski et al., 2000). These ET_B knockout rats, which have a 4 week postnatal mortality, serve as models for human Hirschsprung disease. Previous studies have shown that brain ET_B receptor expression is particularly high immediately after birth, but drops down to lower levels by postnatal day 21 (Briyal et al., 2012b). The locations of these receptors and their correlation, or lack thereof, with CNS growth factors during these crucial stages of development remain to be determined.

[0006] While it is clear that ET_B receptors are needed for normal CNS development, it remains uncertain which cells or pathways they exert a protective or proliferative influence on. Previous studies have shown that selective stimulation of ET_B receptors produces neuroprotection against oxidative stress and a significant reduction in infarct volume in the brains of adult rats subjected to cerebral ischemia (Leonard et al., 2011; 2012). It was also found that protection and recovery from the ischemic condition was at least partially due to an increase in angiogenesis and neurogenesis within 7 days following infarct and treatment with ET_B receptor agonist, IRL-1620 (Leonard and Gulati, 2013). An increase in vascular and nerve growth factors within the brain of IRL-1620-treated infarcted animals coincided with an increase in the level of ET_B receptors.

[0007] Vascular endothelial growth factor (VEGF) is expressed normally in the cerebral microvessels as well as in the neuronal tissue of both neonates and adults (Hoehn et al., 2002). VEGF in the fetal human brain is located on neuroepithelial cells, neuroblasts, radial glial cells and endothelial cells, and its expression appears to be developmentally regulated and correlated with angiogenesis (Virgintino et al., 2003). While VEGF is well known to be necessary for blood vessel growth, recent research has indicated that it also plays a significant role in promoting neurogenesis, neuronal patterning, and neuronal migration (Rosenstein et al., 2010). It has been shown that there is a correlation between VEGF, neuronal growth factor (NGF) and ET_B receptors in the developing brain. ET_B receptors can be stimulated by administering ET_B receptor agonists such as IRL-1620 and growth of the CNS treat diseases can be promoted where CNS has been damaged or has not grown appropriately.

Neurodegenerative diseases

[0008] **Neurodegeneration** is a term for the progressive loss of structure or function of neurons, including death of neurons. Many neurodegenerative diseases

including Amyotrophic lateral sclerosis (ALS), Parkinson's, Alzheimer's, and Huntington's occur as a result of neurodegenerative processes. As research progresses, many similarities appear that relate these diseases to one another on a sub-cellular level. Discovering these similarities offers hope for therapeutic advances that could ameliorate many diseases simultaneously. There are many parallels between different neurodegenerative disorders including atypical protein assemblies as well as induced cell death (Bredesen et al., 2006; Rubinsztein, 2006).

[0009] Alzheimer's disease (AD) is a neurodegenerative disorder characterized by cerebrovascular and neuronal dysfunctions leading to a progressive decline in cognitive functions. Neuropathological hallmarks of AD include beta amyloid ($A\beta$) plaques and neurofibrillary tangles (Johnson et al., 2008). It has long been speculated that cerebrovascular dysfunction contributes to AD. $A\beta$ has been shown to decrease myogenic response, cerebral blood flow (CBF) and vasodilator responses (Han et al., 2008; Niwa et al., 2000; Paris et al., 2004; Shin et al., 2007). Regulation of CBF tends to be impaired in transgenic mice with high intracerebral levels of $A\beta$ (Niwa et al., 2002). Synthetic $A\beta$ has been shown to impair endothelin (ET) dependent relaxation and enhance vasoconstriction *in vivo* and *in vitro* (Niwa et al., 2000; Niwa et al., 2001).

[0010] Several studies have demonstrated an involvement of ET in AD. ET is an endogenous vasoregulatory peptide which targets two main receptors – ET_A and ET_B . ET_A receptors are mainly located on vascular smooth muscle cells and mediate vasoconstriction, whereas ET_B receptors are mainly located on vascular endothelial cells and mediate vasodilatation (Goto et al., 1989; Tsukahara et al., 1994). ET has been demonstrated to be present in the brain and plays an important role in the regulation of cerebral and systemic blood circulation (Gulati et al., 1997; Gulati et al., 1996; Gulati et al., 1995; Rebello et al., 1995a). It was initially demonstrated that ET-1 concentrations in the cerebrospinal fluid of patients with AD were lower compared to control (Yoshizawa et al., 1992), however, subsequent studies indicate that ET-1 like immunoreactivity was significantly increased in the cerebral cortex (frontal and occipital lobes) of patients that suffered from AD compared to control brains (Minami et al., 1995). Brain samples of AD patients obtained post mortem showed increased expression of ET-1 immunoreactivity in astrocytes (Zhang et al., 1994). It has been suggested that ET-1 released from astrocytes may reach the vascular smooth muscle cells and induce vasoconstriction. ET binding sites in the human brains with AD were found to be decreased which could be due to loss of neurons in the cortex (Kohzuki et al., 1995).

[0011] The mechanism by which soluble A β interferes with vascular function is not fully understood. A possible mechanism by which soluble A β interferes with vascular function may be mediated through ET-1 which plays a central role in the regulation of cardiovascular functions and regional blood flow (Gulati et al., 1997; Gulati et al., 1996; Gulati et al., 1995). It was previously found that specific ET_A receptor antagonists (BMS182874 and BQ123) prevent A β induced oxidative stress and cognitive deficits (Briyal et al., 2011). Specific ET_A receptor antagonists reduced escape latencies and also increased preference for the target quadrant. On the other hand, a nonspecific ET_A/ET_B receptor antagonist (TAK-044) did not produce any improvement in spatial memory deficit or loss of preference for the target quadrant (Briyal et al., 2011). This lack of improvement with the non-specific ET_A/ET_B antagonist indicated to us the specific involvement of ET_B receptors in AD.

[0012] ET binding sites in the brain are predominantly of ET_B receptors, and ET_B receptor agonists have been shown to be anti-apoptotic against neurotoxicity of A β (Yagami et al., 2002). Complete deficiency or blockade of ET_B receptors leads to exacerbation of ischemic brain damage, possibly due to the shift in ET vasomotor balance (Chuquet et al., 2002; Ehrenreich et al., 1999). It has been demonstrated that activation of ET_B receptors with intravenous IRL-1620, a highly selective ET_B agonist, results in a significant elevation in CBF in normal rats and reduction in neurological deficit and infarct volume of stroked rats (Leonard et al., 2011; Leonard and Gulati, 2009). It was further found that the efficacy of IRL-1620 in a rat model of stroke was completely antagonized by BQ788 indicating an involvement of ET_B receptors (Leonard et al., 2011; 2012).

Stroke and cerebrovascular disorders

[0013] **Stroke** is the rapid loss of brain function due to disturbance in the blood supply to the brain, which can be due to ischemia or a hemorrhage (Sims and Muyderman, 2009). It is the second leading cause of death and the fourth leading cause of disability worldwide (Mathers et al., 2009; Strong et al., 2007). It is also a predisposing factor for epilepsy, falls and depression (Fisher and Norrving, 2011) and is a foremost cause of functional impairments, with 20% of survivors requiring institutional care after 3 months and 15%-30% being permanently disabled (Steinwachs et al., 2000).

[0014] Stroke is divided into two broad categories: Ischemic strokes, caused by sudden occlusion of arteries supplying the brain, either due to a thrombus at the site of occlusion or formed in another part of the circulation. According to recent data released by the American

Heart Association, 87% of strokes are classified as ischemic (Deb et al., 2010; Feigin et al., 2009; Roger et al., 2012). Hemorrhagic strokes, caused by bleeding from one of the brain's arteries into the brain tissue (subarachnoid hemorrhage) or arterial bleeding in the space between meninges (intra-cerebral hemorrhage).

[0015] The outcome after a stroke depends on the site and severity of brain injury. A very severe stroke can cause sudden death. Stroke affected area of the brain cannot function, which may result in an inability to move one or more limbs on one side of the body, inability to understand or formulate speech, or an inability to see one side of the visual field (Bath and Lees, 2000; Donnan et al., 2008). Early recognition of stroke is most important in order to expedite diagnostic tests and treatments.

[0016] Despite the severity of ischemic stroke, the only currently available FDA-approved pharmacological treatment is recombinant tissue plasminogen activator (rtPA), which dissolves the clot and restores blood flow to the brain. This treatment is complicated by the relatively short window of time between infarct and treatment (3–4 h) and the increased risk of subarachnoid hemorrhage (Micieli et al., 2009). A large number of other agents, broadly classified as neuroprotective and aiming to slow or stop the secondary damage associated with the ischemic cascade following stroke, have shown promise in the initial stages of research but have thus far failed to demonstrate efficacy in clinical trials (Ly et al., 2006). A new approach is therefore needed, one which has the potential to address both the restoration of blood flow and attenuate secondary damage to the penumbral area.

[0017] Following both ischemic stroke and subarachnoid hemorrhage, ET levels in the blood and ET immunoreactivity in the tissues are elevated (Asano et al., 1990; Rebello et al., 1995b; Viossat et al., 1993). A demonstration that the increase in ET levels coincides with a decrease in regional blood flow in the ischemic areas of the brain following experimental stroke led to the investigation of several ET antagonists in the treatment of focal ischemic stroke (Patel et al., 1995). Although some ETA specific and ETA/B non-specific antagonists have shown promise in experimental stroke models, others have not (Barone et al., 2000; Barone et al., 1995; Briyal and Gulati, 2010; Briyal et al., 2007; Briyal et al., 2012a; Gupta et al., 2005; Kaundal et al., 2012; Zhang et al., 2008; Zhang et al., 2005). Overall, this approach has not been useful. It has been demonstrated that ET_B receptors, which increase VEGF and NGF in the brain, are overexpressed at the time of birth and their expression decreases with maturity of the brain (Briyal et al., 2012b). It appears that ET_B receptors present in large number in the CNS play a key role in its development. This fundamental information

demonstrates the possible involvement of ET_B receptor in the brain development to generate neurovascular plasticity of the brain that has been damaged following cerebral ischemia. It was found that stimulation of ET_B receptors with intravenous IRL-1620, a highly selective ET_B agonist, resulted in a significant elevation in cerebral blood flow in normal rats (Leonard and Gulati, 2009). In addition, functional ET_B receptors have been shown to enhance proliferation of neuronal progenitors and to protect against apoptosis in the dentate gyrus, olfactory epithelium, and cortical neurons (Ehrenreich et al., 1999; Laziz et al., 2011; Lee et al., 2003; Yagami et al., 2005). The evidence that a deficiency in ET_B receptors leads to a poorer outcome following cerebral ischemia (Chuquet et al., 2002) and complete deficiency or blockade of ET_B receptors leads to exacerbation of ischemic brain damage (Ehrenreich et al., 1999) led to the investigation of the role of ET_B receptors in a model of ischemic stroke. When a majority of research on ET and stroke thus far has focused on antagonizing ET_A receptors selectively or non-selectively in order to prevent excessive vasoconstriction, the effect of selectively activating ET_B receptors in a focal stroke model was examined (Leonard et al., 2011; 2012; Leonard and Gulati, 2013).

[0018] In clinical practice at present there are two basic treatments, preventive treatment using long term antiplatelet or anticoagulant agents to reduce the risk of stroke, or acute treatment by fibrinolytics. However, less than 2% of patients are able to receive fibrinolytics (Font et al., 2010). Extensive research is being conducted in search of neuroprotective agents for possible use in acute phase of stroke, and of agents that can be used for neurorepair in later stages of stroke.

SUMMARY OF THE INVENTION

[0019] The present invention relates to compositions and methods for treating neuropsychiatric disorders in vertebrates and humans. More specifically, the present invention provides for use of IRL-1620, an endothelin-B receptor agonist, in appropriate doses to be a neuroprotective and a neuroregenerative agent.

[0020] Accordingly, in one aspect the disclosure provides a method of treating a neuropsychiatric disorder comprising administering to a patient in need thereof a therapeutically effective amount of an endothelin-B receptor agonist to treat the neuropsychiatric disorder.

[0021] In some embodiments, the endothelin-B receptor agonist is co-administered with an additional agent to treat the neuropsychiatric disorder. In some embodiments, the additional

agent is selected from the group consisting of an antidepressant, an anti-inflammatory agent, a CNS stimulant, a neuroleptic, and an anti-proliferative agent.

[0022] In additional embodiments, the endothelin-B receptor agonist is selected from the group consisting of IRL-1620, BQ-3020, [Ala^{1,3,11,15}]-Endothelin, Sarafotoxin S6c, endothelin-3, and a mixture thereof.

[0023] The present disclosure contemplates that in further embodiments, the neuropsychiatric disorder is selected from the group consisting of a cerebrovascular disease, stroke, cerebral ischemia, cerebral hemorrhage, head trauma, brain injury, a brain tumor, multiple sclerosis and demyelinating diseases, dementia, vascular dementia, Alzheimer's disease, Parkinson's disease, Huntington's disease, ataxia, motor neuron disease, Amyotrophic lateral sclerosis, drug intoxication, alcoholism, chronic brain infections, brain abscess, white matter disease, Binswanger's disease, Moyamoya disease, perinatal hypoxia, cerebral asphyxia, intracranial birth injury, congenital malformation of the brain, mood disorders, and depression.

[0024] In some embodiments, the endothelin-B receptor agonist is administered at a dose ranging from 0.0001 to 0.5 mg/kg. In further embodiments, the endothelin-B receptor agonist is administered repeatedly at intervals of 1 to 6 hours after every two to five days.

[0025] In any of the embodiments of the disclosure, it is contemplated that the patient is a mammal. In some embodiments, the mammal is a human.

[0026] In another aspect, the disclosure provides a composition comprising (a) an endothelin-B receptor agonist, (b) an agent used to treat a neuropsychiatric disorder, and optionally (c) an excipient.

[0027] The disclosure also provides, in an additional aspect, an article of manufacture comprising (a) a packaged composition comprising an endothelin-B receptor agonist and an agent for a neuropsychiatric disorder; (b) an insert providing instructions for a simultaneous or sequential administration of the endothelin-B receptor agonist and the agent for the neuropsychiatric disorder to treat a patient in need thereof; and (c) a container for (a) and (b). In some embodiments, the endothelin-B receptor agonist is IRL-1620.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] **Figure 1.** Structure of IRL-1620 (Suc-Asp-Glu-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp; SEQ ID NO: 1).

[0029] Figure 2. Expression of vascular endothelial growth factor and endothelin B receptors in the vasculature of the postnatal rat brain. **A)** Representative images of blood vessels in the rat cortex at postnatal days 1, 7, 14 and 28, stained for VEGF (red) and ET_B receptors (green). Scale bar = 10 μ m. **B)** Intensity of VEGF in the rat brain vasculature at postnatal days 1, 7, 14 and 28. **C)** Number of VEGF-positive vessels per 20 μ m-thick rat brain slice at postnatal days 1, 7, 14 and 28. **D)** Intensity of ET_B receptors in the rat brain vasculature at postnatal days 1, 7, 14 and 28. *P<0.05 vs. day 1; #P<0.01 vs. day 7. Values are expressed as mean \pm SEM. 30 male pups were divided into following groups: Day 1 (N=10); Day 7 (N=10); Day 14 (N=5); Day 28 (N=5).

[0030] Figure 3. Expression of nerve growth factor and endothelin B receptors in the cortex and subventricular zone of the postnatal rat brain. **A)** Representative images of the rat cortex at postnatal days 1, 7, 14 and 28, stained for NGF (red) and ET_B receptors (green). Scale bar = 100 μ m. **B)** Representative images of the rat SVZ at postnatal days 1, 7, 14 and 28, stained for NGF (red) and ET_B receptors (green). Scale bar = 100 μ m. **C)** Intensity of NGF in the cortex and SVZ of the rat brain at postnatal days 1, 7, 14 and 28. **D)** Number of NGF-positive cells per 100 μ m² in the cortex and SVZ of the rat brain at postnatal days 1, 7, 14 and 28. **E)** Intensity of ET_B receptors in the cortex and SVZ of the rat brain at postnatal days 1, 7, 14 and 28. *P<0.001 vs. day 1; #P<0.01 vs. day 7. Values are expressed as mean \pm SEM. 30 male pups were divided into following groups: Day 1 (N=10); Day 7 (N=10); Day 14 (N=5); Day 28 (N=5).

[0031] Figure 4. Effect of ET_B receptor agonist, IRL-1620, on vascular endothelial growth factor and ET_B expression in the postnatal rat brain. Rat pups were administered either saline (control; N=5) or IRL-1620 (5 μ g/kg, IV; N=5) on postnatal day 21. Pups were then sacrificed on postnatal day 28 and brains removed for analysis. **A)** Representative images of blood vessels in the control and IRL-1620-treated rat cortex at postnatal day 28, stained for VEGF (red) and ET_B receptors (green). Scale bar = 10 μ m. **B)** Intensity of VEGF in the rat brain vasculature at postnatal day 28. **C)** Number of VEGF-positive vessels per 20 μ m-thick rat brain slice at postnatal day 28. **D)** Intensity of ET_B receptors in the rat brain vasculature at postnatal day 28. *P<0.05 vs. Control. Values are expressed as mean \pm SEM.

[0032] Figure 5. Effect of ET_B receptor agonist, IRL-1620, on nerve growth factor and ET_B expression in the postnatal rat brain. Rat pups were administered either saline (control; N=5) or IRL-1620 (5 μ g/kg, IV; N=5) on postnatal day 21. Pups were then sacrificed on postnatal day 28 and brains removed for analysis. **A)** Representative images of the cortex of

control and IRL-1620-treated rat brains at postnatal day 28, stained for NGF (red) and ET_B receptors (green). Scale bar = 100 μ m. **B)** Representative images of the SVZ of control and IRL-1620-treated rat brains at postnatal day 28, stained for NGF (red) and ET_B receptors (green). Scale bar = 100 μ m. **C)** Intensity of NGF in the cortex and SVZ of the rat brain at postnatal day 28. **D)** Number of NGF-positive cells per 100 μ m² in the cortex and SVZ of the rat brain at postnatal day 28. **E)** Intensity of ET_B receptors in the cortex and SVZ of the rat brain at postnatal day 28. *P<0.05 vs. Control. Values are expressed as mean \pm SEM.

[0033] Figure 6. Effect of ET_B receptor agonist, IRL-1620, on protein levels of vascular growth factor, nerve growth factor and ET_B receptors in the postnatal rat brain. Rat pups were administered either saline (control; N=5) or IRL-1620 (5 μ g/kg, IV; N=5) on postnatal day 21. Pups were then sacrificed on postnatal day 28 and brains removed for analysis. **A)** Representative blots of VEGF, ET_B and NGF protein expression in the control and IRL-1620 treated rat brains at postnatal day 28 with either β -tubulin or β -actin as protein loading controls. Lane 1 = Control; Lane 2 = IRL-1620. **B)** Fold change in the expression of VEGF in the rat brain at postnatal day 28. **C)** Fold change in the expression of ET_B receptors in the rat brain at postnatal day 28. **D)** Fold change in the expression of NGF in the rat brain at postnatal day 28. *P<0.05 vs. Control. Values are expressed as mean \pm SEM.

[0034] Figure 7. Effect of an ET_B receptor agonist, IRL-1620, in presence and absence of an ET_B receptor antagonist, BQ788, on malondialdehyde (MDA) (A), reduced glutathione (GSH) (B) and superoxide dismutase (SOD) (C) levels in A β induced oxidative stress in the rat brain. Values are expressed as mean \pm SEM. *p<0.0001 compared to sham; #p<0.001 compared to A β + vehicle (N=6).

[0035] Figure 8. Effect of an ET_B receptor agonist, IRL-1620, in presence and absence of an ET_B receptor antagonist, BQ788, on the escape latency (A) and path length (B) on each training day of the water maze task in non-diabetic rats. The animals were submitted to four daily trials to find a hidden platform for 4 training days. Values were expressed as mean \pm S.E.M. *p<0.001 compared to sham; #p<0.001 compared to A β + vehicle (N=6).

[0036] Figure 9. Effect of an ET_B receptor agonist, IRL-1620, in presence and absence of an ET_B receptor antagonist, BQ788, in the water maze probe trial task. Time spent in each quadrant in the probe trial in non-diabetic rats (A). Representative trajectories of each group during probe trial in the water maze task (B). Values were expressed as mean \pm S.E.M. *p<0.001 compared to sham; #p<0.001 compared to A β + vehicle (N=6).

[0037] Figure 10. Effect of an ET_B receptor agonist, IRL-1620, in presence and absence of an ET_B receptor antagonist, BQ788, on the escape latency (A) and path length (B) on each training day of the water maze task in diabetic rats. The animals were submitted to four daily trials to find a hidden platform for 4 training days. Values were expressed as mean \pm S.E.M. * $p < 0.001$ compared to sham; # $p < 0.001$ compared to A β + vehicle (N=6).

[0038] Figure 11. Effect of an ET_B receptor agonist, IRL-1620, in presence and absence of an ET_B receptor antagonist, BQ788, in the water maze probe trial task. Time spent in each quadrant in the probe trial in diabetic rats (A). Representative trajectories of each group during probe trial in the water maze task (B). Values were expressed as mean \pm S.E.M. * $p < 0.001$ compared to sham; # $p < 0.001$ compared to A β + vehicle (N=6).

[0039] Figure 12: Two mm coronal sections of brains stained with TTC to visualize the infarct area 7 days post middle cerebral artery occlusion (red indicates normal tissue and white indicates infarct tissue). Representative slices from groups are shown. IRL-1620 (5 μ g/kg, IV) or isotonic saline (1 ml/kg, IV) was injected at 2, 4, and 6 hr post MCAO. BQ-788 (1 mg/kg, IV) was administered once 15 min prior to the first injection of IRL-1620 or vehicle.

[0040] Figure 13: Effect of ET_B receptor agonist, IRL-1620, in presence and absence of BQ788 on 7 day survival of rats undergoing either sham surgery or middle cerebral artery occlusion.

[0041] Figure 14: Binding affinity (K_d) and receptor density (B_{max}) of ET_B receptors in the left and right cerebral hemisphere in male Sprague Dawley rats (A) 24-hours and (B) 7 days following MCAO. Values are expressed as Mean \pm S.E.M, N=4 each group. No significant change in K_d or B_{max} was observed between left and right hemispheres in both sham (control) and stroke (MCAO) groups.

[0042] Figure 15: Effect of ET_B receptor agonist, IRL-1620 (3 doses of 5 μ g/kg, i.v., at 2, 4 and 6 hours post ischemia), and antagonist, BQ788 (1 mg/kg, i.v.), on glial fibrillary acidic protein (GFAP) post middle cerebral artery occlusion. A. Representative 30 μ m-thick ischemic brain slice stained for the ET_B receptor (green) and GFAP (red). Scale bar = 2000 μ m. Bottom panel shows number of reactive astrocytes (GFAP+ cells) per 100 μ m² in the cortex, striatum, and subventricular zone of middle cerebral artery occluded rats at 24 h after infarct. * $P < 0.001$ vs. sham. # $P < 0.0001$ vs. MCAO + vehicle. @ $P < 0.01$ vs. MCAO + IRL-1620; and number of reactive astrocytes (GFAP+ cells) per 100 μ m² in the cortex, striatum,

and subventricular zone of middle cerebral artery occluded rats at 1 w after infarct. Values are expressed as mean \pm SEM (n=5/group). *P<0.001 vs. sham.

[0043] Figure 16: Effect of ET_B receptor agonist, IRL-1620 (three doses of 5 μ g/kg, i.v., at 2, 4 and 6 h post ischemia), and antagonist, BQ788 (1 mg/kg, i.v.), on neuronal nuclei (NeuN) post middle cerebral artery occlusion. (A) Representative image of the cortex of an IRL-1620-treated animal 1 week following MCAO, stained for the ET_B receptor (green) and NeuN (red). Scale bar = 100 μ m. (B) Representative image of the striatum of an IRL-1620-treated animal 1 week following MCAO, stained for the ET_B receptor (green) and NeuN (red). Scale bar=10 μ m. (C) Number of neuronal nuclei per 100 μ m² in the cortex, striatum, and subventricular zone of middle cerebral artery occluded rats at 24 h after infarct. *P<0.05 vs. sham. #P<0.01 vs. MCAO+vehicle. @P<0.0001 vs. MCAO+IRL-1620. (D) Number of neuronal nuclei per 100 μ m² in the cortex, striatum, and subventricular zone of middle cerebral artery occluded rats at 1 week after infarct. Values are expressed as mean \pm SEM (n=5/group). *P<0.0001 vs. sham. #P<0.0001 vs. MCAO+vehicle. @P<0.0001 vs. MCAO+IRL-1620.

[0044] Figure 17: Effect of ET_B receptor agonist, IRL-1620 (3 doses of 5 μ g/kg, i.v., at 2, 4 and 6 hours post ischemia), and antagonist, BQ788 (1 mg/kg, i.v.), on proliferating cells post middle cerebral artery occlusion. Representative image of the cortex of an IRL-1620-treated animal 1 week following MCAO depicting a cerebral blood vessel, stained for the ET_B receptor (green) and BrdU (red). Scale bar = 100 μ m. Bottom panel shows number of proliferating cells (BrdU+) per 100 μ m² in the cortex, striatum, and subventricular zone of middle cerebral artery occluded rats at 1 week after infarct. Values are expressed as mean \pm SEM (n=5/group). *P<0.01 vs. sham. #P<0.0001 vs. MCAO + vehicle. @P<0.0001 vs. MCAO + IRL-1620.

[0045] Figure 18: Effect of ET_B receptor agonist, IRL-1620 (3 doses of 5 μ g/kg, i.v., at 2, 4 and 6 hours post ischemia), and antagonist, BQ788 (1 mg/kg, i.v.), on vascular endothelial growth factor (VEGF) post middle cerebral artery occlusion. A. Representative images of blood vessels in the rat cortex 24 h following MCAO, stained for the ET_B receptor (green) and VEGF (red). Rows: 1. Sham; 2. MCAO + vehicle; 3. MCAO + IRL-1620; 4. MCAO + BQ788 + vehicle; 5. MCAO + BQ788 + IRL-1620. Scale bar = 10 μ m. B. Representative images of blood vessels in the rat cortex 1 week following MCAO, stained for the ET_B receptor (green) and VEGF (red). Rows same as in (A 24 h). Scale bar = 10 μ m.

[0046] Figure 19: Effect of ET_B receptor agonist, IRL-1620 (3 doses of 5 µg/kg, i.v., at 2, 4 and 6 hours post ischemia), and antagonist, BQ788 (1 mg/kg, i.v.), on vascular endothelial growth factor (VEGF) post middle cerebral artery occlusion. Upper panel shows number of VEGF+ vessels per 30 µm brain slice middle cerebral artery occluded rats at 24 h after infarct. *P<0.05 vs. sham. #P<0.01 vs. MCAO + vehicle. @P<0.05 vs. MCAO + IRL-1620. The lower panel shows number of VEGF+ vessels per 30 µm brain slice middle cerebral artery occluded rats at 1 week after infarct. Values are expressed as mean ± SEM (n=5/group). *P<0.01 vs. sham. #P<0.01 vs. MCAO + vehicle. @P<0.05 vs. MCAO + IRL-1620.

[0047] Figure 20: Effect of ET_B receptor agonist, IRL-1620 (3 doses of 5 µg/kg, i.v., at 2, 4 and 6 hours post ischemia), on protein levels of vascular endothelial growth factor (VEGF) post middle cerebral artery occlusion. A. Representative blot of VEGF protein levels in the rat brain 24 hours post MCAO with β-tubulin as a loading control. Lane 1 – sham (LH), Lane 2 – sham (RH), Lane 3 – MCAO + Vehicle (LH), Lane 4 – MCAO + Vehicle (RH), Lane 5 – MCAO + IRL-1620 (LH), Lane 6 – MCAO + IRL-1620 (RH). LH = left hemisphere, RH = right hemisphere. B. Representative blot of VEGF protein levels in the rat brain 1 week post MCAO with β-tubulin as a loading control, with lane distribution the same as in (A). C. Expression of VEGF protein levels in the rat brain 24 hours following MCAO. D. Expression of VEGF protein levels in the rat brain 1 week following MCAO. Values are expressed as mean ± SEM (n=5/group). *P<0.001 vs. Sham. #P<0.01 vs MCAO + Vehicle.

[0048] Figure 21: Effect of ET_B receptor agonist, IRL-1620 (3 doses of 5 µg/kg, i.v., at 2, 4 and 6 hours post ischemia), and antagonist, BQ788 (1 mg/kg, i.v.), on nerve growth factor (NGF) post middle cerebral artery occlusion. A. Representative image of the cortex of an IRL-1620-treated animal 1 week following MCAO, stained for the ET_B receptor (green) and NGF (red). Scale bar = 10 µm. B. Number of NGF+ cells per 100µm² in the cortex, striatum, and subventricular zone of middle cerebral artery occluded rats at 1 week after infarct. Values are expressed as mean ± SEM (n=5/group). #P<0.0001 vs. MCAO + vehicle. @P<0.0001 vs. MCAO + IRL-1620.

[0049] Figure 22: Effect of ET_B receptor agonist, IRL-1620 (3 doses of 5 µg/kg, i.v., at 2, 4 and 6 hours post ischemia), on protein levels of nerve growth factor (NGF) post middle cerebral artery occlusion. A. Representative blot of NGF protein levels in the rat brain 24 hours post MCAO with β-tubulin as a loading control. Lane 1 – sham (LH), Lane 2 – sham

(RH), Lane 3 – MCAO + Vehicle (LH), Lane 4 – MCAO + Vehicle (RH), Lane 5 – MCAO + IRL-1620 (LH), Lane 6 – MCAO + IRL-1620 (RH). LH = left hemisphere, RH = right hemisphere. B. Representative blot of NGF protein levels in the rat brain 1 week post MCAO with β -tubulin as a loading control, with lane distribution the same as in (A). C. Expression of NGF protein levels in the rat brain 24 hours following MCAO. D. Expression of NGF protein levels in the rat brain 1 week following MCAO. Values are expressed as mean \pm SEM (n=5/group). *P<0.01 vs. Sham.

DETAILED DESCRIPTION OF THE INVENTION

[0050] The present disclosure provides compositions and methods for treating a neuropsychiatric disorder. It is disclosed herein that an endothelin-B receptor agonist functions as neuroprotective and a neuroregenerative agent. The compositions and methods of the disclosure generally relate to administering an endothelin-B receptor agonist to a patient in need thereof to treat a neuropsychiatric disorder.

[0051] Accordingly, in one aspect the disclosure provides a method of treating a neuropsychiatric disorder comprising administering to a patient in need thereof a therapeutically effective amount of an endothelin-B receptor agonist to treat the neuropsychiatric disorder.

[0052] In some embodiments, the endothelin-B receptor agonist is selected from the group consisting of IRL-1620, BQ-3020, [Ala^{1,3,11,15}]-Endothelin, Sarafotoxin S6c, endothelin-3, and a mixture thereof.

[0053] In some embodiments, the present disclosure contemplates that ET_B receptor agonists such as IRL-1620 (Figure 1) can be used to treat various neuropsychiatric disorders such as cerebrovascular diseases, stroke, cerebral ischemia, cerebral hemorrhage, head trauma, brain injury, brain tumors, multiple sclerosis and demyelinating diseases, dementia, vascular dementia, Alzheimer's disease, Parkinson's disease, Huntington's disease, ataxias, motor neuron disease, Amyotrophic lateral sclerosis, drug intoxication, alcoholism, chronic brain infections, brain abscess, white matter disease, Binswanger's disease, Moyamoya disease, perinatal hypoxia, cerebral asphyxia, intracranial birth injury, congenital malformation of the brain, mood disorders, and depression.

[0054] In some embodiments, the endothelin-B receptor agonist is co-administered with an additional agent to treat the neuropsychiatric disorder. In some embodiments, the additional

agent is selected from the group consisting of an antidepressant, an anti-inflammatory agent, a CNS stimulant, a neuroleptic, and an anti-proliferative agent.

General additional agents

[0055] Examples of antidepressants, CNS stimulants and neuroleptic agents useful in the methods and compositions of the disclosure include, but are not limited to Abilify, Adapin, Adderall, Alepam, Alertec, Aloperidin, Alplax, Alprax, Alprazolam, Alviz, Alzolam, Amantadine, Ambien, Amisulpride, Amitriptyline, Amoxapine, Amfebutamone, Anafranil, Anatensol, Ansial, Ansiced, Antabus, Antabuse, Antideprin, Anxiron, Apo-Alpraz, Apo-Primidone, Apo-Sertral, Aponal, Apozepam, Aripiprazole, Aropax, Artane, Asendin, Asendis, Asentra, Ativan, Atomoxetine, Aurorix, Aventyl, Axoren, Baclofen, Beneficat, Benperidol, Bimaran, Bioperidol, Biston, Brotopon, Bespar, Bupropion, Buspar, Buspimen, Buspinol, Buspirone, Buspisal, Cabaser, Cabergoline, Calepsin, Calcium carbonate, Calcium carbimide, Calmax, Carbamazepine, Carbatrol, Carbolith, Celexa, Chloralduurat, Chloralhydrat, Chlordiazepoxide, Chlorpromazine, Cibalith-S, Cipralext, Citalopram, Clomipramine, Clonazepam, Clozapine, Clozaril, Concerta, Constan, Convulext, Cylert, Cymbalta, Dapotum, Daquiran, Daytrana, Defanyl, Dalmane, Damixane, Demolox, Depad, Depakene, Depakote, Depixol, Desyrel, Dostinex, dextroamphetamine, Dexedrine, Diazepam, Didrex, Divalproex, Dogmatyl, Dolophine, Droperidol, Desoxyn, Edronax, Effectin, Effexor (Efexor), Eglonyl, Einalon S, Elavil, Elontril, Endep, Epanutin, Epitol, Equetro, Escitalopram, Eskalith, Eskaziny, Eskazine, Etrafon, Eukystol, Eunerpan, Faverin, Fazaclo, Fevarin, Finlepsin, Fludecate, Flunanthate, Fluoxetine, Fluphenazine, Flurazepam, Fluspirilene, Fluvoxamine, Focalin, Gabapentin, Geodon, Gladem, Glianimon, Guanfacine, Halcion, Halomonth, Haldol, Haloperidol, Halosten, Imap, Imipramine, Imovane, Janimine, Jatroneural, Kalma, Keselan, Klonopin, Lamotrigine, Largactil, Levomepromazine, Levoprome, Leponex, Lexapro, Libotryp Libritabs, Librium, Linton, Liskantin, Lithane, Lithium, Lithizine, Lithobid, Lithonate, Lithotabs, Lorazepam, Loxapac, Loxapine, Loxitane, Ludiomil, Lunesta, Lustral, Luvox, Lyrica, Lyogen, Manegan, Manerix, Maprotiline, Mellaril, Melleretten, Melleril, Melneurin, Melperone, Meresa, Mesoridazine, Metadate, Methamphetamine, Methotrimeprazine, Methylin, Methylphenidate, Minitran, Mirapex, Mirapexine, Moclobemide, Modafinil, Modalina, Modecate, Moditen, Molipaxin, Moxadil, Murelax, Myidone, Mylepsinum, Mysoline, Nardil, Narol, Navane, Nefazodone, Neoperidol, Neurontin, Nipolept, Norebox, Normison, Norpramine, Nortriptyline, Novodorm, Nitrazepam, Olanzapine, Omca, Oprymea, Orap, Oxazepam, Pamelor, Parnate, Paroxetine,

Paxil, Peluces, Pemoline, Pergolide, Permax, Permitil, Perphenazine, Pertofrane, Phenelzine, Phenytoin, Pimozide, Piportil, Pipotiazine, Pragmarel, Pramipexole, Pregabalin, Primidone, Prolift, Prolixin, Promethazine, Prothipendyl, Protriptyline, Provigil, Prozac, Prysoline, Psymion, Quetiapine, Ralozam, Reboxetine, Redeptin, Resimatil, Restoril, Restyl, Rhotrimine, riluzole, Risperdal, Risperidone, Rispolept, Ritalin, Rivotril, Rubifen, Rozerem, Sediten, Seduxen, Selecten, Serax, Serenace, Serepax, Serenase, Serentil, Seresta, Serlain, Serlift, Seroquel, Seroxat, Sertan, Sertraline, Serzone, Sevinol, Sideril, Sifrol, Sigaperidol, Sinequan, Sinqualone, Siquan, Sirtal, Solanax, Solian, Solvex, Songar, Stazepin, Stelazine, Stilnox, Stimuloton, Strattera, Sulpiride, Sulpiride Ratiopharm, Sulpiride Neurazpharm, Surmontil, Symbyax, Symmetrel, Tafil, Tavor, Taxagon, Tegretol, Telesmin, Temazepam, Temesta, Temposil, Terfluzine, Thioridazine, Thiothixene, Thombran, Thorazine, Timonil, tissue plasminogen activator (tPA), Tofranil, Tradon, Tramadol, Tramal, Trancin, Tranax, Trankimazin, Tranquinal, Tranlycypromine, Trazalon, Trazodone, Trazonil, Trialodine, Trevilor, Triazolam, Trifluoperazine, Trihexane, Trihexyphenidyl, Trilafon, Trimipramine, Triptil, Trittico, Troxal, Tryptanol, Tryptomer, Ultram, Valium, Valproate, Valproic acid, Valrelease, Vasiprax, Venlafaxine, Vestra, Vigicer, Vivactil, Wellbutrin, Xanax, Xanor, Xydep, Zaleplon, Zamhexal, Zeldox, Zimovane, Zispin, Ziprasidone, Zolarem, Zoldac, Zolofit, Zolpidem, Zonalon, Zopiclone, Zotepine, Zydis, and Zyprexa.

Anti-Inflammatory Agents

[0056] Any agents having anti-inflammatory effects can be used in the present invention. The anti-inflammatory agent can be a steroidal anti-inflammatory agent, a nonsteroidal anti-inflammatory agent, or a combination thereof. In some embodiments, anti-inflammatory agents include, but are not limited to, alclofenac, alclometasone dipropionate, algestone acetone, alpha amylase, amcinafal, amcinafide, amfenac sodium, amiprilose hydrochloride, anakinra, anirolac, anitrazafen, apazone, balsalazide disodium, bendazac, benoxaprofen, benzydamine hydrochloride, bromelains, broperamole, budesonide, carprofen, cicloprofen, cintazone, cliprofen, clobetasol propionate, clobetasone butyrate, clopirac, cloticasone propionate, cormethasone acetate, cortodoxone, deflazacort, desonide, desoximetasone, dexamethasone dipropionate, diclofenac potassium, diclofenac sodium, diflorasone diacetate, diflumidone sodium, diflunisal, difluprednate, diftalone, dimethyl sulfoxide, drocinonide, endrysone, enlimomab, enolicam sodium, epirizole, etodolac, etofenamate, felbinac, fenamole, fenbufen, fenclofenac, fenclorac, fendosal, fempipalone, fentiazac, flazalone, fluazacort, flufenamic acid, flumizole, flunisolid acetate, flunixin, flunixin meglumine,

fluocortin butyl, fluorometholone acetate, fluquazone, flurbiprofen, fluretofen, fluticasone propionate, furaprofen, furobufen, halcinonide, halobetasol propionate, halopredone acetate, ibufenac, ibuprofen, ibuprofen aluminum, ibuprofen piconol, ilonidap, indomethacin, indomethacin sodium, indoprofen, indoxole, intrazole, isoflupredone acetate, isoxepac, isoxicam, ketoprofen, lofemizole hydrochloride, lomoxicam, loteprednol etabonate, meclofenamate sodium, meclofenamic acid, meclorison dibutyrate, mefenamic acid, mesalamine, meseclazone, methylprednisolone suleptanate, morniflumate, nabumetone, naproxen, naproxen sodium, naproxol, nimazone, olsalazine sodium, orgotein, orpanoxin, oxaprozin, oxyphenbutazone, paranyline hydrochloride, pentosan polysulfate sodium, phenbutazone sodium glycerate, pirfenidone, piroxicam, piroxicam cinnamate, piroxicam olamine, pirprofen, prednazate, prifelone, prodolic acid, proquazone, proxazole, proxazole citrate, rimexolone, romazarit, salcolex, salnacedin, salsalate, sanguinarium chloride, seclazone, sermetacin, sudoxicam, sulindac, suprofen, talmetacin, talniflumate, talosalate, tebufelone, tenidap, tenidap sodium, tenoxicam, tesicam, tesimide, tetrydamine, tiopinac, tixocortol pivalate, tolmetin, tolmetin sodium, triclone, triflumidate, zidometacin, zomepirac sodium, aspirin (acetylsalicylic acid), salicylic acid, corticosteroids, glucocorticoids, tacrolimus, pimecorlimus, prodrugs thereof, co-drugs thereof, and combinations thereof.

Anti-Proliferative Agents

[0057] Any agents having anti-proliferative effects can be used in the present invention. The anti-proliferative agent can be a natural proteinoous agent such as a cytotoxin or a synthetic molecule. In some embodiments, the active agents include antiproliferative substances such as actinomycin D, or derivatives and analogs thereof (synonyms of actinomycin D include dactinomycin, actinomycin IV, actinomycin I₁, actinomycin X₁, and actinomycin CO, all taxoids such as taxols, docetaxel, and paclitaxel, paclitaxel derivatives, all olimus drugs such as macrolide antibiotics, rapamycin, everolimus, structural derivatives and functional analogues of rapamycin, structural derivatives and functional analogues of everolimus, FKBP-12 mediated mTOR inhibitors, biolimus, prodrugs thereof, co-drugs thereof, and combinations thereof). Representative rapamycin derivatives include 40-O-(3-hydroxy)propyl-rapamycin, 40-O-[2-(2-hydroxy)ethoxy]ethyl-rapamycin, or 40-O-tetrazole-rapamycin, 40-epi-(N-1-tetrazolyl)-rapamycin (ABT-578 manufactured by Abbot Laboratories, Abbot Park, Ill.), prodrugs thereof, co-drugs thereof, and combinations thereof.

[0058] The present disclosure contemplates that in further embodiments, the neuropsychiatric disorder is selected from the group consisting of a cerebrovascular disease, stroke, cerebral ischemia, cerebral hemorrhage, head trauma, brain injury, a brain tumor, multiple sclerosis and demyelinating diseases, dementia, vascular dementia, Alzheimer's disease, Parkinson's disease, Huntington's disease, ataxia, motor neuron disease, Amyotrophic lateral sclerosis, drug intoxication, alcoholism, chronic brain infections, brain abscess, white matter disease, Binswanger's disease, Moyamoya disease, perinatal hypoxia, cerebral asphyxia, intracranial birth injury, congenital malformation of the brain, mood disorders, and depression.

[0059] According to the disclosure, the endothelin-B receptor agonist may be administered at a dose ranging from 0.0001 to 0.5 mg/kg. In further embodiments, the endothelin-B receptor agonist is administered at a dose ranging from about 0.0001 to about 0.5 mg/kg, or from about 0.0001 to about 0.4 mg/kg, or from about 0.0001 to about 0.3 mg/kg, or from about 0.0001 to about 0.2 mg/kg, or from about 0.0001 to about 0.1 mg/kg, or from about 0.001 to about 0.5 mg/kg, or from about 0.001 to about 0.4 mg/kg, or from about 0.001 to about 0.3 mg/kg, or from about 0.001 to about 0.2 mg/kg, or from about 0.001 to about 0.1 mg/kg, or from about 0.01 to about 0.5 mg/kg, or from about 0.01 to about 0.4 mg/kg, or from about 0.01 to about 0.3 mg/kg, or from about 0.01 to about 0.2 mg/kg, or from about 0.01 to about 0.1 mg/kg, or from about 0.0005 to about 0.5 mg/kg, or from about 0.0005 to about 0.4 mg/kg, or from about 0.0005 to about 0.3 mg/kg, or from about 0.0005 to about 0.2 mg/kg, or from about 0.0005 to about 0.1 mg/kg. In additional embodiments, the endothelin-B receptor agonist is administered at a dose of at least about 0.0001 mg/kg, or at least about 0.0002 mg/kg, or at least about 0.0005 mg/kg, or at least about 0.001 mg/kg, or at least about 0.002 mg/kg, or at least about 0.005 mg/kg, or at least about 0.007 mg/kg, or at least about 0.01 mg/kg, or at least about 0.02 mg/kg, or at least about 0.03 mg/kg, or at least about 0.04 mg/kg, or at least about 0.05 mg/kg, or at least about 0.06 mg/kg, or at least about 0.07 mg/kg, or at least about 0.08 mg/kg, or at least about 0.09 mg/kg, or at least about 0.1 mg/kg, or at least about 0.2 mg/kg, or at least about 0.3 mg/kg, or at least about 0.4 mg/kg. In still further embodiments, the endothelin-B receptor agonist is administered at a dose of less than about 0.0001 mg/kg, or less than about 0.0002 mg/kg, or less than about 0.0005 mg/kg, or less than about 0.001 mg/kg, or less than about 0.002 mg/kg, or less than about 0.005 mg/kg, or less than about 0.007 mg/kg, or less than about 0.01 mg/kg, or less than about 0.02 mg/kg, or less than about 0.03 mg/kg, or less than about 0.04 mg/kg, or less than about 0.05 mg/kg,

or less than about 0.06 mg/kg, or less than about 0.07 mg/kg, or less than about 0.08 mg/kg, or less than about 0.09 mg/kg, or less than about 0.1 mg/kg, or less than about 0.2 mg/kg, or less than about 0.3 mg/kg, or less than about 0.4 mg/kg, or less than about 0.5 mg/kg.

[0060] The endothelin-B receptor agonist, in various embodiments, is administered to a patient repeatedly at intervals of 1 to 6 hours. In some embodiments, the endothelin-B receptor agonist is administered to the patient every 1 to 5 hours, or every 1 to 4 hours, or every 1 to 2 hours, or every hour, or every 2 hours, or every 3 hours, or every 4 hours, or every 5 hours, or every 6 hours. In further embodiments, the endothelin-B receptor agonist is administered to the patient every two to five days, or every three to five days, or every two days, or every three days, or every four days, or every five days.

Endothelin B receptor ontogeny in the postnatal rat brain

[0061] A decrease in expression of ET_B receptors in the brain of rat pups at postnatal day 28 as measured using immunoblotting technique has been reported (Briyal et al., 2012b). In order to determine the location of these receptors and their potential correlation with vascular and neural growth factors in the developing brain, the brains were immunofluorescently labeled of rat pups at postnatal days 1, 7, 14 and 28 with antibodies for ET_B receptors, VEGF and NGF. The intensity of ET_B receptor staining within the vasculature was significantly higher on day 14 compared to day 1 and day 7 of postnatal age (Figure 2). In contrast, intensity of ET_B staining in the cortex and subventricular zones of developing rat brain decreased significantly at day 14 of postnatal age compared to day 1 and day 7 ($P < 0.0001$; Figure 3). ET_B intensity was found to be similar at postnatal age of 14 and 28 days. These results indicate that there is indeed a decrease in the expression of ET_B receptors within the neural tissue of the developing rat brain, but not in the neurovasculature.

Vascular endothelial growth factor ontogeny in the postnatal rat brain

[0062] VEGF in the vasculature of the postnatal rat brain was evaluated at day 1, 7, 14 and 28 of postnatal age via immunofluorescent labeling. As illustrated in figure 2, the intensity of VEGF staining in the neurovasculature steadily increased from postnatal days 1 through 14. Similarly, the number of VEGF-positive vessels per 20 μm -thick brain slice significantly ($P < 0.0001$) increased from 2.22 ± 0.36 on day 1 to 5.69 ± 0.74 on day 14 of postnatal age (Figure 2). While both VEGF and ET_B intensity and expression within the cerebral vasculature of the developing rat brain increased with age, there was no significant correlation between the two ($r^2 = 0.8279$; $P = 0.0901$).

Nerve growth factor ontogeny in the postnatal rat brain

[0063] Immunofluorescent labeling was used to determine the expression and distribution of NGF in the postnatal rat brain at days 1, 7, 14 and 28. The intensity of staining for NGF in the cortex of the developing rat brain significantly decreased from day 1 to day 7 ($P<0.001$) and again from day 7 to day 14 ($P<0.01$) of postnatal age. There was no significant difference in NGF intensity from postnatal day 14 to day 28 (Figure 3). NGF intensity within the subventricular zone of the postnatal rat brain decreased between days 7 and 14 ($P<0.01$), with no further decline between days 14 and 28 (Figure 3) of postnatal age. Interestingly, average number of cells staining positive for NGF did not significantly alter during the course of experiment in either the cortex or SVZ. There was, however, a significant correlation between a decrease in ET_B receptors and NGF in the cortex of the developing rat brain with age ($r^2=0.9742$; $P=0.0130$). These results indicate that, as the rat brain matures the overall expression of NGF declines. This decrease may be correlated with the drop in ET_B receptors within the neuronal tissue.

Effect of IRL-1620 on endothelin B receptors in the postnatal rat brain

[0064] Administration of ET_B receptor agonist, IRL-1620, on postnatal day 21 resulted in a significant increase in ET_B receptors in the 28-day old rat brain as compared to the control group. Overall, protein expression of ET_B receptors, as measured using immunoblotting technique, increased in the animals who had received IRL-1620 (Figure 6). Upon immunofluorescent labeling of the brain slices, it was found that intensity of ET_B receptor staining was significantly higher in both the cerebral vasculature (Figure 2) and the cortex (Figure 3) of IRL-1620-treated animals as compared to control ($P<0.05$). These results suggest that selective stimulation of ET_B receptors during neonatal development may result in an upregulation of these receptors.

Effect of IRL-1620 on vascular endothelial growth factor in the postnatal rat brain

[0065] As seen in Figure 2, VEGF increases in the cerebral vasculature throughout development in rats. Selective stimulation of ET_B receptors using agonist IRL-1620 leads to a further increase in both the intensity of VEGF staining and the number of VEGF-positive vessels ($P<0.05$; Figures 4) within the postnatal rat brain when compared to control animals of the same age. These results were confirmed using immunoblotting technique showing that overall VEGF expression is significantly enhanced within the postnatal brains of rats treated

with IRL-1620 ($P < 0.05$; Figure 6). These findings suggest that selective stimulation of ET_B receptors during development enhances cerebrovascular angiogenesis.

Effect of IRL-1620 on nerve growth factor in the postnatal rat brain

[0066] Immunofluorescent labeling of NGF significantly diminishes by postnatal day 14 in the rat brain. Administration of ET_B receptor agonist, IRL-1620, on postnatal day 21 did not alter either intensity of NGF staining nor number of NGF-positive cells within the cortex or subventricular zones of rats as compared to control (Figure 5). Similarly, overall expression of NGF within the postnatal rat brain as measured via immunoblotting technique was comparable in both control and treated groups (Figure 6). Selective ET_B receptor stimulation does not appear to have any significant effect on NGF levels within the rat brain during postnatal development.

Experimental procedure

Animals

[0067] Timed pregnant female Sprague-Dawley rats (Harlan, Indianapolis, IN) were caged singly in a room controlled for ambient temperature (23 ± 1 °C), humidity ($50 \pm 10\%$) and a 12 h light/dark cycle (6:00 am – 6:00 pm). Food and water were available ad libitum. All animal care and use for procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Midwestern University. In order to avoid variation due to hormonal changes, male pups only were separated and utilized for this study. The pups were euthanized via decapitation on postnatal day 1, 7, 14 and 28. The brains were aseptically removed and processed for either immunofluorescent labeling or immunoblot analysis of ET_B receptors, VEGF and NGF.

Study design and agent administration

[0068] After 21 days of gestation, 70 male and 65 female pups were born to 10 pregnant female rats. 30 male pups were randomly selected for Study I, and divided into 4 groups as follows: Group 1 = male pups euthanized on postnatal day 1 ($N=10$); Group 2 = male pups euthanized on postnatal day 7 ($N=10$); Group 3 = male pups euthanized on postnatal day 14 ($N=5$); Group 4 = male pups euthanized on postnatal day 28 ($N=5$). An additional 20 male pups were randomly selected for Study II, and divided into 2 groups: Control ($N=10$) and IRL-1620-treated ($N=10$). Pups in Study II received 3 doses of either isotonic saline (1 ml/kg) or ET_B receptor agonist IRL-1620 (5 μ g/kg; American Peptide Co, Inc., Sunnyvale,

CA) on postnatal day 21. The doses were given intravenously at 2 hour intervals. All pups were weighed and evaluated for developmental and behavioral characteristics at postnatal day 1, 7, 14 and 28. Developmental and behavioral characteristics included active vs. sluggish behavior, healthy vs. shedding fur, licking, grooming, aggressive behavior, defecation, urination and wet dog shakes.

Immunofluorescent labeling

[0069] Immunofluorescently labeled antibodies were used to determine the expression of ET_B receptors, VEGF and NGF in the developing rat brain. Male rat pups were euthanized via decapitation, and the brains were removed at postnatal day 1, 7, 14 and 28. The brains were washed in isotonic saline and transferred to a 4% paraformaldehyde (PFA)/NaPO₄ buffer solution for 2 hours to fix the tissue, followed by suspension in a 20% sucrose/4% PFA solution for 48 hours at 4 °C. Brains were then sliced into 20 µm thick coronal sections at - 30 °C using a cryostat (Microtome cryostat HM 505 E; Walldorf, Germany). Sections were processed for immunofluorescent staining as described by Loo, et al. (Loo et al., 2002), with minor modifications. The primary antibody for ET_B receptors was an anti-ET_B receptor antibody raised in sheep against the carboxy-terminal peptide of the rat ET_B receptor (1:200; ab50658; Abcam, Cambridge, MA). Determination of angiogenic and neurogenic markers was performed using antibodies against VEGF (anti-VEGF; 1:500; ab46154; Abcam) and nerve growth factor (anti-NGF; ab6199; Abcam). Sections were washed in PBS and then blocked with 10% v/v serum in PBS containing 0.3% Triton X-100 for 1 h. Sections were then incubated with the primary antibody overnight at 4 °C, and again washed with PBS and incubated with the appropriate secondary antibody for 2 h at room temperature. Double labeling for co-localization was performed sequentially. Sections were rinsed with PBS and mounted on glass slides with Vectashield (Vector Laboratories, Inc., Burlingame, CA). Fluorescence was detected using an inverted fluorescent microscope (Nikon Eclipse TiE, Melville, NY). All images for analysis were taken with the same exposure (300 msec for VEGF and NGF; 800 msec for ET_B) and objective (Plan Fluor 10x Ph1DL) settings with a multichannel ND acquisition using NIS Elements BR imaging software (Nikon Instruments, Inc., Melville, NY).

Immunofluorescent analysis

[0070] Analyses for NGF were performed specifically in the cortex and SVZ of the rat brain. Overlaying a grid of 100 x 100 µm squares on each image, the number of cells

staining positively for NGF was determined in six randomly selected, non-congruent $100\ \mu\text{m}^2$ sections per brain slice in each area. All cells falling at least 50% inside the $100\ \mu\text{m}^2$ area were counted. For the evaluation of angiogenesis, the total number of VEGF-positive blood vessels was determined per brain slice. Fluorescent intensity for each marker was measured using the unaltered images with NIS Elements BR imaging software (Nikon Instruments, Inc., Melville, NY).

Immunoblotting

[0071] Protein levels of VEGF, NGF and ET_B receptors in the postnatal rat brain of animals in Study II were estimated using immunoblotting technique. Animals were decapitated and the brains were removed on postnatal day 28, which is 7 days post administration of either saline or ET_B receptor agonist, IRL-1620. The tissue was homogenized in 10x (w/v) RIPA lysis buffer, and protein concentration was determined according to the Lowry method, using bovine serum albumin as standard (Lowry et al., 1951). Protein (60 μg) was denatured in Laemmli sample buffer and resolved in 10% SDS-PAGE, and then transferred onto nitrocellulose membrane. After blocking, the membranes were incubated with rabbit polyclonal anti-VEGF (1:1000; Abcam, Cambridge, MA), anti-NGF (1:500; Abcam, Cambridge, MA), or anti- ET_B (1:1000; Sigma-Aldrich) antibodies overnight at 4°C , followed by 1.5 hours incubation with by HRP-conjugated secondary antibodies (1:2000; Cell Signaling Technology, Inc., MA) at room temperature. β -tubulin (1:2000; Cell Signaling Technology, Inc. MA) or β -actin (1:10000; Sigma-Aldrich) were used as loading controls. The labeled proteins were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) using the Kodak Gel Logic 1500 Imaging System (Carestream Health Inc., New Haven, CT). Protein expression was analyzed using Image J (NIH) software.

Statistical analysis

[0072] A power analysis was conducted using GraphPad InStat-2.00. The power was set to 80% ($\beta=0.8$) and the level of significance (α) used was 0.05. The power analysis indicated that a sample size of 5 per group was sufficient to achieve the desired power. Data are presented as mean \pm S.E.M. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc comparison test was used for intergroup comparison. Unpaired t-test was used for comparison of postnatal day 28 control vs. IRL-1620 groups. A *P* value of less than

0.05 was considered to be significant. The statistical analysis was processed using GraphPad Prism 6.02 (GraphPad, San Diego, CA).

[0073] The goal of the present study was to determine the ontogeny of ET_B receptors, VEGF and NGF in the postnatal rat brain, as well as to investigate whether or not selective stimulation of ET_B receptors during postnatal development would result in changes in the expression of either the receptors or growth factors. In confirmation of previous studies (Briyal et al., 2012b), it was found that ET_B receptors appear to decrease in the neuronal tissue with postnatal age. Interestingly, both ET_B receptor and VEGF intensity increased within the cerebral vasculature from postnatal day 1 to day 14. NGF, on the other hand, decreased concurrently with ET_B receptors in the cortex and SVZ of the rat brain from postnatal day 1 to day 14. Selective stimulation of ET_B receptors via intravenous IRL-1620 on postnatal day 21 led to a significant increase in ET_B receptors and VEGF, but not NGF. These results suggest that, while the ontogeny of ET_B receptors may be related to both vascular and neuronal growth factors within the developing brain, stimulation of ET_B receptors during postnatal development exerts more influence over angiogenesis than neurogenesis.

[0074] While the rat model is commonly used in ontological studies, it is important to recognize that gestation and development, particularly with regards to the brain, differ between rodents and humans. It has been reported that 16.7 rat days are equivalent to 1 human year (Quinn, 2005). Extrapolating this information out onto the timeline of the present study provides the following information: postnatal day 1 = 21 human days; postnatal day 7 = 5 human months; postnatal day 14 = 10 human months; and postnatal day 28 = 20 human months. This is significant as rapid brain growth for the human begins at the end of the second trimester, peaks at birth and then decreases over the next several years. Rats, however, with a gestational period of only 21 days, experience the most rapid rise in brain growth and development within the first 10 postnatal days (Gil-Mohapel et al., 2010). This period is roughly equivalent to the third trimester in human brain development.

[0075] The period of time evaluated by the present study, postnatal rat days 1 to 28, coincide with the first 2 years of equivalent human brain development. As expected, the highest levels of neuronal growth factor along with ET_B receptors were noted in the cortex and SVZ on the first day following birth. These levels decreased significantly with increasing brain maturity from day 1 to day 14. No significant change was noted between days 14 and 28. A decrease in ET_B receptor protein expression with the whole rat brain by

postnatal day 21 has been reported (Briyal et al., 2012b). The present data shed light on earlier reports of low levels of neuronal progenitors and increased CNS disturbances in ET_B deficient rat pups (Ehrenreich et al., 2000; Riechers et al., 2004; Vidovic et al., 2008). A significant co-relation was found between the declines in intensity of NGF and ET_B receptor staining in the cerebral cortex of developing brain; however, when IRL-1620 was administered on postnatal day 21, to stimulate ET_B receptors, this co-relation was lost and an increase in ET_B receptor staining was not accompanied with an increase in NGF staining in the cerebral cortex. It is possible that there is a regulatory mechanism that initiates a decrease in ET_B receptor and NGF staining as the brain matures and this regulatory mechanism does not allow IRL-1620 to produce any pharmacologically induced increase in ET_B receptor and NGF staining of the brain that is close to maturity.

[0076] ET_B receptor stimulation, therefore, does not appear to increase neurogenesis during late-stage CNS development, although it has been shown to enhance this process during adult neurovascular repair processes (Leonard and Gulati, 2013) indicating a loss of regulatory mechanism following cerebral ischemia. Selective stimulation of ET_B receptors did, however increase the overall expression of ET_B within the whole brain as measured via immunoblotting technique as well as the intensity of ET_B receptor staining of the cerebral vasculature of the developing rat brain, suggesting that such stimulation does enhance angiogenesis during the late postnatal period.

[0077] Overall, both VEGF and ET_B intensity within the cerebral vasculature increased throughout the period studied. VEGF is a potent angiogenic factor essential for CNS vascularization, development and repair. Previous research has indicated the VEGF is restricted mainly to cortical neurons early in development, but then switches to maturing glial cells around the blood vessels as the vascular bed begins to stabilize (Ogunshola et al., 2000). While VEGF expression within the neuronal tissue was not specifically determined, the results demonstrate an increase in VEGF around the vasculature as the brain develops. In the earlier stages of CNS development, when NGF levels are high, VEGF may serve as a promoter of neurogenesis, neuronal migration and neuroprotection (Rosenstein et al., 2010). Similar effects are seen in the adult brain following ischemic injury, with increased levels of VEGF promoting cerebrovascular repair (Gora-Kupilas and Josko, 2005; Nowacka and Obuchowicz, 2012). Indeed, previous studies have shown that selective stimulation of ET_B receptors following permanent cerebral ischemia leads to an increase in both VEGF and ET_B expression, coincident with neuroprotection and cerebrovascular repair (Leonard and Gulati,

2013). In the present study, selective stimulation of ET_B receptors at postnatal day 21 resulted in increases in VEGF and ET_B expression both in the cerebral vasculature and the whole brain. These results serve to both confirm the relationship between ET_B receptors and VEGF and to highlight their importance in the developing brain.

[0078] Hypoxia-ischemia brain damage during the neonatal period is one of the main factors in brain dysfunction (Li et al., 2008). Premature infants, in particular, often experience episodes of hypoxia-ischemia which can lead to reduced cortical growth and development. These impairments may continue through childhood and adolescence, and can cause dysfunction within the neural micro circuitry leading to epilepsy, neurodevelopmental disorders and psychiatric illnesses (Malik et al., 2013). Episodes of hypoxia-ischemia within the brain are known to increase hypoxia-inducible transcription factor-1, which in turn upregulates VEGF (Trollmann et al., 2008). It has been shown that selective ET_B receptor stimulation upregulates both ET_B receptors and VEGF in the brains of both normal neonates and adult rats subjected to cerebral ischemia. It is possible that early treatment with a selective ET_B receptor agonist may enhance VEGF and neuroprotection, thereby enabling the brains of infants suffering from hypoxic damage to repair this neuronal damage.

[0079] The present study indicates that selective stimulation of ET_B receptors enhances VEGF within the developing rat brain. While no similar significant upregulation of NGF was noted in the present study, a correlation in the ontogeny of ET_B receptors and NGF was observed within the cortex and SVZ of postnatal rat pups. It appears that both ET_B receptors and NGF are important in the early phase of development of the CNS. As studies have shown that selective ET_B receptor stimulation is capable of enhancing cerebrovascular repair in the adult brain following ischemia, it would be of interest to determine whether or not similar treatment could significantly improve repair mechanisms within the developing brain subjected to ischemia.

Studies in animal model of Alzheimer's disease

[0080] Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 280-310g were allowed to acclimate for at least 4 days before use. Animals were housed in a room with controlled temperature (23±1°C), humidity (50±10%), and light (6:00 A.M. to 6:00 P.M.). Food and water were available continuously. Animal care and use for experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of

Midwestern University. All anesthetic and surgical procedures were in compliance with the guidelines established by the IACUC of Midwestern University.

Agents and experimental design

[0081] Amyloid- β (1-40) (Tocris Bioscience, Ellisville, MO, USA), IRL-1620 [N-Succinyl-[Glu⁹, Ala^{11,15}] endothelin 1] (American Peptide Co, Inc., Sunnyvale, CA, USA) and BQ788 (American Peptide Co, Inc., Sunnyvale, CA, USA) were dissolved in sterile saline and all the solutions were freshly prepared before injections. Ketamine (Butler Animal Health Supply, Dublin, OH, USA) was administered at a dose of 100 mg/kg, intraperitoneally (i.p.), and xylazine (Lloyd Laboratories, Shenandoah, IA, USA) was administered at a dose of 10 mg/kg, i.p. Rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and a lateral cerebral ventricle was cannulated by placing the rat in a stereotaxic (David Kopf Instruments, Tujunga, California, USA) instrument and fixing the cannula (coordinates: 1.0 mm lateral, 1.5 mm caudal to bregma and 4.0 mm deep from the bone). Cannula (Plastics One, Roanoke, VA, USA) was anchored with dental acrylic to three screws placed in the skull. The animals were allowed to recover from surgery for at least seven days. After 7 days, rats were treated with vehicle, A β (1-40), ET_B receptor agonist and/or antagonist in the lateral cerebral ventricles using the implanted cannula. Vehicle, A β or ET_B receptor agonist and antagonist were injected in a volume of 5 μ l over a period of 5 minutes. A β (1-40) was used because it is highly soluble compared to A β (1-42) and induces endothelial dysfunction of both cerebral and systemic blood vessels in addition to memory deficit (Nitta et al., 1994; Niwa et al., 2000; Smith et al., 2004; Weller et al., 1998). Agents were delivered using 10 μ l Hamilton syringe and agent treatments were carried out individually using separate 10 μ l syringe. IRL-1620 was administered 1 hour after A β injection whereas BQ788 was administered 15 minutes prior to IRL-1620 injection. At the end of the experiment, placement of cannula was confirmed by injecting methylene blue dye (5 μ l) and observing the site and extent of staining. All experiments were performed on day 15 (Briyal et al., 2011). Water maze testing was performed from day 15 to day 19 after which animals were euthanized. Animals used for oxidative stress measurements were euthanized on day 15 without being subjected to any behavioral testing. Diabetes mellitus type 2 was induced in rats belonging to the diabetic group by administering freshly prepared streptozotocin at the dose of 45 mg/kg, i.p. Streptozotocin was dissolved in 0.01 M sodium citrate buffer of pH 4.3. On day 3, blood glucose was obtained from the rat-tail and tested for hyperglycemia

using the SureStep Complete Blood Glucose monitor kit. Rats with blood glucose levels above 11.1 mM were considered diabetic. Diabetic and non-diabetic animals were randomly divided into five groups (6 rats per group) (i) Sham, (ii) A β + Vehicle, (iii) A β + IRL-1620, (iv) A β + BQ788 (v) A β + BQ788 + IRL-1620. A β (1-40) was administered intracerebroventricularly (i.c.v.) (20 μ g in 3 equally divided doses i.e. 6.67 μ g were injected 3 times for a total of 20 μ g dose) on day 1, 7, and 14. Specific ET_B receptor agonist, IRL-1620 (3 μ g) and specific ET_B receptor antagonist, BQ788 (10 μ g) were administered i.c.v. daily for 14 days starting from day 1 of A β injection. The doses of IRL-1620 and BQ788 were selected on the basis of previous studies conducted in our laboratory (Leonard et al., 2011).

Estimation of oxidative stress markers

[0082] Malondialdehyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD) were estimated on day 15 in the rat brains. Rats were decapitated and the brains quickly removed, cleaned with chilled saline and stored at -80 °C. The biochemical analysis was performed within 48 hours.

Measurement of lipid peroxidation

[0083] MDA, a marker of lipid peroxidation, was measured spectrophotometrically (Ohkawa et al., 1979). Briefly, the whole brain of each animal was removed separately and was homogenized with 10 times (w/v) in 0.1M sodium phosphate buffer (pH 7.4). Acetic acid 1.5 ml (20 %), pH 3.5, 1.5 ml thiobarbituric acid (0.8 %) and 0.2 ml sodium dodecyl sulfate (8.1 %) were added to 0.1 ml of processed tissue sample. The mixture was then heated at 100 °C for 60 minutes. The mixture was cooled, and 5 ml of butanol:pyridine (15:1 % v/v) and 1 ml of distilled water were added. After centrifugation at 4,000 rpm for 10 minutes, the organic layer was withdrawn and absorbance was measured at 532 nm using a spectrophotometer.

Measurement of Glutathione

[0084] Glutathione was measured spectrophotometrically (Ellman, 1959). Briefly, whole brain was homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). This homogenate was then centrifuged with 5 % trichloroacetic acid to separate the proteins. To 0.1 ml of supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5'5 dithiobis (2-nitrobenzoic acid) (DTNB) and 0.4 ml of double distilled water was added. The mixture was vortexed and the absorbance read at 412 nm within 15 min.

Measurement of Superoxide dismutase

[0085] SOD was estimated as described by Kakkar et al (Kakkar et al., 1984). Briefly, whole brain was homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). The reagents sodium pyrophosphate buffer 1.2 ml (0.052 M) pH 8.3, 0.1 ml phenazine methosulphate (186 μ M), 0.3 ml nitro blue tetrazolium (300 μ M) and 0.2 ml NADH (780 μ M) were added to 0.1 ml of processed tissue sample. The mixture was then incubated for 90 min at 30 °C. Then 4 ml of n-butanol and 1 ml of acetic acid were added. The mixture was shaken vigorously. After centrifugation at 4,000 rpm for 10 minutes, the organic layer was withdrawn and absorbance was measured at 560 nm using a spectrophotometer. Protein was estimated using Lowry's method (Lowry et al., 1951).

Morris water maze (MWM) test for cognitive impairment

[0086] Spatial learning and memory of animals were tested in a MWM (Morris, 1984). A circular water tank (132 cm diameter, 60 cm height, painted white was filled with water (25 ± 2 °C) to a depth of 40 cm, the water was rendered opaque by the addition of non-fat milk. The pool was divided into four equal quadrants, labeled north, south, east, and west. A circular, white escape platform (10 cm in diameter) was submerged approximately 2 cm below the surface of the water, 10 cm off the edge of the tank at a position designated as quadrant II (target quadrant). A video camera was mounted on the ceiling in the center of the pool. The escapes latency and swimming path length was monitored with a Videomex tracking system and data were collected using Videomex Water Maze Software (Columbus Instruments, Ohio, USA).

[0087] The platform remained in the same quadrant during the entire acquisition phase experiments and removed in probe trial. Acquisition trials (4 trials per day for 4 days) were started by placing rat in a pool facing the wall of the tank from different randomly chosen start positions, and time required to find the invisible platform was recorded. A trial lasted until the rat found the platform or until 60 seconds had elapsed and an inter-trial interval of approximately 30 seconds. If rat did not find the platform within 60 seconds, it was guided to the platform and placed on it for 60 seconds. Time to reach the platform (latency in seconds) and swimming path length (in centimeters) was measured. After completion of the fourth trial on each day, the rat was dried and returned to its home cage. Twenty four hours after the final acquisition trial, the platform was removed from the pool and a probe trial lasting 60 seconds was performed; the time spent in the target quadrant was recorded. Time spent in the

target quadrant indicated the degree of memory consolidation which had taken place after learning.

Statistical analysis

[0088] Results were expressed as Mean \pm S.E.M. In acquisition trials of Morris water maze following parameters were recorded: escape latency (time required to reach the platform from the releasing point in seconds), and path length (distance traveled by rat from the release point to reach platform in centimeters). Analysis of variance (ANOVA) was conducted on these data, with group as the between-subject factor and with repeated measures such as trial and day as within subject factors. Post hoc analysis (Tukey's test) was used to determine significance between the groups. For probe trial data, time spent in quadrant II were recorded and analyzed by one way ANOVA and post hoc analysis by Bonferroni's test. Oxidative stress measures were analyzed by one way ANOVA followed by post hoc analysis using Bonferroni's test. All analysis was carried out using GraphPad Prism Statistical Software, version 5.00 (GraphPad, San Diego, CA, USA). $P < 0.05$ represents level of significance.

Effect of diabetes mellitus type 2 on rats

[0089] Diabetic rats were sluggish and had decreased locomotion as compared to non-diabetic rats. However, diabetic and non-diabetic rats had similar performance in Morris water maze tests (Fig. 8-11). Figure 7 illustrates that there was no difference in oxidative stress parameters between diabetic and non-diabetic rats.

Effect of ET_B receptor agonist and antagonist on oxidative stress parameters in A β -treated non-diabetic and diabetic rats

[0090] To determine the involvement of ET_B receptors in A β induced changes in oxidative stress parameters, malondialdehyde, reduced glutathione and superoxide dismutase levels in the brains of sham and A β treated rats were measured following administration of vehicle, IRL-1620 or BQ788+IRL-1620 (Fig. 1).

Effect on brain malondialdehyde levels

[0091] Brain levels of malondialdehyde (MDA) were measured to determine the effect of ET_B receptor stimulation on lipid peroxidation following A β treatment (Fig. 7A). As expected, levels of MDA were significantly ($p < 0.0001$) higher in A β treated rats for both

non-diabetic and diabetic groups compared to sham groups. MDA level for non-diabetic A β treated animals was 516.13 ± 14.02 nmol/g wet tissue which was greater compared to sham (112.1 ± 1.84 nmol/g wet tissue) animals. In diabetic rats, MDA level was 531.58 ± 9.02 nmol/g wet tissue in A β treated while it was 114.32 ± 2.05 nmol/g wet tissue in sham animals. MDA levels were significantly ($P < 0.001$) reduced in IRL-1620 treated rats compared to vehicle treated A β rats for both non-diabetic (278.47 ± 8.55 nmol/g wet tissue) and diabetic (315.09 ± 5.25 nmol/g wet tissue) animals. Administration of BQ788 prior to IRL-1620, blocked IRL-1620 induced change in MDA levels and the levels were similar to those seen in vehicle treated rats.

Effect on brain reduced glutathione levels

[0092] Reduced glutathione (GSH) levels in A β treated non-diabetic and diabetic animals were significantly ($P < 0.0001$) lower than those of sham operated animals. The mean GSH level for non-diabetic and diabetic A β treated groups were 102.5 ± 5.96 and 81.2 ± 4.33 μ g/g wet tissue, respectively, while that in sham rats it was 239.1 ± 8.0 μ g/g wet tissue. Treatment with IRL-1620 significantly ($P < 0.001$) increased levels of GSH in the brains of A β treated non-diabetic and diabetic rats (192.74 ± 6.26 and 166.42 ± 6.63 μ g/g wet tissue, respectively) (Fig. 7B). Pretreatment with BQ788 blocked the positive effect of IRL-1620 treatment on GSH levels (81.2 ± 5.49 μ g/g wet tissue; $P < 0.001$).

Effect on brain superoxide dismutase levels

[0093] The levels of superoxide dismutase (SOD) in the brains of vehicle treated non-diabetic (13.23 ± 0.53 U/mg protein) and diabetic (11.07 ± 0.54 U/mg protein) A β rats were significantly ($P < 0.0001$) lower than those of sham operated group (35.22 ± 1.43 U/mg protein). Administration of IRL-1620 significantly improved SOD levels in non-diabetic and diabetic rats (22.26 ± 1.16 and 21.4 ± 1.65 U/mg protein, respectively) (Fig. 7C). Similar to GSH, SOD levels were significantly ($P < 0.001$) lower when non-diabetic and diabetic A β animals were pretreated with BQ788 prior to IRL-1620 administration (15.32 ± 0.44 and 16.52 ± 0.45 U/mg protein, respectively).

Effect of ET_B receptor agonist and antagonist on memory deficit in A β treated non-diabetic and diabetic rats

[0094] No significant difference was seen between non-diabetic and diabetic rats in water maze acquisition (Fig. 8 and 10) and probe trial test (Fig. 9 and 11). Rats treated with A β

took significantly ($p < 0.0001$) longer time (escape latency) to find the platform. Sham group improved their performance in the hidden platform test as indicated by a decrease in escape latencies across successive days (day effect, $F_{(3,100)} = 3.968$, $p < 0.001$). There was a significant difference between escape latencies on day 1, 2, 3 and 4 in A β rats treated with vehicle compared to sham ($F_{(3,59)} = 8.273$, $p < 0.0001$). However, when ET_B receptor agonist, IRL-1620 was administered to A β treated rats, escape latency decreased significantly on day 3 and 4 of training when compared to A β rats treated with vehicle ($F_{(4,59)} = 19.602$, $p < 0.001$). IRL-1620 significantly improved cognitive impairment caused by A β treatment in rats. On the other hand, administration of an ET_B receptor antagonist, BQ788, blocked the improvement in escape latency produced by IRL-1620 in A β rats (Fig. 8 and 10). A difference in escape latency is also shown in representative trajectories of each group during an acquisition trial in the water maze task (Fig. 8 and 10). The distance traveled by a rat to reach the platform (path length) decreased across days ($F_{(3,51)} = 76.3$, $p < 0.0001$), and there was also a significant day \times group interaction ($F_{(12,100)} = 8.88$, $p < 0.0001$) indicating differences among groups within each trial day. There was a significant difference between sham, A β rats treated with vehicle and A β rats treated with IRL-1620 ($F_{(2,57)} = 24.2$, $p < 0.01$); post hoc analysis showed that A β rats treated with vehicle group swam longer path lengths as compared to sham group ($p < 0.001$). A β rats treated with IRL-1620 group swam significantly ($p < 0.001$) shorter path lengths as compared to vehicle group indicating beneficial effects of IRL-1620 treatment (Fig. 8 and 10). A β produced significant impairment of cognitive function in rats which could be improved with ET_B receptor agonist, and was blocked by specific ET_B receptor antagonist.

[0095] Removal of platform from the target quadrant (quadrant II) resulted in a general tendency to swim preferentially in the target quadrant as opposed to other quadrants (probe trial). Therefore, time spent in quadrant II was compared for all the groups in order to observe effect of agents on memory retention. Time spent in other quadrants, specifically, does not reflect degree of memory consolidation and was therefore not subjected to analysis. In probe trial, time spent in the target quadrant was significantly decreased in rats treated with A β compared to sham treated rats indicating memory deficit in A β treated rats.

Administration of ET_B receptor agonist, IRL1620, significantly ($p < 0.0001$) increased time spent in the target quadrant compared to vehicle treated A β rats (Fig. 9 and 11).

Administration of an ET_B receptor antagonist, BQ788, to A β treated rats did not produce any improvement in time spent in the target quadrant. Difference in time spent in the target quadrant is shown in representative trajectories of each group during probe trial in the water

maze task (Fig. 9 and 11). A β produced significant memory deficit in rats which significantly improved with ET_B receptor agonist treatment but was blocked by specific ET_B receptor antagonist.

[0096] The purpose of this study was to determine the effect of selective ET_B receptor stimulation by IRL-1620 on functional recovery following experimentally induced cognitive impairment by intracerebroventricular injections of A β in adult diabetic and non-diabetic rats. The current findings suggest that IRL-1620 produced a significant preventative effect in A β induced cognitive impairment in both diabetic and non-diabetic rats. In order to confirm that the effects of IRL-1620 were specific to stimulation of ET_B receptors, a selective ET_B receptor antagonist, BQ788, was used to block the effect of IRL-1620. A β treatment produced a significant increase in oxidative stress markers in non-diabetic and diabetic rats and treatment with selective ET_B receptor agonist, IRL-1620, significantly reduced oxidative stress markers. The reduction in oxidative stress marker induced by IRL-1620 was blocked by pretreatment with specific ET_B receptor antagonist, BQ788, in A β non-diabetic and diabetic rats.

[0097] The incidence of diabetes mellitus and AD increases with age, and the incidence of AD is significantly higher in patients with diabetes mellitus (Janson et al., 2004). Pathologically, both are associated with altered glucose homeostasis and extracellular accumulation of amyloid proteins. This suggests that there are common underlying mechanisms such as cross-seeding of amyloid proteins or metabolic dysfunction. Therefore, the effect of IRL-1620 was studied in both non-diabetic and diabetic rats treated with A β .

[0098] The prevalence of dementia, particularly of AD type is increasing and it is one of the most significant neurodegenerative disorders in the elderly. Recent studies support that metabolic and vascular dysfunctions are involved in pathology and progression of AD. Vascular alterations, with impairment of glucose utilization and blood flow changes, occur with and prior to AD diagnosis (Baquer et al., 2009; Casadesus et al., 2007; Meier-Ruge et al., 1994). These changes precede cognitive impairment and exacerbate underlying AD pathology. The discovery and prevention of vascular dysfunction could lead to new strategies to prevent or halt the progression of AD. Closely linked with vascular changes in AD is A β , the major protein component of senile plaques in AD brains. An elevated level of A β in the brain is one of the prominent features of AD (Hardy and Selkoe, 2002). Tissues that produce the most A β are the brain and skeletal muscles, both of which possess high metabolism and well-developed vascular networks (Cirrito et al., 2005; Ethell, 2010).

Vascular damage and reactive gliosis are co-localized with amyloid deposits in AD brains, suggesting that vasculature may be a clinically significant site of AD pathology (Suo et al., 1998).

[0099] ET system has long been known to play an important role in the regulation of cerebral blood circulation. Several studies have demonstrated involvement of ET in AD. Due to highly potent vasoconstriction caused by ET-1 via ET_A receptors, it was postulated that administration of an ET antagonist would decrease the damage associated with AD. ET_A receptor antagonists, BQ123 and BMS182874, demonstrated reduced oxidative stress and improve the learning and memory deficit following A β treatment in rats. However, a combined ET_{A/B} receptor antagonist had no beneficial effect (Briyal et al., 2011). This led us to investigate the involvement of ET_B receptors in AD. In fact, the ET binding sites in the brain are predominantly of ET_B receptors, and ET_B receptor agonists act as anti-apoptotic factor against the neurotoxicity of A β (Yagami et al., 2002). Elevation of A β is directly implicated in vascular pathology, and vascular dysfunction in AD is characterized by disruption of vascular architecture including lower capillary density and reduced blood flow (Bell and Zlokovic, 2009; de la Torre, 1994; Iadecola et al., 2009; Zlokovic, 2008). Conversely, activation of endothelial ET_B receptors is known to elicit vasodilatation, and previous studies in our lab have indicated that this leads to an increase in CBF in normal rats (Leonard and Gulati, 2009), indicating a possible role of ET_B agonists in the treatment and prevention of AD.

[0100] In the present study, A β treated rats received either vehicle or IRL-1620 for 14 days. On day 15, animals were evaluated for cognitive impairment and their brains were removed for analysis of oxidative stress markers. Treatment with IRL-1620 effectively reduced oxidative stress as measured by decreased levels of malondialdehyde (MDA) and increased levels of reduced glutathione (GSH) and superoxide dismutase (SOD) compared to vehicle treated group. Blockade of ET_B receptors with BQ788, on the other hand, resulted in an increase in oxidative stress. Increased levels of MDA along with decreased levels of antioxidants GSH and SOD are all hallmarks of oxygen free radical generation occurring as an early event in AD pathology (Cutler et al., 2004; Nunomura et al., 2001). The etiology of AD is thought to be complex and initiates a variety of biochemical reactions leading to excess intracellular Ca²⁺, glutamate excitotoxicity, production of reactive oxygen species, and eventual apoptosis. Agents that target these events in order to slow or prevent irreversible injury are labeled as neuroprotective. Oxidative stress and A β are related to each other

(Hensley et al., 1994; Mark et al., 1997). Oxidative stress also contributes to vascular dysfunction. It has been reported that oxidative damage during pathogenesis of AD may be directly due to A β (Murray et al., 2007; Murray et al., 2005). It is disclosed herein that the ET_B receptor agonist, IRL-1620, decreased the oxidative stress markers that were increased by A β . It appears that A β can produce vasoconstriction and an increase oxidative stress, both of which could be mediated through ET. Stimulation of endothelial ET_B receptors is known to elicit vasodilatation, and previous studies in our lab have indicated that this leads to an increase in CBF. Hence, ET_B receptor agonists may be quite effective in preventing the damage due to A β in AD.

[0101] Thus, it is disclosed herein that stimulation of ET_B receptors following A β treatment leads to functional recovery. Behavioral studies were conducted using MWM to determine whether ET_B receptor agonists are able to improve the impairment of learning and memory caused by A β . It was found that A β produced a significant impairment in spatial memory as evidenced by significantly longer escape latencies and no preference for the quadrant which previously contained the platform in the probe trial. Other researchers have also shown learning and memory deficits due to A β (Ahmed et al., 2010; Lopes et al., 2010; Tsukuda et al., 2009). It is shown herein that the specific ET_B receptor agonist, IRL-1620, significantly improved the spatial memory deficit caused by A β treatment. On the other hand, antagonism of ET_B receptors with BQ788, given prior to either vehicle or IRL-1620, resulted in learning and memory deficit similar to those seen in vehicle group. These results suggest that the improvement seen with IRL-1620 is due to selective stimulation of the ET_B receptors. The observed functional deficits coincided with changes observed in oxidative stress markers.

[0102] Activation of ET_B receptors with IRL-1620 is known to cause cerebral vasodilatation and increased blood flow through release of nitric oxide (NO) (Kitazono et al., 1995; Leonard and Gulati, 2009; Tirapelli et al., 2005). Previous findings have revealed that cerebral neurovascular dysfunction in relation to bioavailability of NO formed by endothelial NO contributes to cognitive decline and neurodegeneration in AD (de la Torre et al., 2003). NO also plays an obligatory role in the regulation of CBF and cell viability and in the protection of nerve cells in AD (Toda et al., 2009). Recent studies have demonstrated that endothelial NO stimulation and increased CBF via pharmacological means enhance angiogenesis (Chen et al., 2007; Ding et al., 2008). Along these lines, the ET_B receptor has been shown to enhance the formation of new blood vessels through eNOS (Goligorsky et al.,

1999). Previous reports using an ET_B receptor deficient model have indicated that this receptor promotes neuronal survival and decreases apoptosis in the hippocampus, dentate gyrus, and olfactory epithelium (Ehrenreich, 1999; Laziz et al., 2011; Riechers et al., 2004). Both induction of eNOS and direct anti-apoptotic neuronal effects of ET_B receptor activation may play a role in reduction of oxidative stress and improvement in behavioral recovery following AD.

[0103] In conclusion, reduction in oxidative stress and improvement in cognitive impairment following ET_B receptor agonist, IRL-1620, and attenuation of these effects by a specific ET_B receptor antagonist, BQ788, in the current study indicates that ET_B receptors may be a new therapeutic target for neuroprotection in AD.

Studies in animal model of stroke

[0104] Effect of IRL-1620 on levels of brain endothelin receptors in middle cerebral artery occluded rats: ET_B receptors are present in large number in the CNS and appear to play a key role in its development. It has been demonstrated that ET_B receptors in the brain are overexpressed at the time of birth and their expression decreases with maturity of the brain (Briyal et al., 2012b). It has also been shown that acute ischemic phase is followed by an intense sprouting of neurons and capillaries (Carmichael, 2006; Murphy and Corbett, 2009) along with activation of glial cells to create an environment for neuronal growth and plasticity (Hermann and Zechariah, 2009; Zhang and Chopp, 2009). A regenerative response will be pharmacologically activated in the ischemic brain by stimulating ET_B receptors. Stimulation of ET_B receptors by IRL-1620 has been shown to provide neuroprotective effect in MCAO rats (Leonard et al., 2011; 2012; Leonard and Gulati, 2013). ET_A receptors are increased in the infarcted hemisphere at 24 hours post ischemia and subsequently return to normal levels by one week, on the other hand a significant increase in ET_B receptor expression occurs after 1 week only in the infarcted hemisphere of rats treated with IRL-1620 (Leonard et al., 2012). It is contemplated that IRL-1620 not only stimulates ET_B receptors but in longer periods increases the number and affinity of these receptors.

[0105] Effect of IRL-1620 on neurological deficit following focal cerebral ischemia: In a preliminary study, the effect of selectively activating ET_B receptors by IRL-1620 following permanent middle cerebral artery occlusion in rats was determined. Twenty-four hours after middle cerebral artery occlusion, there was a significantly ($P<0.001$) higher neurological deficit and poor motor function compared to sham-operated rats, indicative of neurological

impairment following induction of cerebral ischemia. Animals treated with IRL-1620 showed significant improvement in all neurological and motor function tests when compared with vehicle-treated. In a longer term study, cerebral ischemia resulted in a distinct loss of motor coordination as measured by the foot fault error and rota rod tests at 1, 4, and 7 days post infarction. Whereas vehicle-treated occluded rats performed worse with each assessment, animals treated with ET_B receptor agonist, IRL-1620, showed minimal deficit at day one following occlusion, and improved over the course of 7 days. Pretreatment with ET_B receptor antagonist, BQ-788, followed by either vehicle or IRL-1620 resulted in significantly more deficits than both sham-operated ($P<0.001$) or IRL-1620 ($P<0.05$) treatment, indicating that the improvement observed with IRL-1620 is specific to the stimulation of ET_B receptors (Leonard et al., 2011; 2012).

[0106] Effect of IRL-1620 on binding characteristics of ET_B receptors following focal cerebral ischemia: Changes in binding characteristics of ET_B receptors were determined in the brain, 1 and 7 days following MCAO. MCAO was produced in rats and binding studies were performed using [¹²⁵I]-IRL-1620 (specific activity 2200 Ci/mmol) as the radioligand and cold IRL-1620 (0-32 nM) as displacer. Non-specific binding was determined using 1 μM concentration of IRL-1620. K_d and B_{max} values were calculated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego). Binding characteristics (K_d and B_{max}) were not altered at 24 hours post MCAO. However, a significant decrease in K_d values of ET_B receptor binding in both left and right hemispheres was observed 7 days post-MCAO. The decrease in K_d in the right (ischemic) hemisphere was significantly ($P<0.001$) greater compared to left (non-ischemic) hemisphere. B_{max} was increased in both left and right hemispheres with the right hemisphere showing a significantly ($P<0.001$) greater increase compared to the left hemisphere (Figure 14). It can be concluded that an increase in the density and affinity of ET_B receptors on the 7th day of cerebral ischemia is an attempt to provide neuroprotection of ischemic brain.

[0107] Effect IRL-1620 on infarct volume in middle cerebral artery occluded rats: Middle cerebral artery occlusion for 7 days resulted in an infarct volume of $177.06 \pm 13.21 \text{ mm}^3$ in vehicle-treated rats. Administration of IRL-1620 significantly reduced infarct volume ($54.06 \pm 14.12 \text{ mm}^3$; $P<0.05$) as compared with vehicle. Infarct volumes did not reduce when ET_B receptor antagonist, BQ-788, was given with either vehicle or IRL-1620 (Fig. 12). A substantial edema was noted in the vehicle-treated animals, with the infarcted hemisphere $9.73 \pm 1.26\%$ larger than the contralateral hemisphere, whereas IRL-1620-treated animals

showed no significant edema, with infarcted hemisphere only $1.51 \pm 1.81\%$ larger than non-infarcted hemisphere. Conversely, blockade of the ET_B receptor with BQ-788 followed by either vehicle or IRL-1620 treatment significantly increased edema (17.02 ± 3.17 and $17.97 \pm 5.17\%$, respectively, $P < 0.01$) (Leonard et al., 2012).

[0108] Effect of IRL-1620 on 7 day survival of rats following focal cerebral ischemia:

Studies were also conducted to determine the effect of stimulating ET_B receptors using a selective agonist, IRL-1620, in rats with middle cerebral artery occlusion (MCAO). It was found that there was no mortality in sham treated rats throughout the 7 day observation period. However, MCAO rats in the vehicle treated group presented with 38% mortality by 7th day. On the other hand, MCAO rats treated with IRL-1620 showed no mortality throughout the 7 day period. However, MCAO rats treated with an ET_B receptor antagonist, BQ-788+vehicle or BQ-788+IRL-1620, showed 25% mortality during the 7 day observation (Figure 13) (Leonard et al., 2012). Preliminary results and supporting literature have prompted the investigation of the mechanism involved in neuroprotective and neurorestorative effects of stimulating ET_B receptors in rats with cerebral ischemia.

[0109] Effect of IRL-1620 on angiogenesis and neurogenesis following cerebral ischemia in rats:

Angiogenesis and neurogenesis are the driving forces for the neurovascular remodeling that is essential post-stroke to restore normal brain function (Hawkins and Davis, 2005). VEGF is an endogenous protein known for its ability to promote angiogenesis and enhance vascular permeability. Under hypoxic conditions such as cerebral ischemia, VEGF expression is induced in neurons, astrocytes and endothelial cells via hypoxia-inducible factor-1 (HIF-1) (Breier and Risau, 1996). Once expressed, VEGF initiates both direct and indirect neuroprotective actions, inhibiting apoptosis, stimulating neurogenesis and angiogenesis, increasing glucose uptake and activating antioxidants (Gora-Kupilas and Josko, 2005). It has been shown that intracerebroventricular (i.c.v.) administration of an ET_B receptor agonist in normal rats stimulates production of VEGF and activates VEGF receptors in the brain, while, in cultured astrocytes, this agonist increases VEGF-A mRNA as well as BrdU incorporation (Koyama et al., 2012; Koyama et al., 2011). ET_B receptor agonist, IRL-1620, has been shown in our previous studies, to provide significant neuroprotection at both 24 hours and 1 week following permanent cerebral ischemia. Therefore, the neuroprotective and neurorestorative effect following stimulation of ET_B receptors in cerebral ischemic rats was studied (Leonard and Gulati, 2013). At 24 hours post occlusion, it was found that IRL-1620 treatment increased ET_B receptor expression and

preserved neuronal numbers in the cortex, striatum and subventricular zone (SVZ) of the ischemic rat brain (Figures. 15-22). IRL-1620 also enhanced the number of blood vessels labeled with vascular endothelial growth factor (VEGF) when compared to vehicle treatment (Figures 18-20). By 1 week following MCAO, VEGF-positive vessels/30 μm brain slice in the IRL-1620 group numbered 11.33 ± 2.13 versus 4.19 ± 0.79 in the vehicle group ($P < 0.01$), indicating an increase in angiogenesis. Additionally, animals receiving IRL-1620 displayed an increased number of proliferating cells ($P < 0.0001$) and cells positively staining for NGF ($P < 0.0001$) in the infarcted brain. NGF-positive cells in the cortex, striatum and SVZ of IRL-1620 treated animals numbered 2.29 ± 0.31 , 2.08 ± 0.26 , and 3.05 ± 0.38 per 100 μm^2 , respectively, demonstrating a significant increase in neurogenesis as compared to the vehicle group, which averaged less than 1 NGF-positive cell per 100 μm^2 (Figures 21 and 22). Pretreatment with ET_B antagonist, BQ-788, blocked the effects of IRL-1620 treatment, confirming the role of ET_B receptors in the neurovascular remodeling actions of IRL-1620. Results of the present study indicate that IRL-1620, administered on the day of infarct, is neuroprotective and enhances angiogenic and neurogenic remodeling following cerebral ischemia (Leonard and Gulati, 2013).

[0110] ETB receptor agonist, IRL-1620, in the treatment of ischemic stroke: It is disclosed herein that specific ETA receptor antagonists prevent $\text{A}\beta$ induced increase in expression of ETA receptors, oxidative stress and cognitive deficits. However, it was observed that when a combined ETA/B receptor antagonist was used, the beneficial effects were lost (Briyal et al., 2011). These findings led to the investigation of the role of ET_B receptors in CNS disorders. ET_B receptors are present in large number in the CNS and appear to play a key role in its development. It has been demonstrated that ET_B receptors in the brain are overexpressed at the time of birth and their expression decreases with maturity of the brain (Briyal et al., 2012b). It has also been shown that damaged brain exhibits a re-emergence of childhood organizational patterns, reminiscent of an ontogenetic state and is primed for recovery. However, endogenous remodeling of the CNS is not sufficient to restore neurological function. It has been found that ET_B receptor agonist, IRL-1620 [Suc-[Glu9,Ala11,15]-Endothelin-1(8-12)], can increase the expression of ET_B receptors in the CNS. An increase in ET_B receptors can produce reduction in apoptosis and promote angiogenesis and neurogenesis. It has been shown that expression of ET_B receptors is increased in neurons, glia, and macrophages following ischemia. Additionally, studies demonstrate that ET_B receptor activation enhanced proliferation of neurons and inhibit

apoptosis. A regenerative response was pharmacologically activated in damaged brain by stimulating ET_B receptors. ET_B receptor stimulation, via selective ET_B agonist, IRL-1620, significantly improved neurological deficit, motor functions, and oxidative stress markers and decreased infarct volume following ischemia in rats (Leonard et al., 2011; 2012). ET_B receptor agonist, IRL-1620, provides significant neuroprotection at both 24 hours (Leonard et al., 2011) and 1 week (Leonard et al., 2012) following permanent cerebral ischemia and reduced infarct volume by 83.66% in acute study and 69.49% in chronic study. IRL-1620 treatment increased ET_B receptor expression and preserved neuronal numbers in the cortex, striatum and subventricular zone (SVZ) of the ischemic rat brain. IRL-1620 also enhanced the number of blood vessels labeled with vascular endothelial growth factor (VEGF) when compared to vehicle treatment (Leonard and Gulati, 2013). Thus, IRL-1620 administered intravenously was found to be highly effective in preventing damage following stroke and aids in the neurovascular remodeling of ischemic brain by angiogenesis and neurogenesis (Leonard and Gulati, 2013). Studies further indicate that stimulation of ET_B receptors by IRL-1620 provides neuroprotection (Leonard et al., 2011; 2012), and it can be used as a therapeutic agent for Alzheimer's disease (Briyal et al., 2011). It has been demonstrated that IRL-1620 prevents cognitive impairment and oxidative stress induced by A β (Briyal et al., 2011). It is contemplated that enhancement of possible survival mechanisms through stimulation of ET_B receptors by IRL-1620 leads to a better recovery following cerebral ischemia. Most of the stroke patients show substantial neurological improvement (Dimyan and Cohen, 2011) indicating endogenous restorative mechanisms. Hence there is a potential to develop pharmacological agents that can stimulate and amplify these mechanisms. The two major approaches that can be used for the treatment of cerebral ischemia are neuroprotection, which requires an acute intervention, and neurorestoration, which can be instituted during the stroke recovery phase (Andres et al., 2011; Bacigaluppi et al., 2009; Liu et al., 2008). Several trials have been conducted or are in progress using pharmacological agents such as amphetamine, methylphenidate, levodopa, sildenafil, serotonin uptake inhibitors, erythropoietin, statins, and granulocyte colony stimulating factor but none involves stimulation of ET_B receptors. It is contemplated herein that stimulation of ET_B receptors produces, in various embodiments, neuroprotection, neurorestoration, or both.

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Table 1: Effect of ET_B receptor agonist, IRL-1620, and antagonist, BQ788, on neurological deficit and motor function post middle cerebral artery occlusion. IRL-1620 (5 μ g/kg, i.v.) or isotonic saline (1 ml/kg, i.v.) was injected at 2, 4, and 6 h post MCAO. BQ788 (1 mg/kg, i.v.) was administered 15 min prior to the first injection of IRL-1620 or vehicle. Values are expressed as mean \pm SEM (n=5-8/group). *P<0.05 vs. sham. #P<0.05 vs. MCAO + vehicle. @P<0.05 vs. MCAO + IRL-1620.

Treatment Groups		Neurological Evaluation (6 point scale)	Grip Test (6 point scale)	Foot Fault Error (%)	Rota Rod Duration (sec)	Distance Traveled (cm)	Vertical Breaks
Sham	Baseline	0 \pm 0	4.00 \pm 0.29	3.96 \pm 0.82	88.89 \pm 9.18	4968 \pm 242	65.62 \pm 3.18
	Day 1	0 \pm 0	4.00 \pm 0.29	4.04 \pm 1.02	144.89 \pm 9.23	3325 \pm 324	37.63 \pm 3.33
	Day 4	0 \pm 0	3.67 \pm 0.24	4.57 \pm 0.91	150.67 \pm 8.80	5323 \pm 474	58.67 \pm 1.53
	Day 7	0 \pm 0	4.00 \pm 0.41	7.04 \pm 2.50	136.67 \pm 19.61	4306 \pm 314	53.67 \pm 12.31
MCAO + Vehicle	Baseline	0 \pm 0	3.89 \pm 0.26	4.56 \pm 0.89	100.33 \pm 7.54	5069 \pm 329	54.56 \pm 7.65
	Day 1	3.11 \pm 0.31*	1.00 \pm 0.29*	57.64 \pm 7.39*	24.33 \pm 5.87*	764 \pm 216*	1.33 \pm 0.53*
	Day 4	2.75 \pm 0.57*	1.25 \pm 0.32*	72.74 \pm 6.63*	32.50 \pm 13.57*	2353 \pm 787*	15.00 \pm 7.53*
	Day 7	2.75 \pm 0.57*	1.50 \pm 0.43*	58.19 \pm 10.85*	50.00 \pm 18.55	2366 \pm 660	20.25 \pm 7.80*
MCAO + IRL-1620	Baseline	0 \pm 0	3.86 \pm 0.46	4.05 \pm 1.31	113.29 \pm 8.34	5073 \pm 334	61.50 \pm 4.94
	Day 1	1.29 \pm 0.36#	2.71 \pm 0.52#	18.85 \pm 6.48#	78.71 \pm 22.59*	1611 \pm 325*	12.75 \pm 4.76*
	Day 4	0.67 \pm 0.22#	2.33 \pm 0.58	14.00 \pm 3.66#	97.33 \pm 2.08	2898 \pm 451	26.33 \pm 2.08
	Day 7	0.67 \pm 0.22#	3.33 \pm 0.22	8.28 \pm 1.09#	123.67 \pm 7.28	3472 \pm 732	34.33 \pm 3.78
MCAO + BQ788	Baseline	0 \pm 0	3.33 \pm 0.33	5.96 \pm 1.75	102.00 \pm 5.14	5141 \pm 285	55.17 \pm 3.74
	Day 1	3.00 \pm 0.58* [@]	0.67 \pm 0.33* [@]	52.12 \pm 11.93* [@]	37.83 \pm 20.87*	1168 \pm 417*	5.33 \pm 2.47*
	Day 4	3.33 \pm 0.62* [@]	0.33 \pm 0.24*	68.89 \pm 12.27* [@]	37.67 \pm 14.92*	1246 \pm 410*	4.33 \pm 1.52*
	Day 7	3.00 \pm 0.82* [@]	1.67 \pm 0.62	75.00 \pm 17.67* [@]	49.67 \pm 17.56	2280 \pm 836	11.00 \pm 3.97*
MCAO + BQ788 + IRL-1620	Baseline	0 \pm 0	4.50 \pm 0.34	4.92 \pm 1.50	127.33 \pm 16.77	5642 \pm 358	48.00 \pm 9.68
	Day 1	3.00 \pm 0.37* [@]	1.67 \pm 0.33*	61.01 \pm 10.82* [@]	51.17 \pm 19.11*	742 \pm 85*	2.00 \pm 1.48*
	Day 4	3.67 \pm 0.47* [@]	1.00 \pm 0.40*	60.26 \pm 14.59* [@]	62.00 \pm 23.25	1223 \pm 414	6.67 \pm 3.42*
	Day 7	3.00 \pm 0.82* [@]	1.33 \pm 0.62*	57.63 \pm 17.63* [@]	54.00 \pm 35.04	2185 \pm 818	17.33 \pm 8.56*

WHAT IS CLAIMED IS:

1. A method of treating a neuropsychiatric disorder comprising administering to a patient in need thereof a therapeutically effective amount of an endothelin-B receptor agonist to treat the neuropsychiatric disorder.
2. The method of claim 1 wherein the endothelin-B receptor agonist is co-administered with an additional agent to treat the neuropsychiatric disorder.
3. The method of claim 2, wherein the additional agent is selected from the group consisting of an antidepressant, an anti-inflammatory agent, a CNS stimulant, a neuroleptic, and an anti-proliferative agent.
4. The method of any one of claims 1-3 wherein the endothelin-B receptor agonist is selected from the group consisting of IRL-1620, BQ-3020, [Ala^{1,3,11,15}]-Endothelin, Sarafotoxin S6c, endothelin-3, and a mixture thereof.
5. The method of any one of claims 1-4 wherein the neuropsychiatric disorder is selected from the group consisting of a cerebrovascular disease, stroke, cerebral ischemia, cerebral hemorrhage, head trauma, brain injury, a brain tumor, multiple sclerosis and demyelinating diseases, dementia, vascular dementia, Alzheimer's disease, Parkinson's disease, Huntington's disease, ataxia, motor neuron disease, Amyotrophic lateral sclerosis, drug intoxication, alcoholism, chronic brain infections, brain abscess, white matter disease, Binswanger's disease, Moyamoya disease, perinatal hypoxia, cerebral asphyxia, intracranial birth injury, congenital malformation of the brain, mood disorders, and depression.
6. The method of any one of claims 1-5 wherein the endothelin-B receptor agonist is administered at a dose ranging from 0.0001 to 0.5 mg/kg.
7. The method of any one of claims 1-6 wherein the endothelin-B receptor agonist is administered repeatedly at intervals of 1 to 6 hours after every two to five days.

8. A composition comprising (a) an endothelin-B receptor agonist, (b) an agent used to treat a neuropsychiatric disorder, and optionally (c) an excipient.

9. An article of manufacture comprising:

(a) a packaged composition comprising an endothelin-b receptor agonist and an agent for treating a neuropsychiatric disorder;

(b) an insert providing instructions for a simultaneous or sequential administration of the endothelin-b receptor agonist and the agent for the neuropsychiatric disorder to treat a patient; and

(c) a container for (a) and (b).

10. The method of claim 9, wherein the endothelin-B receptor agonist is IRL-1620.

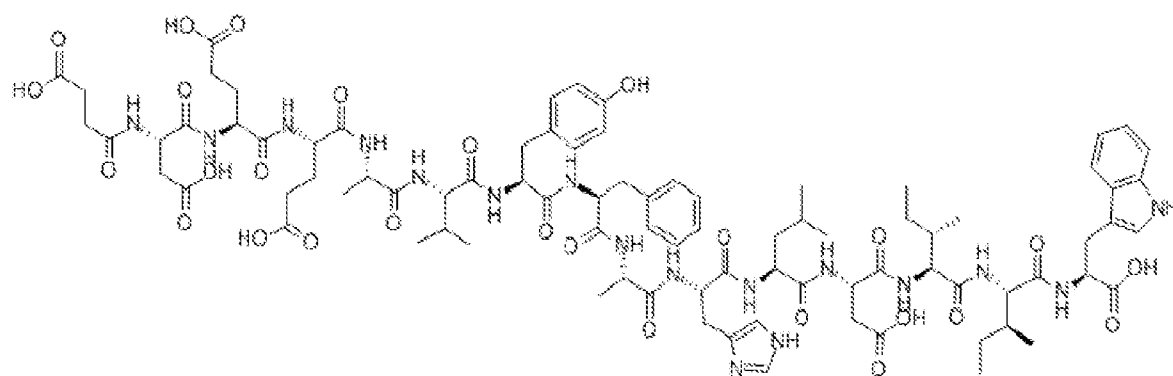
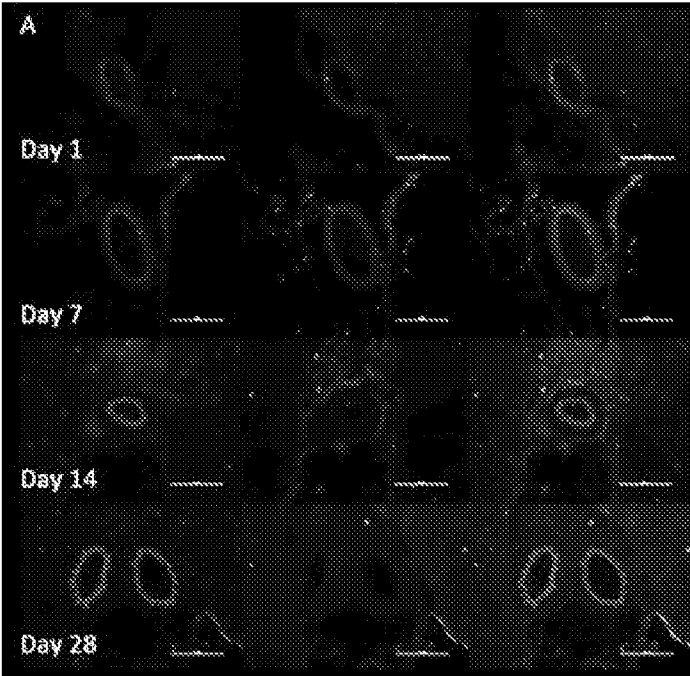


FIGURE 1



FIGURES 2A

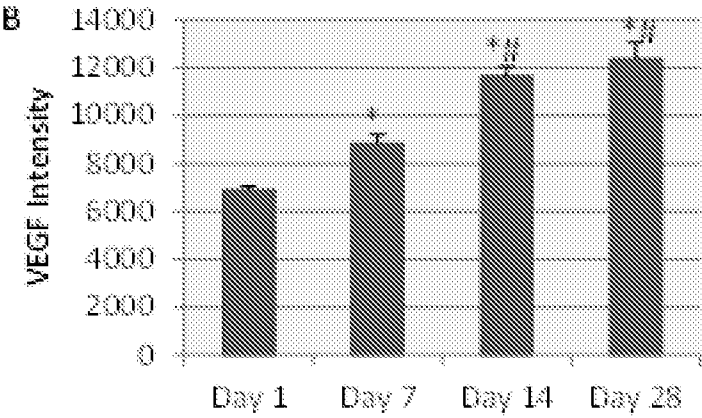


FIGURE 2B

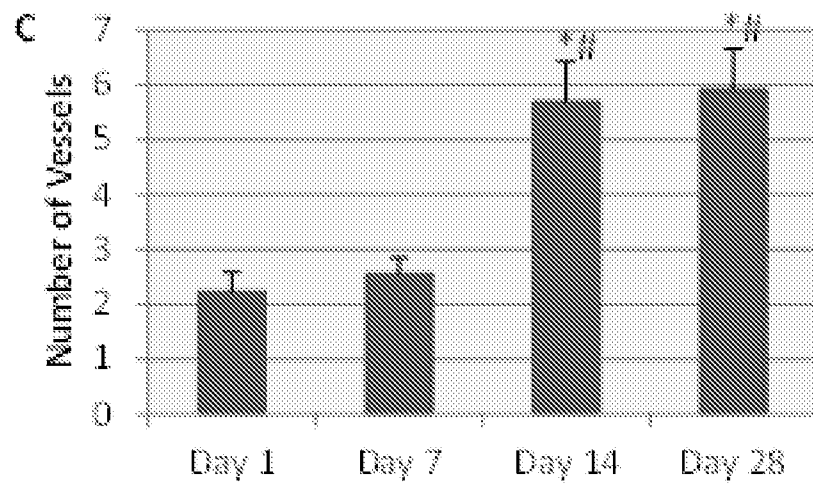


FIGURE 2C

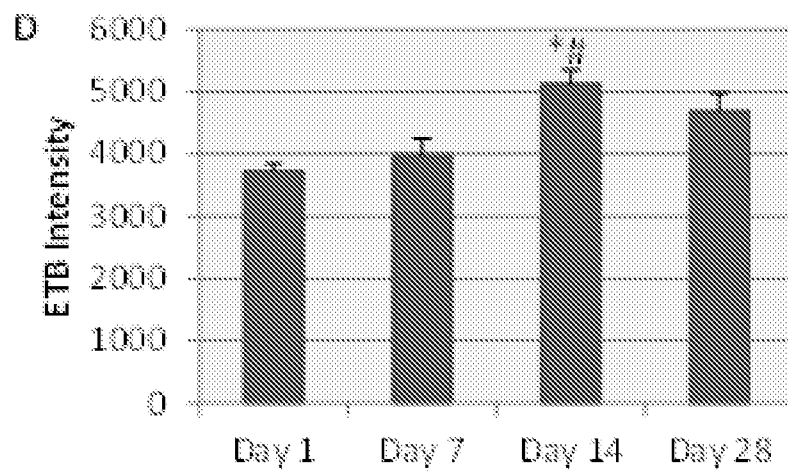


FIGURE 2D

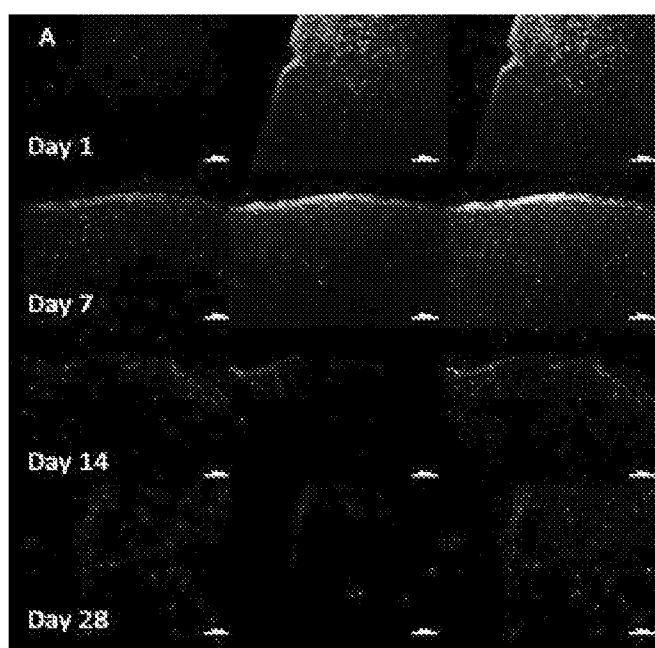


FIGURE 3A

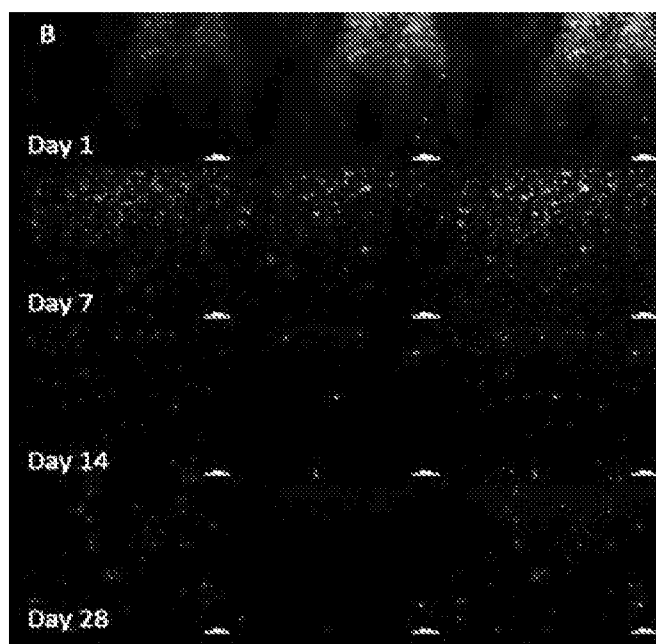


FIGURE 3B

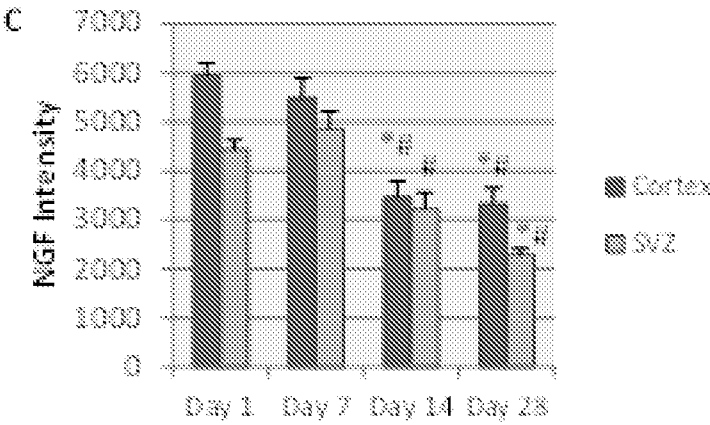


FIGURE 3C

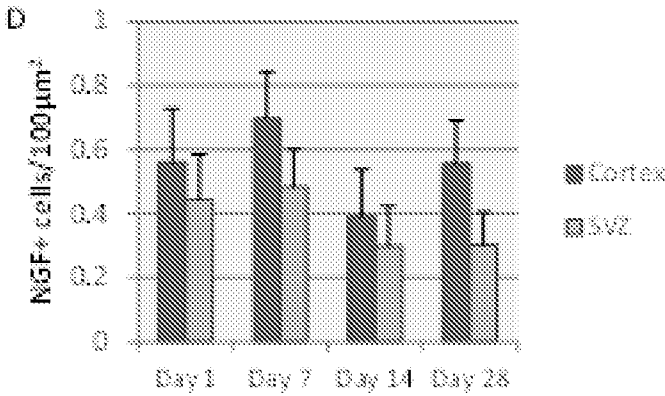


FIGURE 3D

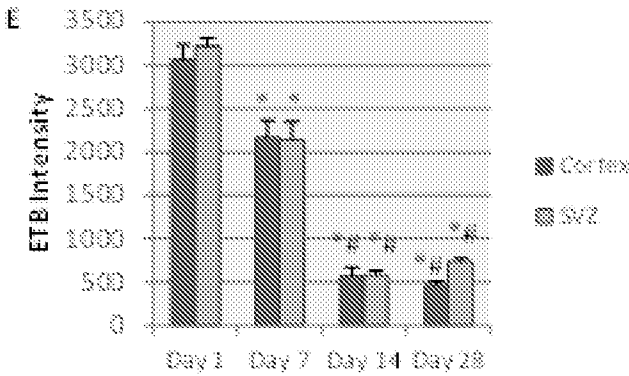


FIGURE 3E

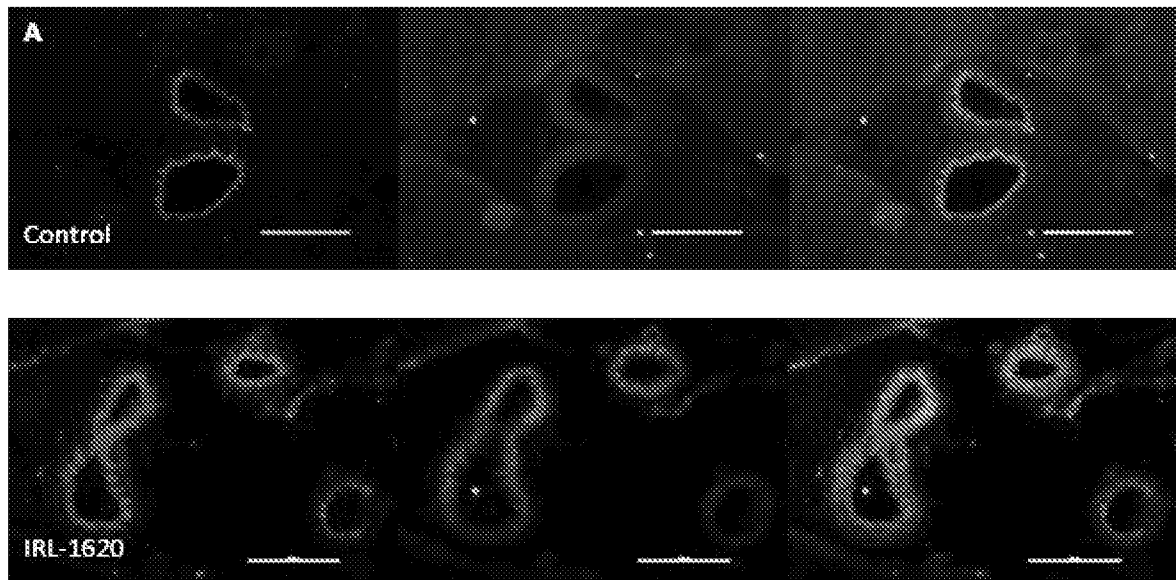


FIGURE 4A

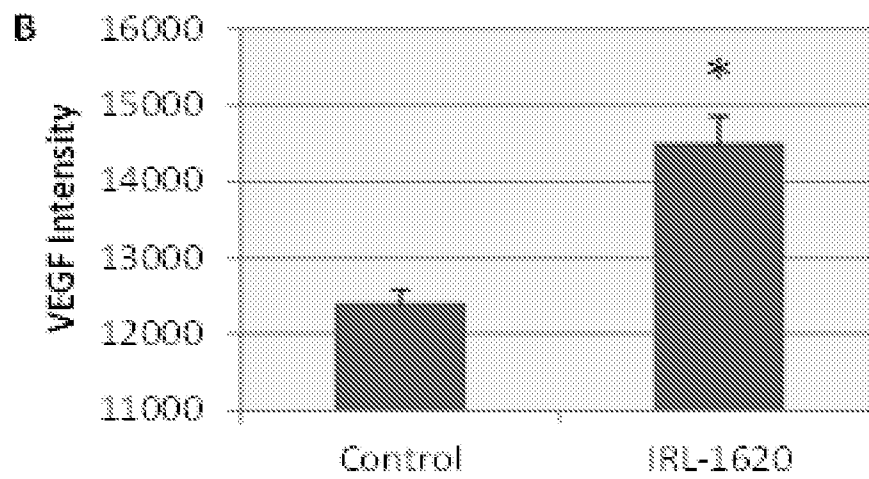


FIGURE 4B

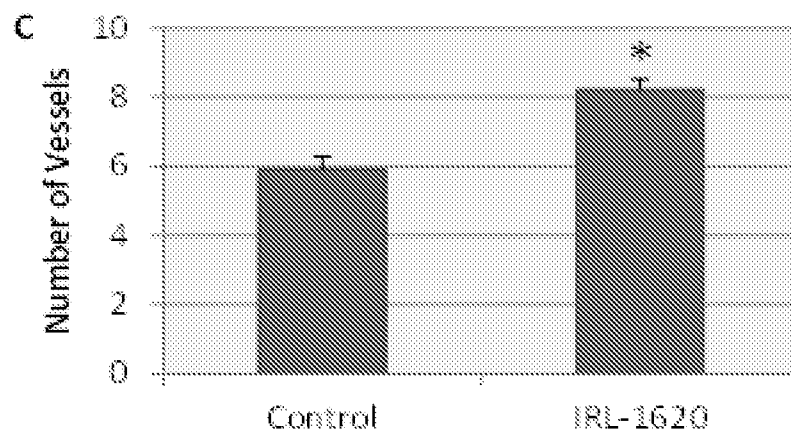


FIGURE 4C

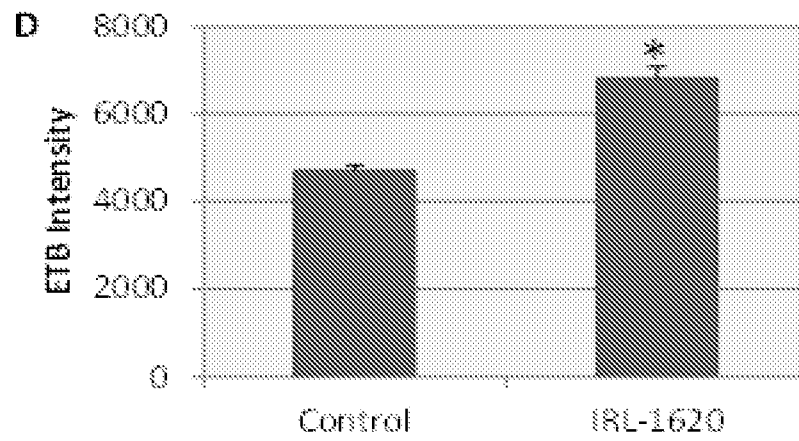


FIGURE 4D

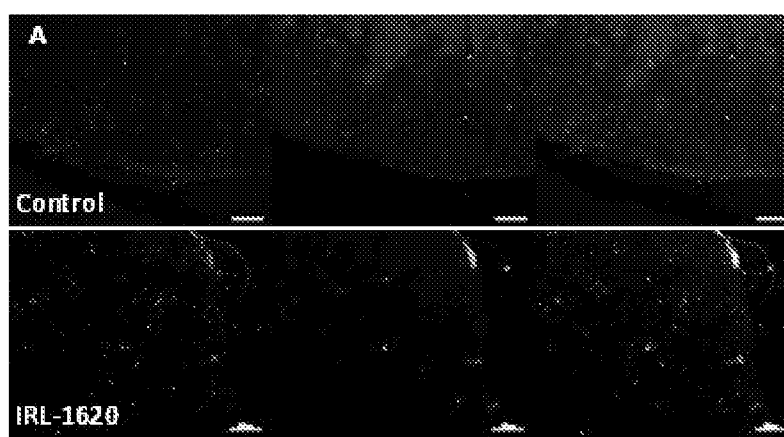


FIGURE 5A

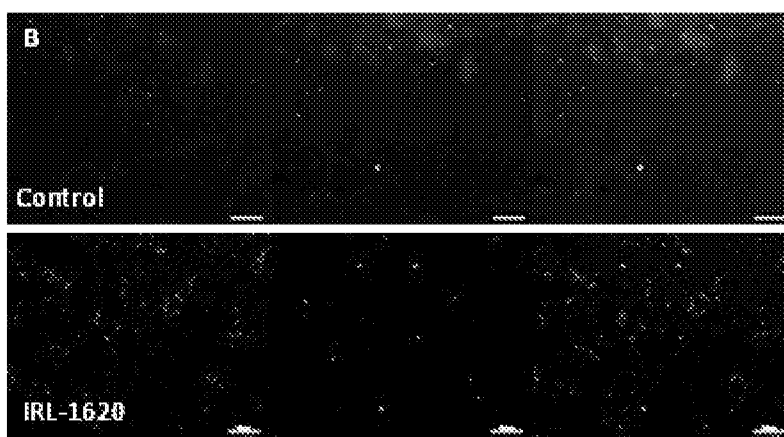


FIGURE 5B

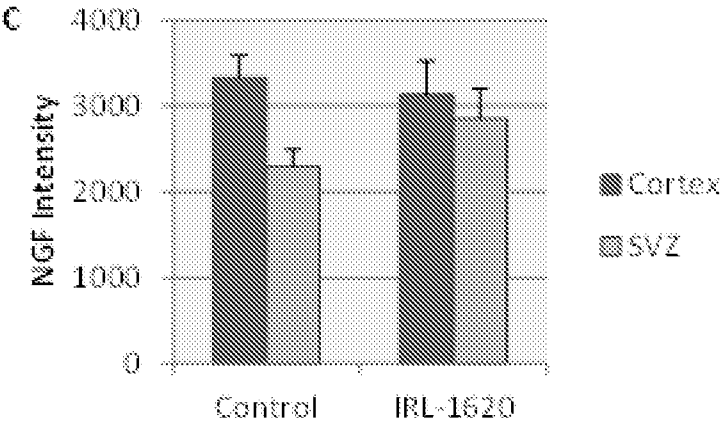


FIGURE 5C

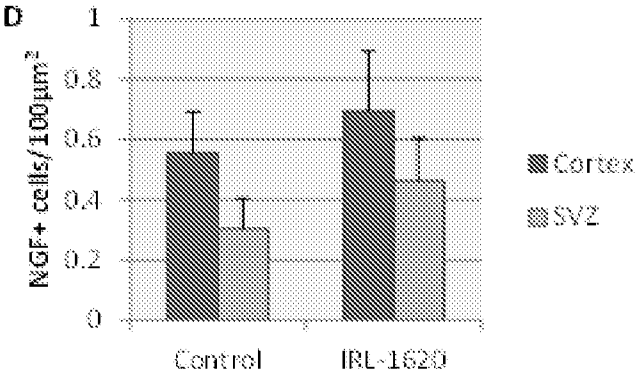


FIGURE 5D

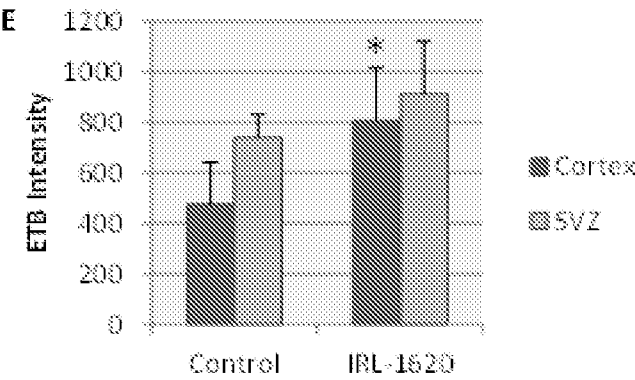


FIGURE 5E

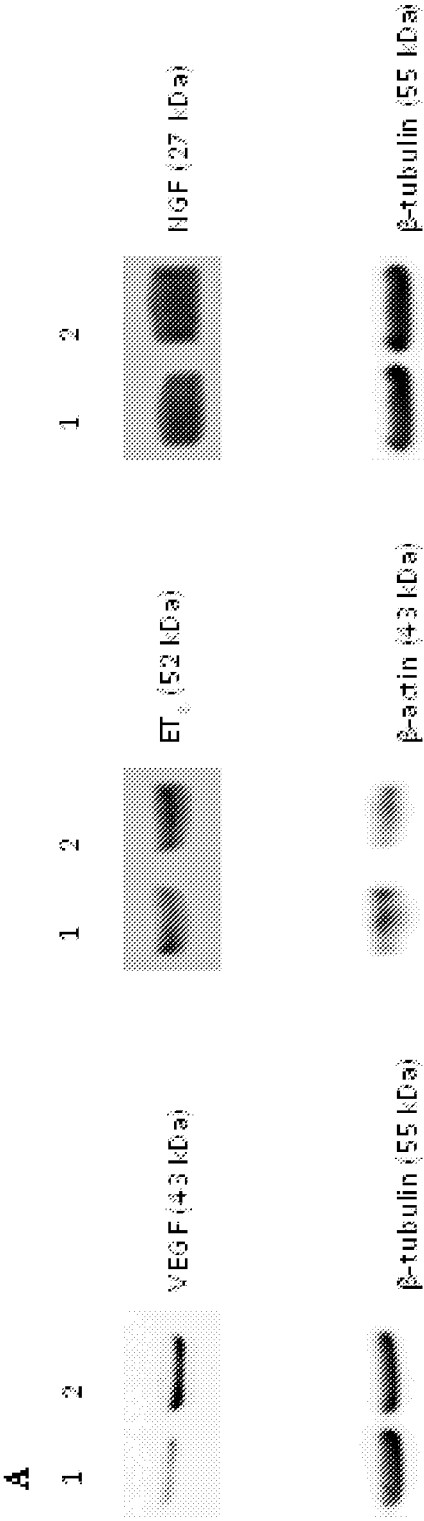


FIGURE 6A

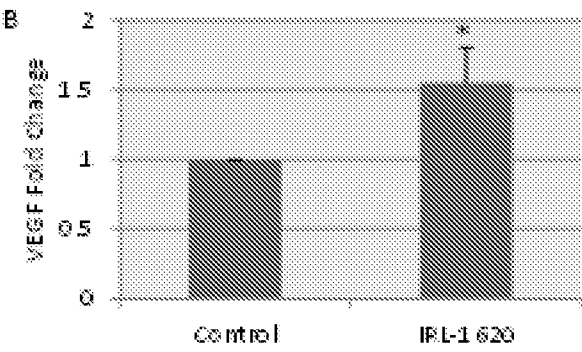


FIGURE 6B

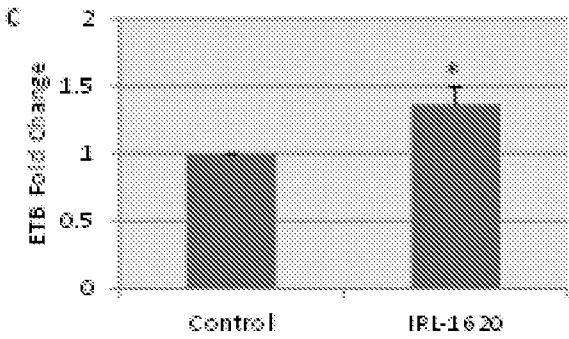


FIGURE 6C

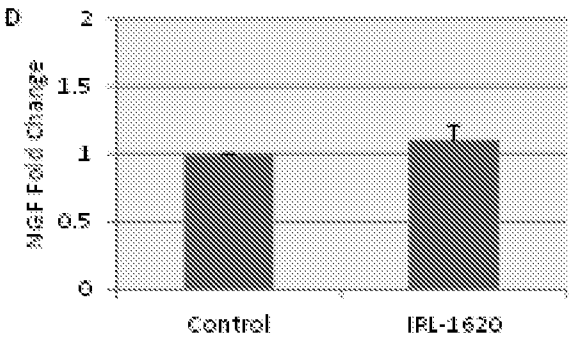


FIGURE 6D

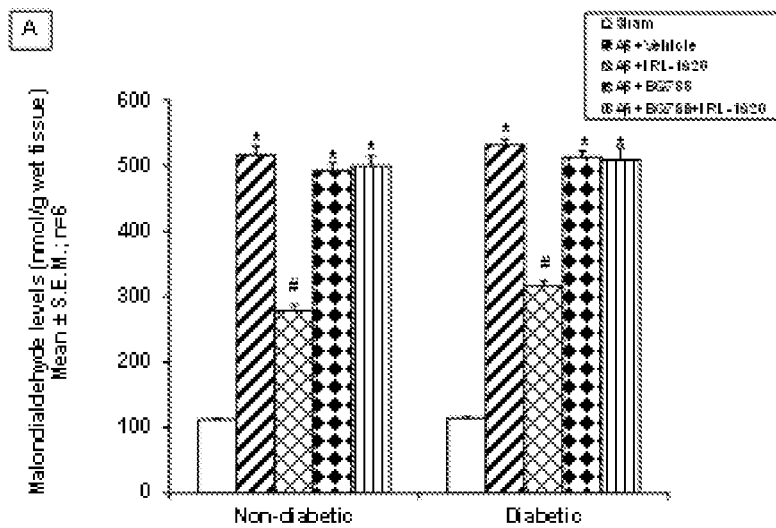


FIGURE 7A

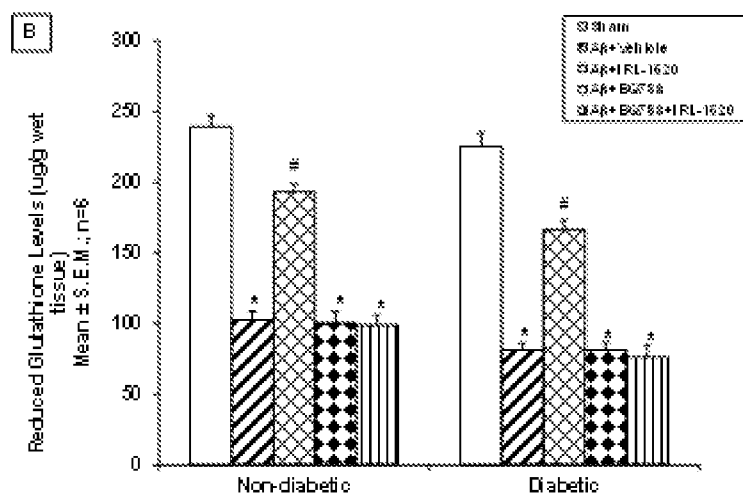


FIGURE 7B

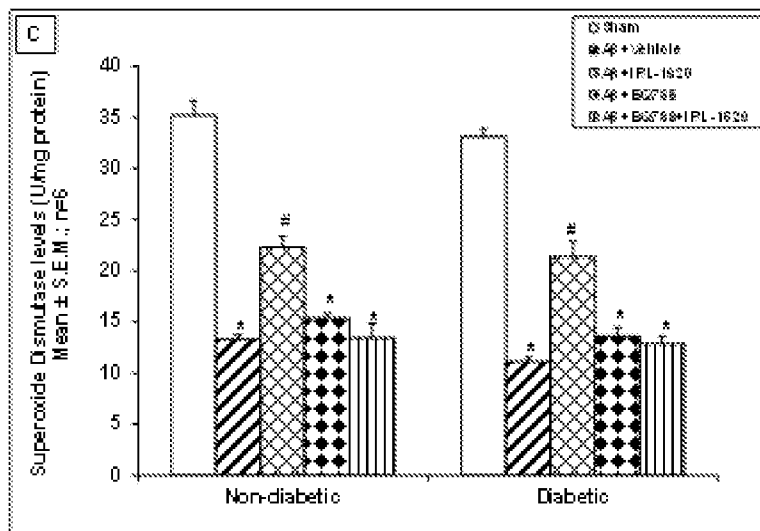


FIGURE 7C

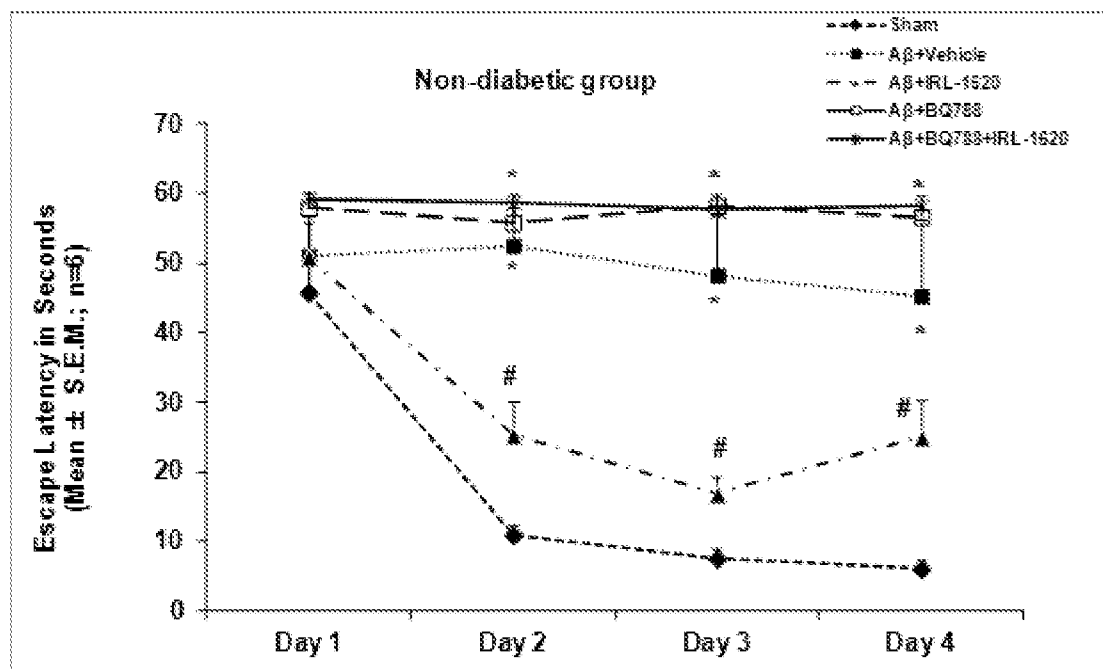


FIGURE 8A

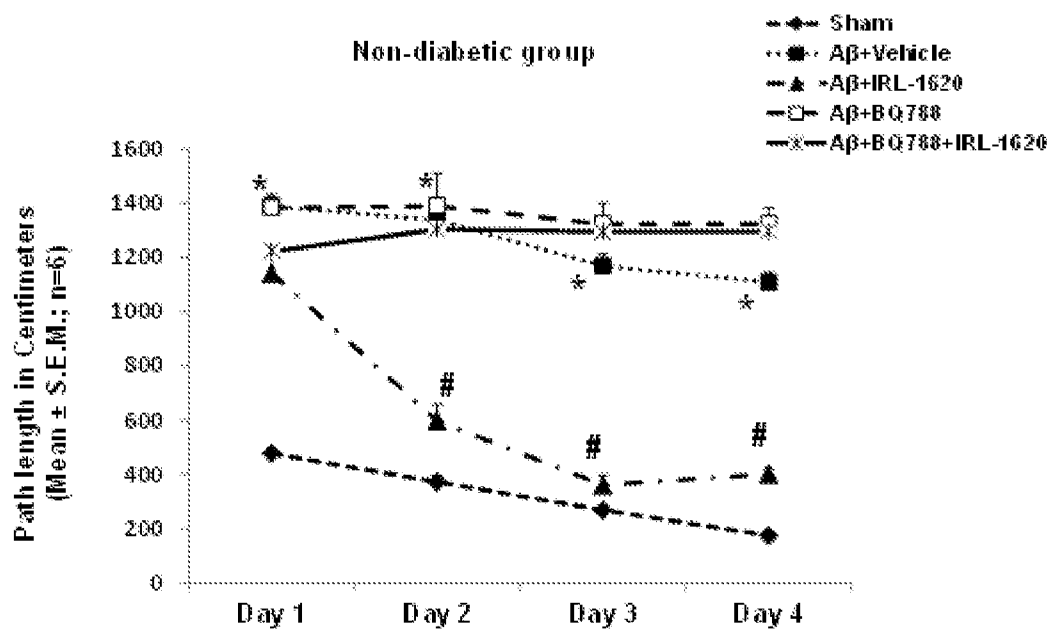


FIGURE 8B

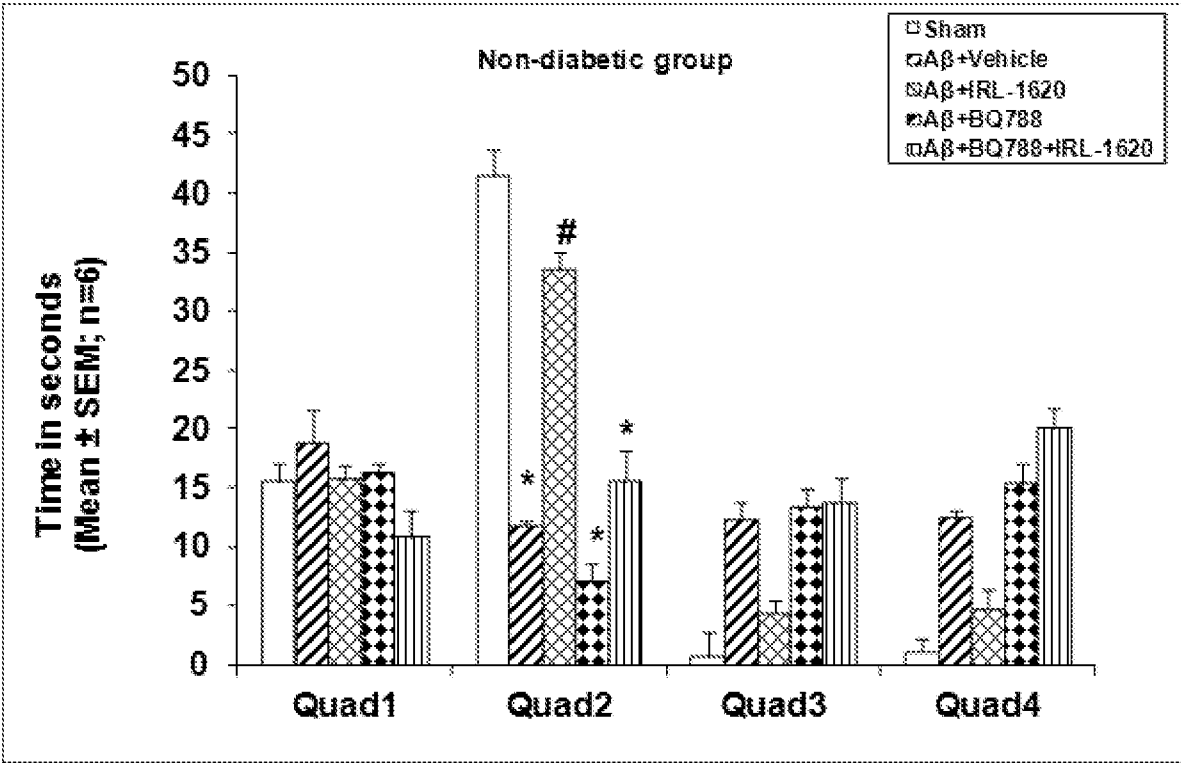


FIGURE 9A

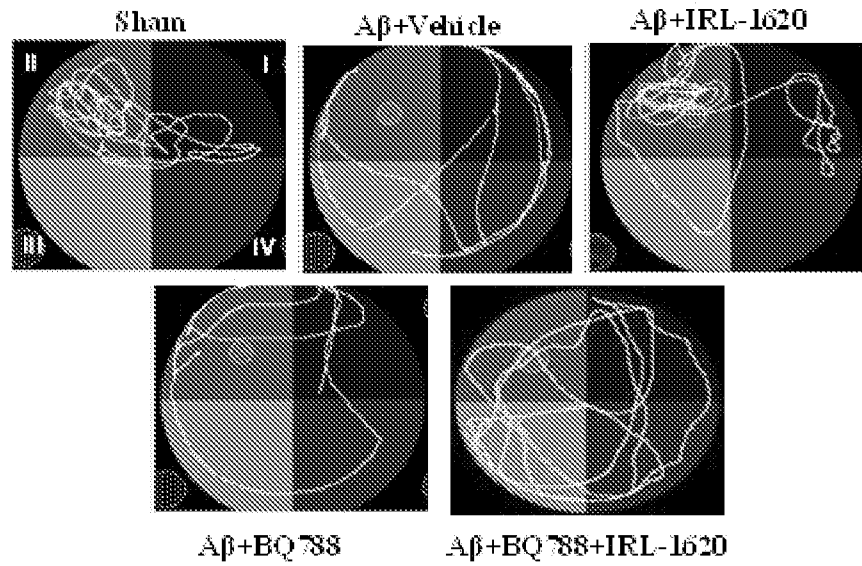


FIGURE 9B

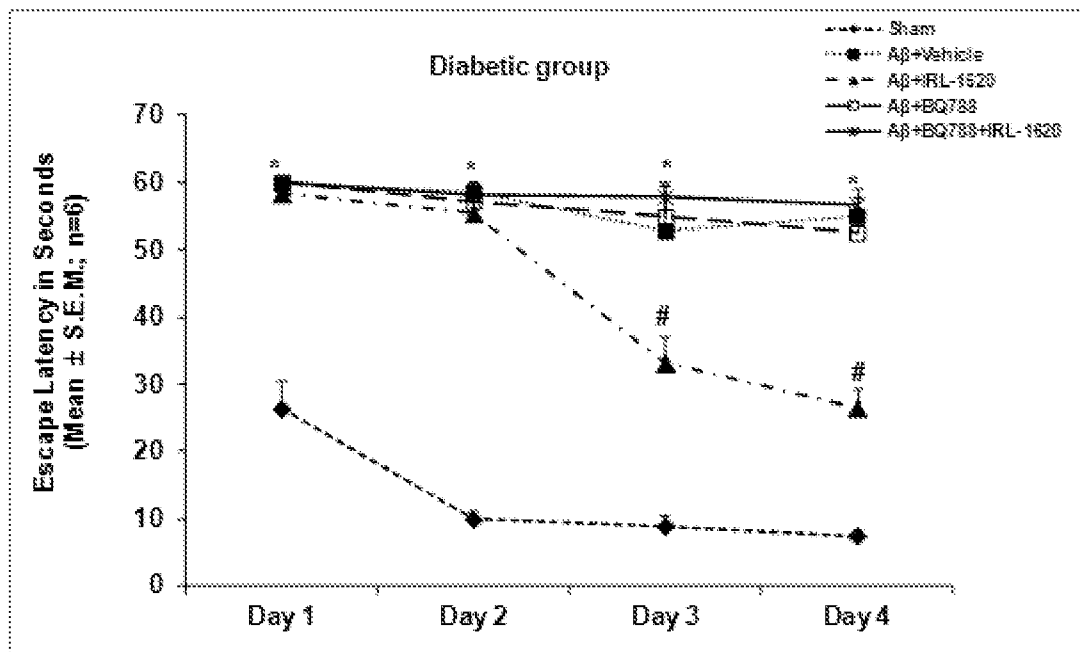


FIGURE 10A

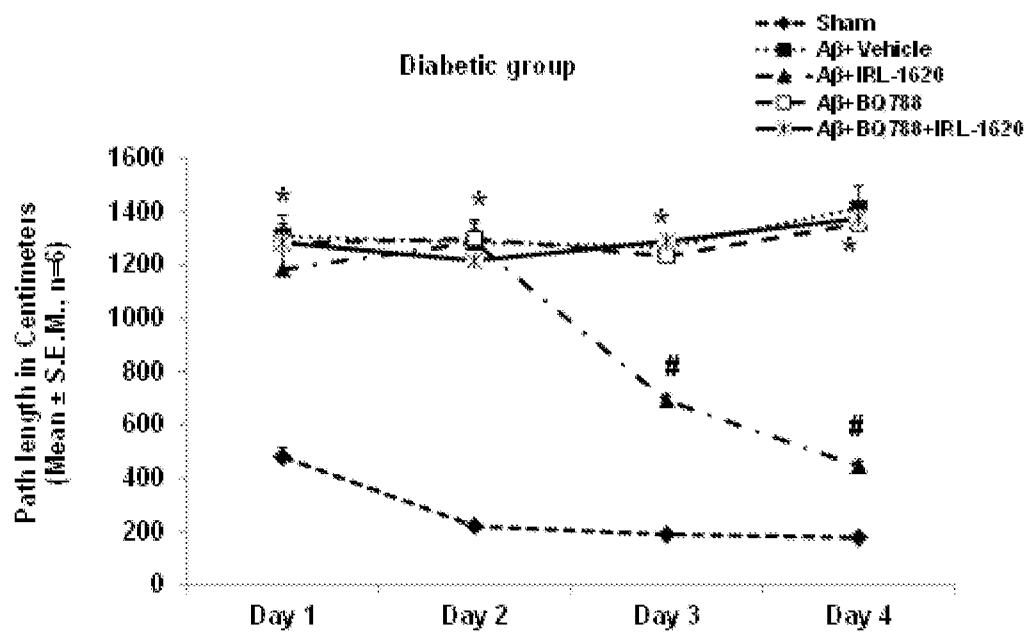


FIGURE 10B

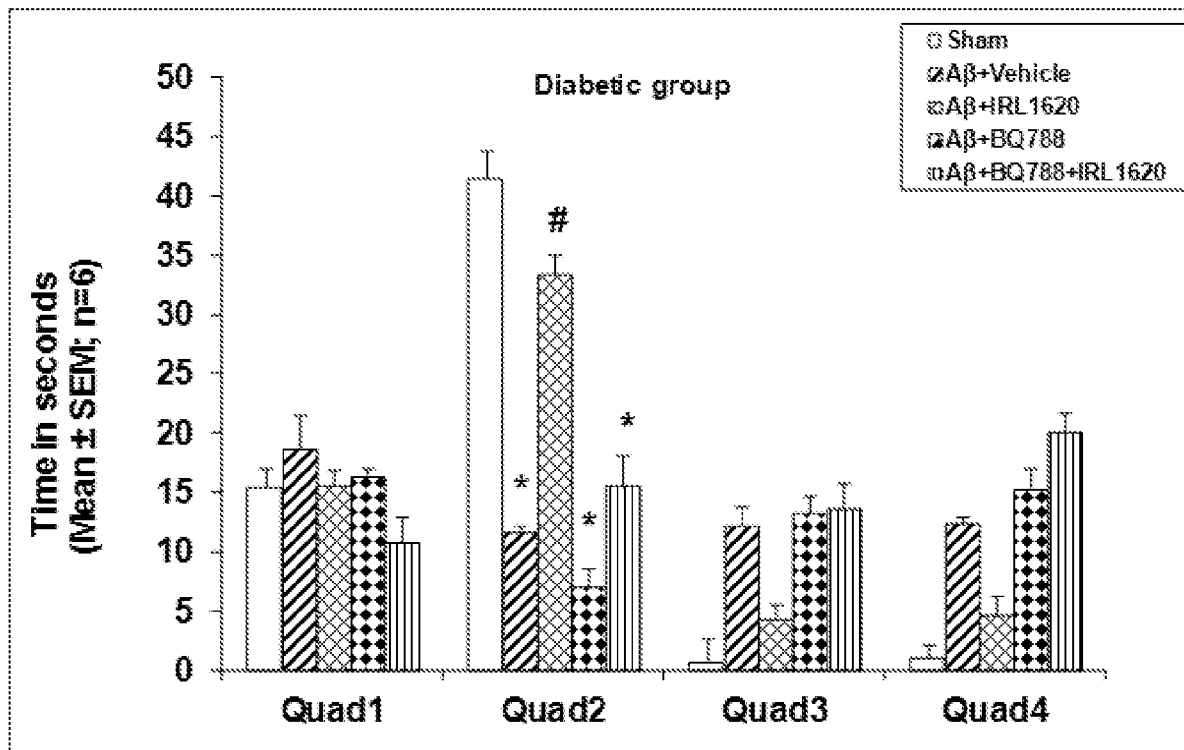


FIGURE 11A

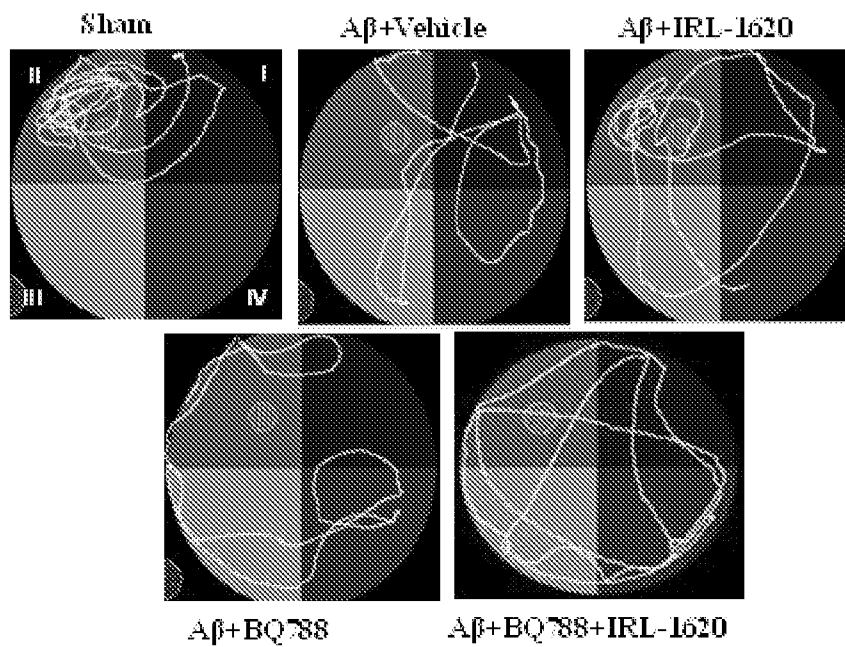


FIGURE 11B

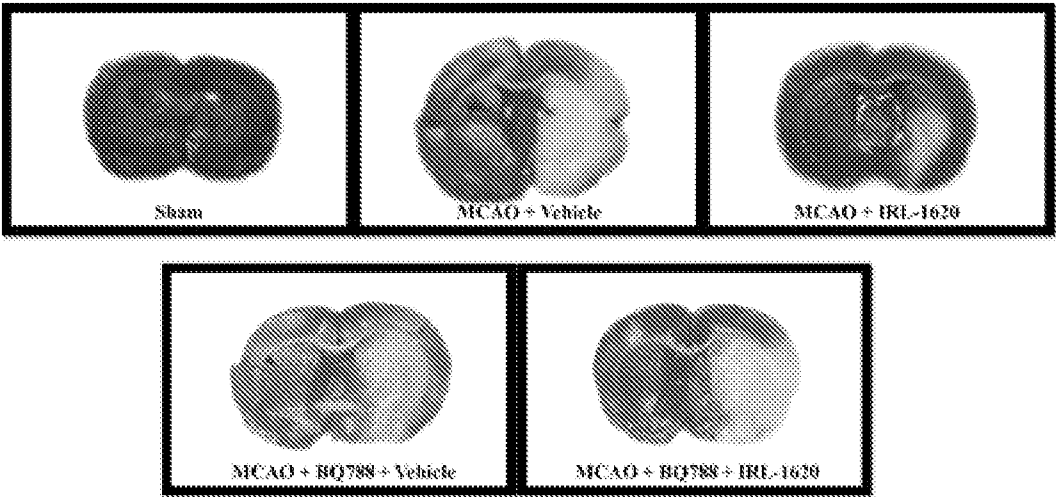


FIGURE 12

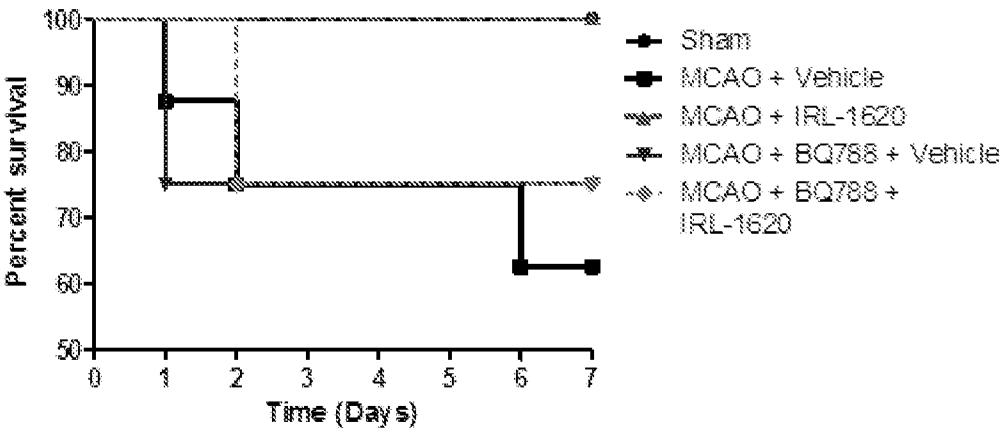


FIGURE 13

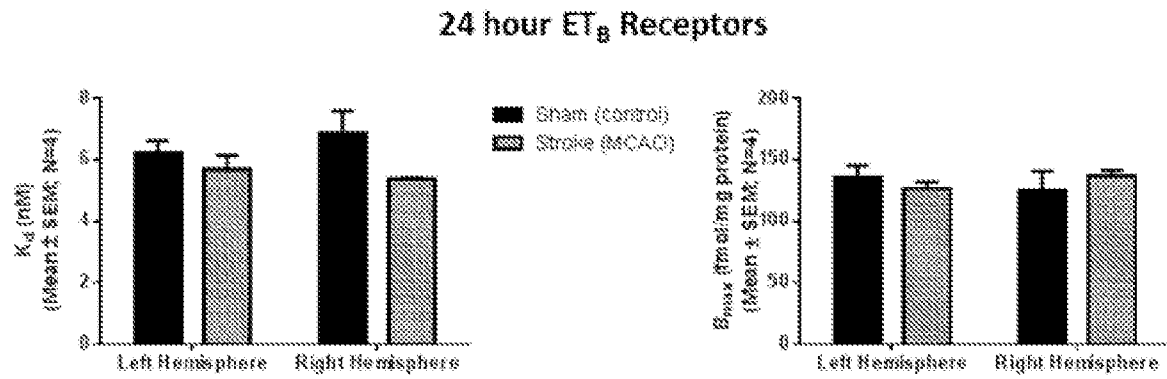


FIGURE 14A

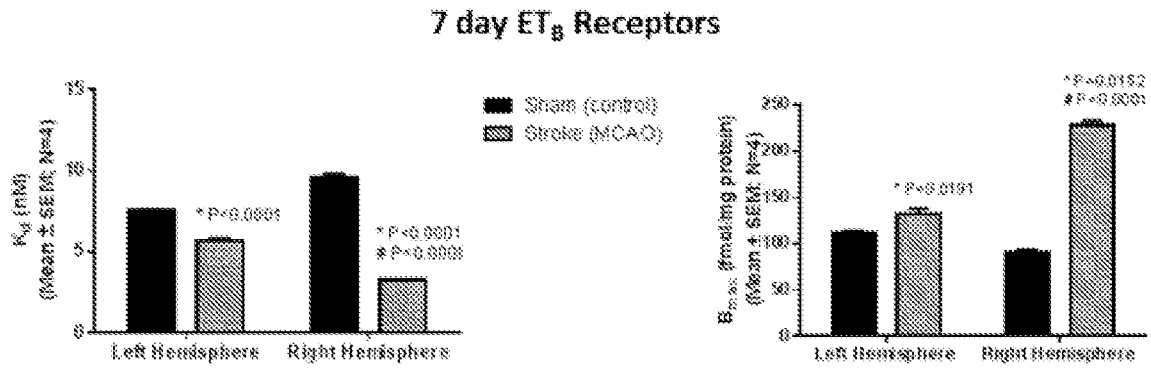


FIGURE 14B

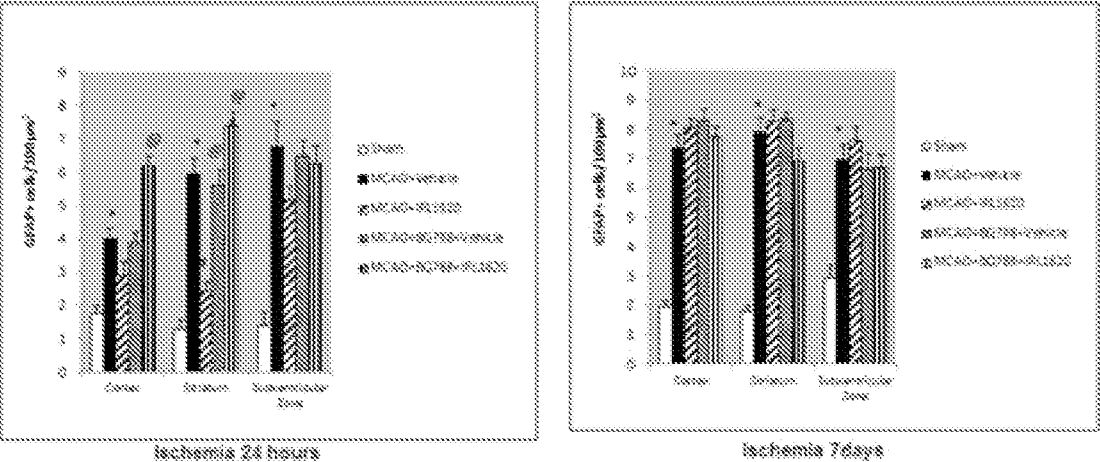
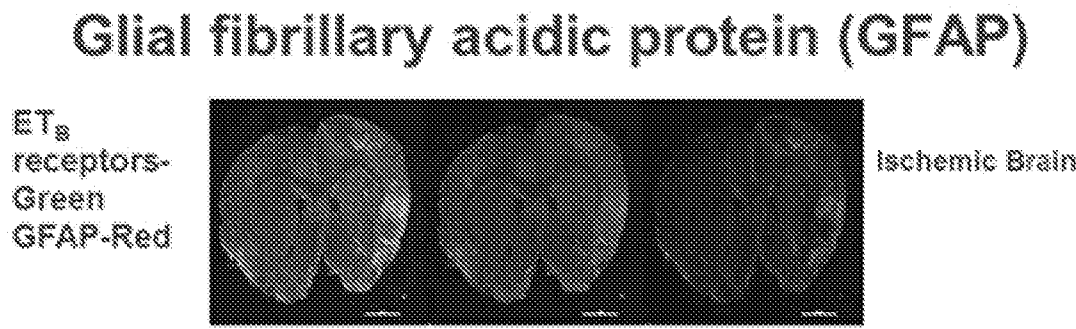


FIGURE 15

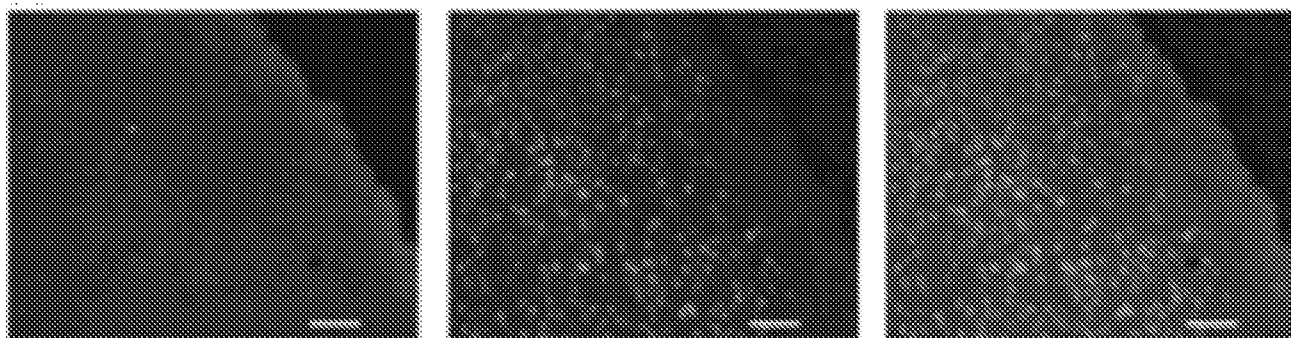


FIGURE 16A

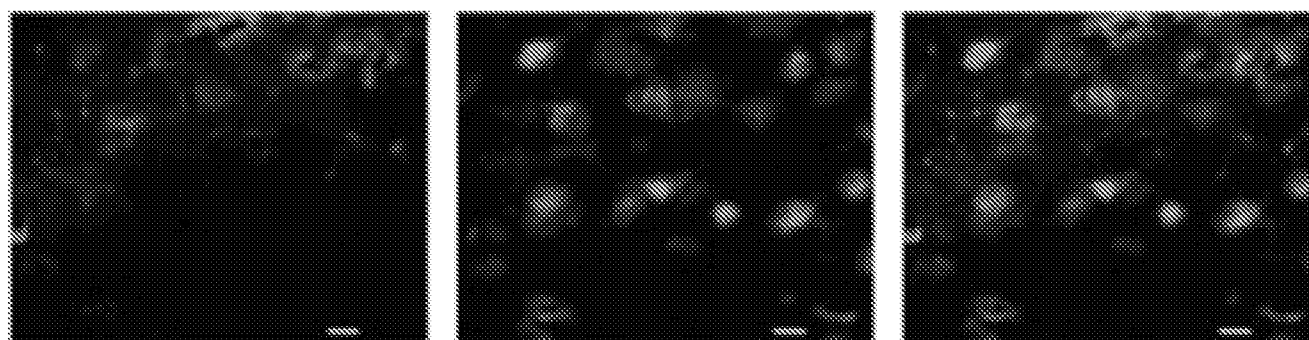


FIGURE 16B

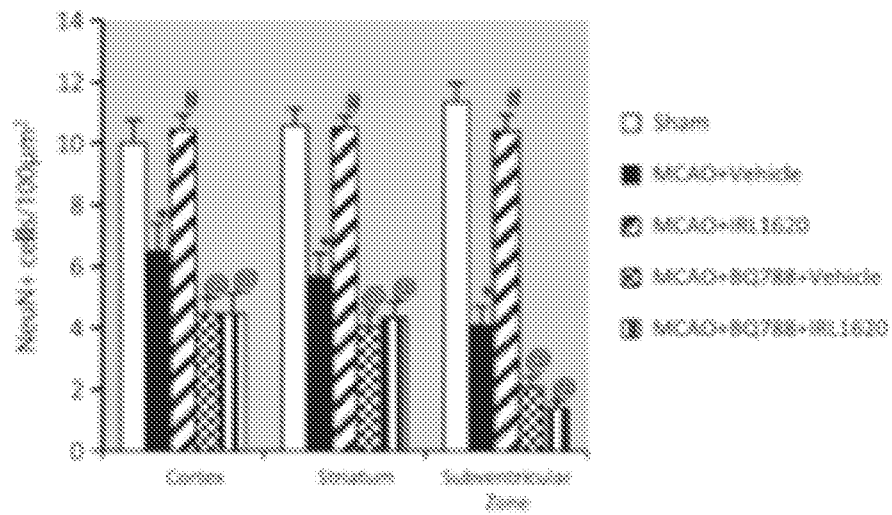


FIGURE 16C

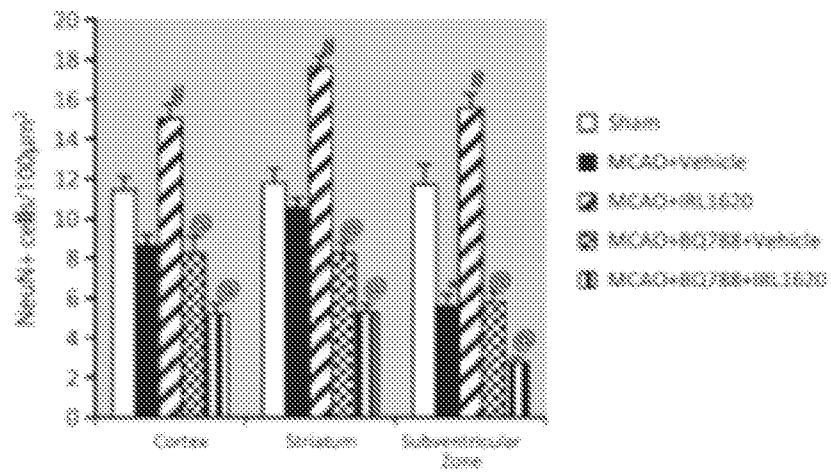


FIGURE 16D

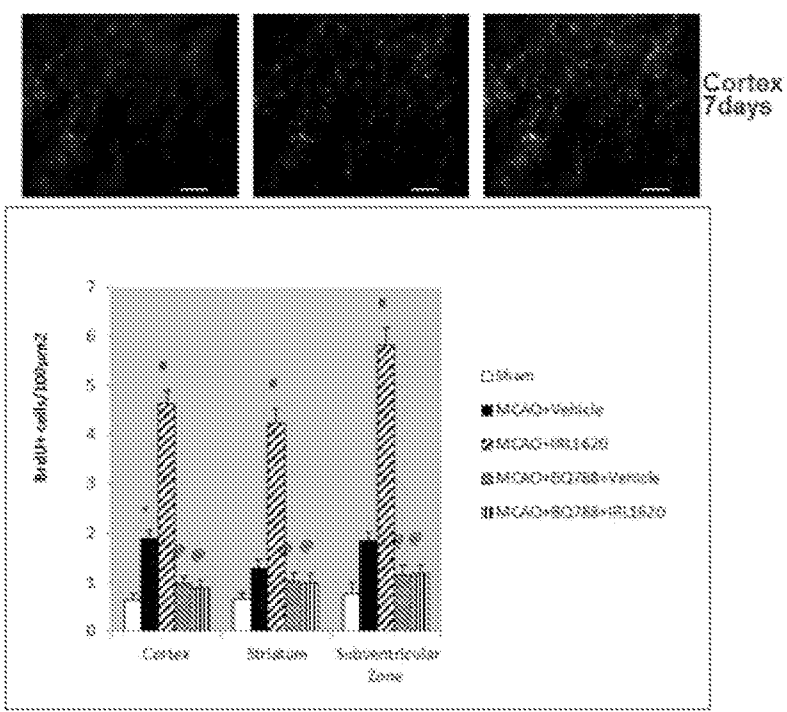
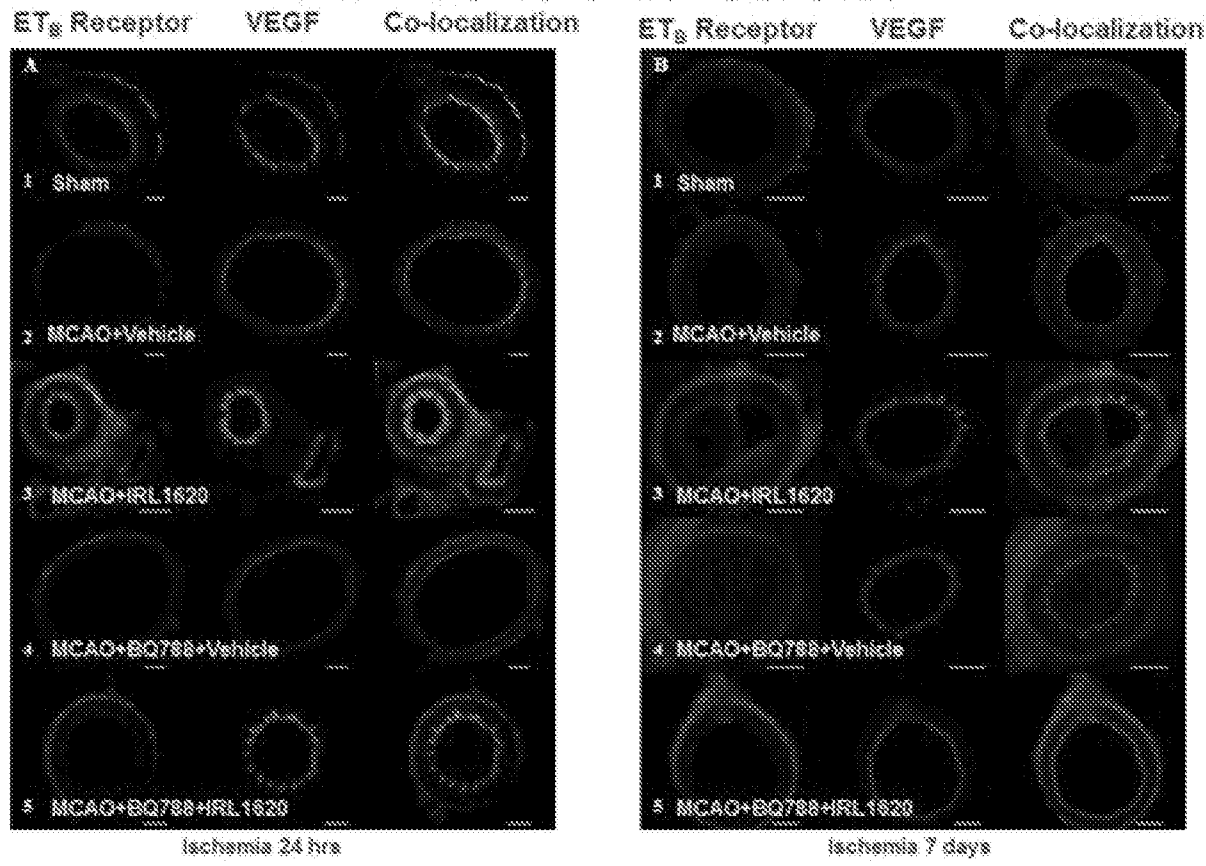


FIGURE 17

IRL-1620 and Stroke



FIGURES 18A AND 18B

IRL-1620 and Stroke

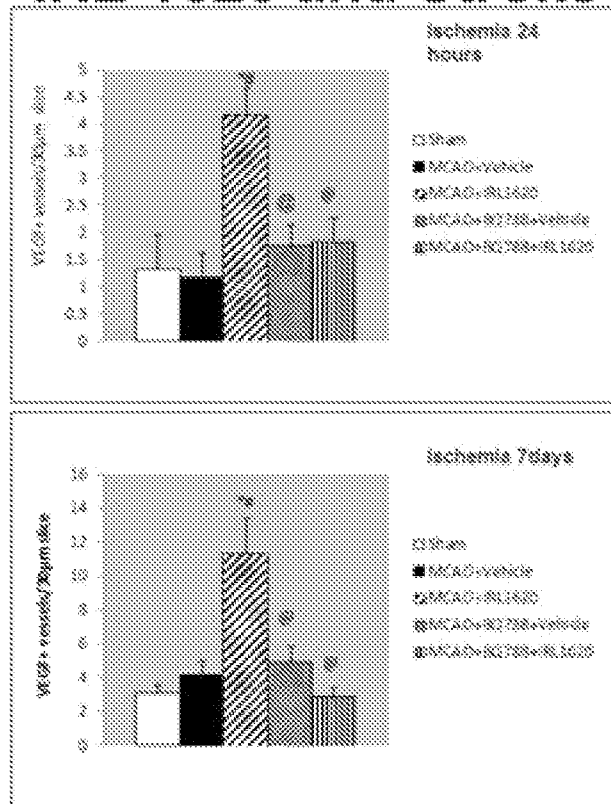


FIGURE 19

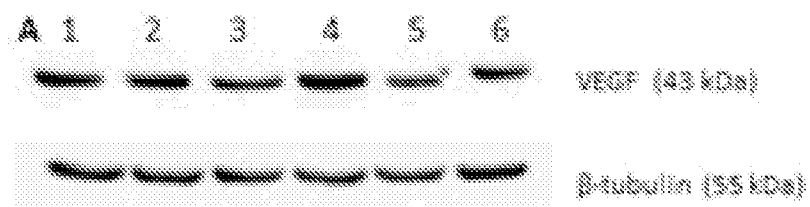


FIGURE 20A

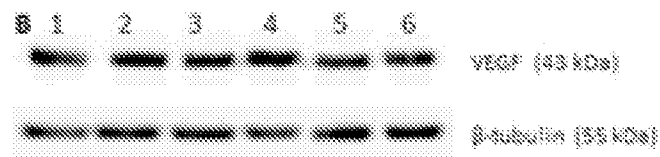


FIGURE 20B

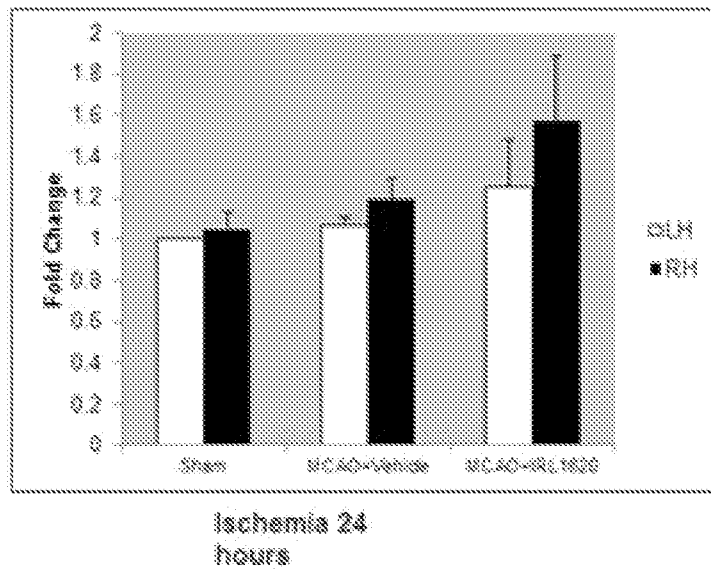


FIGURE 20C

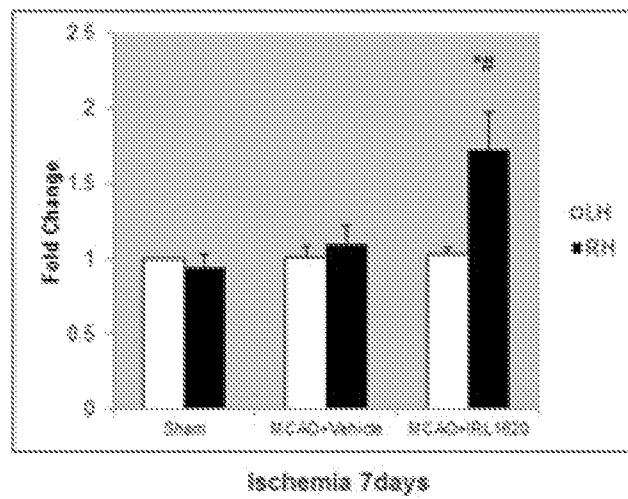


FIGURE 20D

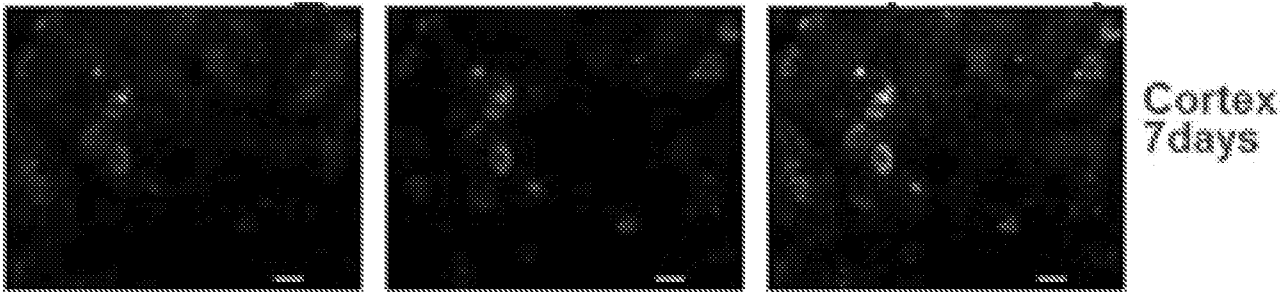


FIGURE 21A

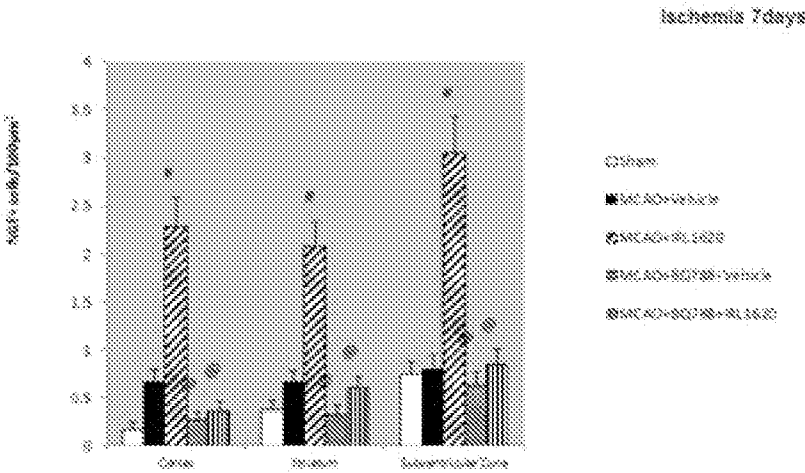


FIGURE 21B



FIGURE 22A

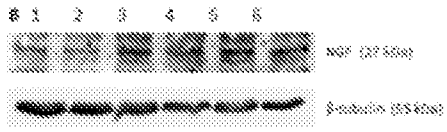
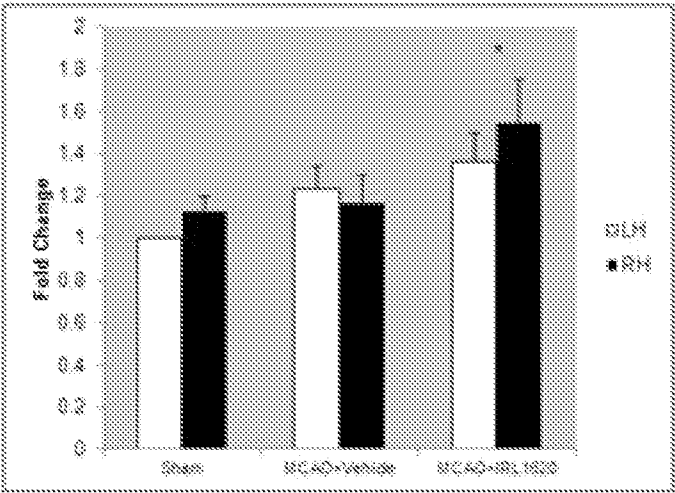
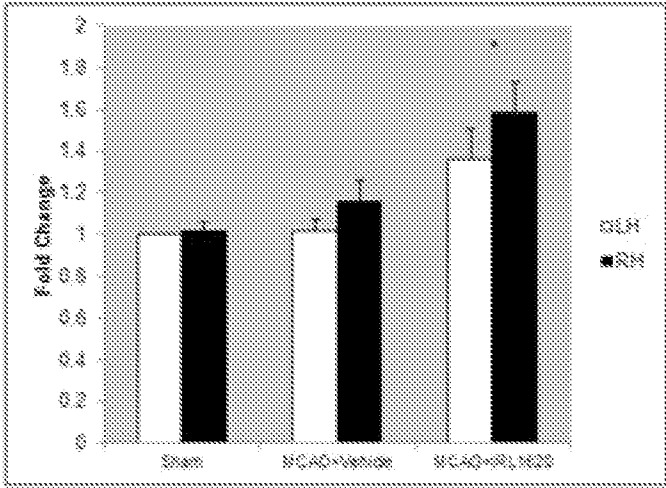


FIGURE 22B



ischemia 24 hours

FIGURE 22C



ischemia 7 days

FIGURE 22D