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(54) **Title:** METHODS AND COMPOSITIONS COMPRISING ANTAGONISTS OF TYPE 1 INTERFERON-MEDIATED SIGNALLING FOR REDUCING A NEUROINFLAMMATORY RESPONSE IN THE CENTRAL NERVOUS SYSTEM FOLLOWING A STROKE

(57) **Abstract:** The present disclosure relates to the amelioration of symptoms associated with a neuropathological inflammatory response component of a stroke event within the central nervous system (CNS) in a subject. Pharmaceutical formulations comprising antagonists of Type 1 interferon-mediated signalling or antagonists of interferon alpha receptor 1 mediated signalling; and methods and medical protocols for reducing neuroinflammation following a stroke are disclosed.



METHODS AND COMPOSITIONS COMPRISING ANTAGONISTS OF TYPE 1
INTERFERON-MEDIATED SIGNALLING FOR
REDUCING A NEUROINFLAMMATORY RESPONSE IN THE CENTRAL NERVOUS
SYSTEM FOLLOWING A STROKE

FILING DATA

- 5 [0001] This application is associated with Australian Patent Application No. 2010905239
filed 26 November 2010 and Australian Patent Application No. 2011902499, filed 24 June
2011, the entire contents of both of which, are incorporated herein by reference.

FIELD

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[0002] The present disclosure relates generally to the amelioration of symptoms associated
with a neuropathological inflammatory response component of a stroke event within the
central nervous system (CNS) in a subject. Agents, medicaments and pharmaceutical
compositions useful in the management of stroke are also enabled herein as are diagnostic
15 assays.

BACKGROUND

- [0003] Bibliographic details of the publications referred to by author in this specification
20 are collected alphabetically at the end of the description.

[0004] Reference to any prior art in this specification is not, and should not be taken as, an
acknowledgment or any form of suggestion that this prior art forms part of the common
general knowledge in any country.

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- [0005] The immune system and the central nervous system (CNS) have long been
considered separate systems with limited interaction. However, recent findings have shown
support for a potential relationship between the two systems. This occurrence has been
most evident in situations of neural injury, specifically ischemia-reperfusion pathologies
30 (Zierath *et al.*, *Neurocrit Care* 12(2):274-284, 2010). The widely accepted view was that
the CNS is immune-privileged as the blood brain barrier (BBB) prevents communication

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with peripheral immune cells (such as macrophages and lymphocytes). This view is now being re-shaped with findings of CNS and immune system communication in neural injury without the disruption of the BBB (Kaur and Ling, *Curr Neurovasc Res* 5(1):71-81, 2008). The mechanism of this CNS neuroinflammation is unknown, thus posing a new approach to investigate neuropathologies demonstrating distinct neuroinflammation.

[0006] Inflammation is a complex biological response initiated to remove a foreign pathogen from a given host. It is characterized by initial pathogen recognition, subsequent tissue oedema and multiple infiltrating cell types. Most notably, macrophages are recruited to ingest and remove foreign matter. The pathogens responsible can be venoms, parasites, allergens and DNA/RNA fragments released from necrotic cells. Initially, this immune response is protective, removing the pathogen, but an exaggerated response can promote detrimental overcompensation. This results from excess differentiation resulting in hematopoietic cell infiltration and release of chemokines and cytokines. These cells adhere to endothelium through intracellular adhesion molecule-1 (ICAM-1) and infiltrate the inflamed tissue (Caprio *et al.*, *Circ Res* 102(11):1359-1367, 2008). Although this is considered a peripheral event, this is also relevant to many neuropathologies including Alzheimer's disease (Akiyama *et al.*, *Neurobiol Aging* 21(3):383-421, 2000; Rogers *et al.*, *Glia* 40(2):260-269, 2002) which exhibit neuroinflammation within the CNS.

[0007] CNS ischemia-reperfusion injuries show characteristic macrophage and leukocyte recruitment resulting in production of various cytokines; however, the response is primarily initiated by activated resident microglia (Banati *et al.*, *Glia* 7(1):111-118, 1993; Giulian and Vaca., *Stroke* 24(12 Suppl):184-190, 1993). Amongst others, mRNA and chaperone proteins released from necrotic cells, act as ligands for Toll-like receptors (TLRs) [Kariko *et al.*, *J Biol Chem* 279(13):12542-12550, 2004; Ohashi *et al.*, *J Immunol* 164(2):558-561, 2000]. Activated microglia secrete pro-inflammatory cytokines including TNF α and interleukins (Block *et al.*, *Neuroreport* 11(5):963-967, 2000; Lambertsen *et al.*, *J Cereb Blood Flow Metab* 25(1):119-145, 2005) alongside chemo-attractants (Shohami *et al.*, *J Neurochem* 53:1541-1546, 1989). The CCL2/3 chemokines lead to the recruitment of macrophages (Cowell *et al.*, *Stroke* 33(3):795-801, 2002), further exacerbating the inflammatory response to the initial ischemia. Upon macrophage infiltration, a

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characteristic cytokine 'storm' develops, which contributes to neurodegeneration.

5 [0008] The inflammatory component of a brain injury contributes to widespread cellular damage in the brain (Greve *et al.*, *Mt Sinai J Med* 76:97-104, 2009). Studies of gene expression changes between control and traumatized brain samples revealed that genes playing a role in inflammation were up-regulated at an early stage after head injury (Crack *et al.*, *J Neural Transm* 116:1-12, 2009). The up-regulation of these genes suggests they play a major role in the secondary injury processes after ischemia-reperfusion injury.

10 [0009] The Type 1 interferons (IFN α , IFN β and IFN ω) play a role in the production of pro-inflammatory cytokines and chemokines (Schindler *et al.*, *J Biol Chem* 282:20059-20063, 2007). Studies have shown an up-regulation in Type 1 interferon gene expression as a result of microglial activation (Field *et al.*, *Brain Behav Immun* 24:996-1007, 2010). Interferons signal through interferon receptors, located widely on cells, including microglia
15 and other immune cells (de Weerd *et al.*, *J Biol Chem* 282:20053-20057, 2007).

[0010] The Type 1 IFN receptor, the IFN α receptor (IFNAR), is composed of IFNAR1 and IFNAR2 subunits.

20 [0011] Upon binding of Type 1 IFN proteins, the IFNAR1 and IFNAR2 subunits dimerize, activating the receptor-bound kinases JAK1 and Tyk2 (Schindler *et al.*, 2007 *supra*). Activation of these proteins recruits Signal Transducers and Activators of Transcription (STAT) proteins, and consequently, STATs are phosphorylated and activated by JAK1 and Tyk2. STAT1 and STAT2 dimerize and form a complex with an intracellular protein,
25 Interferon Regulatory Factor 9 (IRF9) [Leung *et al.*, *Mol Cell Biol* 15:1312-1317, 1995]. This complex translocates to the nucleus, acting as a transcription factor, and up-regulates the expression of anti-viral and anti-proliferative proteins, including pro-inflammatory cytokines (Lloyd *et al.*, *J Neuroinflammation* 5:28, 2008; Wei *et al.*, *J Neuroinflammation* 6:19, 2009).

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[0012] The effects of certain treatments in reducing the neuroinflammatory cascade have been studied. For instance, one study investigated the effects of a small molecule inhibitor,

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Minoxac, on the induction of pro-inflammatory cytokines after a traumatic brain injury (TBI) [Lloyd *et al.*, 2008 *supra*]. The exact mechanism of action of Minoxac is not known, but it is believed to reduce the overproduction of pro-inflammatory cytokines in microglia post-injury, which was seen in this study. In addition to this, in an animal model of
5 epilepsy, administration of Minoxac prevented the chronic activation of microglia, which was linked to neurodegeneration in the long term (Somera-Molina *et al.*, *Epilepsia* 48:1785-1800, 2007).

[0013] Another study looked at the synthetic tripeptide analog (Glypromate) [NNZ-2566]
10 which was found to attenuate the increased expression in pro-inflammatory cytokine levels and attenuate neuropathological events following injury in a rat model of TBI (Wei *et al.*, 2009 *supra*). Glypromate is derived from Insulin-like Growth Factor (IGF-1) and is believed to induce repair of injured brain tissue. In this study, NNZ-2566 particularly suppressed expression of IL-1 β (which is thought to play a significant role in secondary
15 neuronal apoptosis), thus conferring a level of neuroprotection to the tissue.

[0014] These studies illustrate the relevance of blocking certain aspects of the inflammatory response after head injury. However, whilst studies of drug therapies which targeted inflammation in TBI animal models have shown some benefit, when these
20 therapies were put through clinical trials, they were not successful (Menon, *Crit Care Med* 37:5129-5135, 2009). This illustrates the complexity of this pathology.

[0015] There is, therefore, a need to design a therapy which can effectively attenuate secondary neuronal damage resulting from a neuroinflammatory response following
25 stroke.

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SUMMARY

[0016] It is determined herein that stroke leads to inflammation and to neuronal necrosis and apoptosis within the central nervous system (CNS). Such an inflammatory response is referred to herein as "neuroinflammation" which has pathological consequences. The present disclosure teaches a method for ameliorating symptoms of a neuropathological inflammatory response in the CNS and in particular the brain following a stroke event. The method enabled herein is predicated in part on antagonizing Type 1 interferon (IFN)-mediated signalling. The reduction or attenuation of a neuroinflammatory response reduces secondary neuronal damage after stroke.

[0017] Accordingly, an aspect enabled herein is a method for reducing a neuroinflammatory response within the central nervous system (CNS) of a subject following a stroke event, the method comprising administering to the subject an effective amount of an antagonist of Type 1 interferon (IFN)-mediated signalling, for a time and under conditions sufficient to prevent or attenuate neuroinflammation. By "reducing a neuroinflammatory response" includes "ameliorating the symptoms of a neuroinflammatory response".

[0018] Another aspect enabled herein is a method for ameliorating the symptoms of a neuroinflammatory response within the central nervous system (CNS) of a subject following a neurological event or condition, the method comprising administering to the subject an effective amount of an antagonist of Type 1 interferon (IFN)-mediated signalling, for a time and under conditions sufficient to prevent or attenuate neuroinflammation, wherein the neurological event or condition is a stroke event.

[0019] In an embodiment, the present disclosure teaches a method for reducing a neuroinflammatory response within the central nervous system (CNS) of a subject following a stroke event, the method comprising administering to the subject an antagonist of interferon alpha receptor 1 (IFNAR1)-mediated signalling for a time and under conditions sufficient to prevent or ameliorate the symptoms of neuroinflammation.

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[0020] Neuroprotective formulations comprising the Type 1 IFN-mediated signalling antagonist are also contemplated herein, as are diagnostic assays to screen for the level of Type 1 IFN-mediated signalling prior to or following a stroke in the CNS. Aspects disclosed herein are also applicable for the development of stroke protocols for patients
5 who present with symptoms of such a condition.

[0021] The Type 1 IFN-mediated signalling antagonist may target a Type 1 IFN or its receptor or a component thereof. A Type 1 IFN includes IFN α , IFN β and IFN ω . In an embodiment, the antagonist targets the IFN α receptor subunit (IFNAR1) and/or the ability
10 for IFNAR1 to dimerize with IFNAR2. Combination therapy targeting a Type 1 IFN and IFNAR and optionally other components of the neuroinflammatory cascade are also taught herein. The Type 1 IFN-mediated signalling antagonist may be used post-or pre-neurological event. Hence, it may be a therapeutic or prophylactic.

15 [0022] The subject includes a higher order mammal including a human. A human subject may be of any age and includes a fetus.

[0023] Abbreviations used in the subject specification are defined in Table 2.

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TABLE 1

Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	Nucleotide sequence of the IFNAR1 gene
2	Nucleotide sequence of the IFNAR1 AttB1 subclone
3	Nucleotide sequence of the IFNAR1 AttB2 subclone
4	Nucleotide sequence of the IFNAR2 gene
5	Nucleotide sequence of the IFNAR2 AttB1 subclone
6	Nucleotide sequence of the IFNAR2 AttB2 subclone
7	Nucleotide sequence of the GAPDH forward primer
8	Nucleotide sequence of the GAPDH reverse primer
9	Nucleotide sequence of the Allalpha forward primer
10	Nucleotide sequence of the Allalpha reverse primer (1)
11	Nucleotide sequence of the Allalpha reverse primer (2)

TABLE 2
Abbreviations

Abbreviation	Definition
18srRNA	18s ribosomal RNA
ADC	Apparent Diffusion Coefficient
ANOVA	Analysis of Variance
APS	Ammonium Persulphate
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
CCI	Controlled Cortical Impact
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DWI	Diffusion Weighted Imaging
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
H&E	Hematoxylin & Eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid
hulFN	human Type-1 Interferon
IFN	Interferon
IFNAR	Interferon Alpha Receptor
IFNAR1 ^{-/-}	IFNAR1 genetic knock-out
IFNAR2 ^{-/-}	IFNAR2 genetic knock-out
IL	Interleukin
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
IRF	Interferon Regulatory Factor
JAK	Janus Kinase
JAK-1	Janus Associated Kinase 1

Abbreviation	Definition
KO	Knock out (-/-)
LB	Luria Broth
M17	human neuroblastoma M17 cell line
Map2	Neuronal marker indicating microtubule associated proteins
MAR1	Monoclonal antibody against IFNAR1
MCAO	Middle Cerebral Artery Occlusion
MRI	Magnetic Resonance Imaging
MTT	3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NeuN	Neuronal nuclei
NMR	Nuclear Magnetic Resonance
OGD	Oxygen and Glucose Deprivation
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
P-STAT	Phosphorylated-STAT
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RT-PCR	Real-Time Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Means
STAT	Signal Transducers and Activators of Transcription
TBI	Traumatic Brain Injury
TBS-T	Tris Buffered Saline-Tween 20
TE	Tris-EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
TNF	Tumor Necrosis Factor
TNF α	Tumor Necrosis Factor Alpha
Tris	Tris(hydroxymethyl)aminomethane
TYK	Tyrosine Kinase
TYK-2	Tyrosine Kinase 2

BRIEF DESCRIPTION OF THE FIGURES

[0024] Some figures contain color representations or entities. Color photographs are available from the Patentec upon request or from an appropriate Patent Office. A fee may be imposed if obtained from a Patent Office.

[0025] Figure 1 is a graphical representation showing isolated wild-type and IFNAR1^{-/-} C57B/6 neurons subjected to 4 hours oxygen and glucose deprivation (OGD) and 24 hours reperfusion. A subsequent MTT assay showed increased cell viability of IFNAR1^{-/-} neurons at 91.0±4.9% compared to wild-type with 49.8±0.8% (n=6, P<0.05, Student's t-test).

[0026] Figure 2 is a graphical representation showing M17 cells subjected to 3 hours OGD and time-course reperfusion (0, 0.5, 2 and 24 hours), RT-PCR was then performed. IFN α mRNA levels, unlike IFN β , were significantly elevated at 2 hours reperfusion (15.1±2.3 fold change) compared to control. IFN β mRNA levels were only increased at 24 hours reperfusion (2.4±0.1 fold change) compared to control. (n=3, *P.05, One-way ANOVA, Dunnett's Post-hoc test.)

[0027] Figures 3A through C are graphical representations showing M17 cells subjected to 3 hours OGD and time-course reperfusion (0, 0.5, 2 and 24 hours), RT-PCR was then performed. A) Interleukin-6 (IL-6) mRNA levels were significantly increased at 0.5 hour (266.9±10.9 fold change) and 2 hours (194.6±39.4 fold change) reperfusion compared to control. B) Tumor Necrosis Factor α (TNF- α) mRNA levels were elevated at 0 hour (10.0±3.1 fold change) and 0.5 hour (10.1±2.8 fold change) as to control. C) Interleukin-1 β (IL-1 β) mRNA levels showed a significant increase at 24 hours reperfusion (4.6±1.2 fold change) compared to control. (n=3, *P<0.05, Dunnett's Post-hoc test.)

[0028] Figure 4 is a graphical representation showing collated RT-PCR data for each cytokine mRNA levels post-3 hours OGD and time-course reperfusion (0, 0.5, 2 and 24 hours). OGD begins at -4 hours and subsequent reperfusion occurs from 0 hour. IFN α

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mRNA levels increases at 2 hours reperfusion before returning to basal levels. IFN β and IL-1 β mRNA levels only elevate at 24 hours reperfusion. TNF α mRNA levels show immediate increase at 0 and 0.5 hours reperfusion before returning to control levels. IL-6 mRNA levels are elevated at 0.5 and 2 hours reperfusion, and then return to basal levels.

5

[0029] Figure 5 is a graphical representation showing M17 cells transfected with IFNAR1-GFP and IFNAR2-GFP overexpression plasmids and subjected to 3 hours OGD and 24 hours reperfusion. Curiously, M17-IFNAR1 cells demonstrated significant decrease in survival ($21.6 \pm 5.3\%$) compared to wild-type ($44.9 \pm 2.1\%$). Furthermore, M17-IFNAR2
10 cells showed no difference in survival ($39.8 \pm 5.7\%$) compared to wild-type. (n=4, * P<0.05, One-way ANOVA, Dunnett's Post-hoc test.)

[0030] Figure 6 is a representation of the IFNAR1, AttB1 and AttB2 sequences used in Gateway (Registered Trademark) cloning.

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[0031] Figure 7 is a representation of the IFNAR2, AttB1 and AttB2 sequences used in Gateway (Registered Trademark) cloning.

[0032] Figure 8 is a representation of SYBRGreen nucleotide sequences.

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[0033] Figures 9A through C are photographic representations of fluorescent immunohistochemistry showing IFNAR1 localization on primary cultured mouse neurons. Panel A Map-2, Panel B NeuN Panel C-Merge. Note, Map2 is a neuronal marker indicating microtubule associated proteins.

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[0034] Figure 10 is a graphical and photographic representation of IFNAR1^{-/-} (knock out; KO) mice (C57BL/6) show reduced infarct size in a stroke model. The model comprises 2 hours of mid-cerebral artery occlusion followed by 24 hours of reperfusion. IFNAR1^{-/-} mice show a reduced infarct size.

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[0035] Figure 11 is a graphical representation showing that IFNAR1 mAb reduces infarct size in a middle cerebral artery occlusion (MCAO) stroke model. An amount of 0.5mg

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mAb was given iv 1 hour prior to stroke. The infarct volume was assessed 24 hours post-injury. The monoclonal Ab (mAb) is MAR-1.

DETAILED DESCRIPTION

5 [0036] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or method step or group of elements or integers or method steps but not the exclusion of any other element or integer or method step or group of elements or integers or method steps.

10

[0037] Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO). The SEQ ID NOs correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided after the claims.

15

[0038] As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a neuroinflammatory condition" includes a single neuroinflammatory condition, as well as two or more neuroinflammatory conditions; reference to "an antagonist" includes a single antagonist, as well as two or more antagonists; reference to "the disclosure" includes a single and multiple aspects described in the disclosure; and so forth. All aspects disclosed, describe and/or claimed herein are encompassed by term "invention". Such aspects are enabled across the width of the present invention.

20

25 [0039] Type 1 interferons (IFNs) are a super-family of pleiotropic cytokines that induce pro-inflammatory gene transcription *via* the classical JAK/STAT pathway. The Type 1 IFNs include IFN α , IFN β and IFN ω . Certain events or conditions within the central nervous system (CNS) have the capacity to induce a neuroinflammatory response having pathological consequences. Taught herein is the use of an antagonist of Type 1 IFN-mediated signalling within the CNS to attenuate an adverse neuropathological inflammatory response following a stroke event. The attenuation of a neuroinflammatory response leads to a reduction in secondary neuronal damage including neuronal necrosis

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and apoptosis.

[0040] Reference herein to a "type 1 IFN-mediated signalling antagonist" means an antagonist of the activity of a Type 1 IFN, an inhibitor of Type 1 IFN gene expression or translation, an antagonist of a Type 1 IFN receptor activity, function or dimerization or a subunit thereof such as IFNAR1, an inhibitor of Type 2 IFN receptor gene expression and an agent which blocks Type 1 IFN:receptor interaction or any agent which blocks, inhibits or otherwise reduces Type 1 IFN-mediated inflammatory signalling.

10 [0041] An aspect enabled herein is a method for reducing a neuroinflammatory response within the central nervous system (CNS) of a subject following a stroke event, the method comprising administering to the subject an effective amount of an antagonist of Type 1 interferon (IFN)-mediated signalling, for a time and under conditions sufficient to prevent or attenuate neuroinflammation.

15

[0042] A "stroke event" includes any ischemic condition in a blood vessel in the CNS including the brain as well as any blood fluid leakage such as following rupture of a blood vessel. The stroke event may be classified as minor-mild, mild-moderate, moderate, moderate-severe and severe-serious and can result from minor to significant impairment to a subject.

20

[0043] Another aspect of the present disclosure contemplates a method for ameliorating symptoms of a neuroinflammatory response within the central nervous system (CNS) of a subject following a stroke event, the method comprising administering to the subject an effective amount of an antagonist of Type 1 interferon (IFN)-mediated signalling, for a time and under conditions sufficient to prevent or attenuate neuroinflammation.

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[0044] An aspect enabled herein is a method for reducing a neuroinflammatory response within the central nervous system (CNS) of a subject following a stroke event, the method comprising administering to the subject an effective amount of an antagonist of Type 1 interferon (IFN)-mediated signalling, for a time and under conditions sufficient to prevent or attenuate neuroinflammation.

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[0045] A related embodiment provides a method for ameliorating the symptoms associated with a stroke event, the method comprising administering to the subject an effective amount of an antagonist of Type 1 interferon (IFN)-mediated signalling, for a time and under conditions sufficient to reduce or attenuate a neuroinflammatory response within the CNS.

[0046] The present disclosure teaches antagonists of Type 1 IFN-mediated signalling in the form of a compound, agent, chemical agent, pharmacologically agent, medicament, active and drug. The terms "compound", "agent", "chemical agent", "pharmacologically active agent", "medicament", "active" and "drug" are used interchangeably herein to refer to the Type 1 IFN-mediated signalling antagonist which induces a desired pharmacological and/or physiological effect. The desired effect includes reducing Type 1 IFN-mediated signalling *via* the IFN or a receptor subunit such as IFNAR1. The desired physiological effect includes attenuation of a neuropathological inflammatory response including the potential for such an inflammatory response to reach a neuropathological level. The terms also encompass pharmaceutically acceptable and pharmacologically active ingredients of those active agents specifically mentioned herein including but not limited to salts, esters, amides, prodrugs, active metabolites, analogs, mimetics functional equivalents and the like. When the terms "compound", "agent", "chemical agent", "pharmacologically active agent", "medicament", "active", "drug" and "antagonist" are used, then it is to be understood that this includes the active agent *per se* as well as pharmaceutically acceptable, pharmacologically active salt, ester, amide, prodrug, metabolite and analogs thereof.

25

[0047] Reference to the antagonist in these terms includes combinations of two or more actives. A "combination" also includes multi-part such as a two-part composition where the agents are provided separately and given or dispensed separately or admixed together prior to dispensation. Examples include two separate agents wherein one agent down-regulates the activity or level of the Type 1 IFN and the other down-regulates the activity or level of a Type 1 IFN receptor subunit, and in particular IFNAR1. Agents when in combination with an IFN or IFNAR antagonist may also target a down-stream component

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of the Type 1 IFN-mediated signalling cascade.

[0048] The antagonists enabled herein provided systemically cross the blood brain barrier (BBB). This is possible due to the chemical nature of the agent or due to a permealization factor such as a co-factor fused to or formulated with the antagonist. Strategies for accessing the CNS are disclosed in Misra *et al.*, *J Pharm Sci* 6:252-273, 2003.

[0049] The "antagonist" includes a protein, polypeptide or peptide, small chemical molecule, antibody or derivative thereof including an immunoglobulin new antigen receptor (IgNAR) or a genetic molecule.

[0050] A "genetic molecule" is generally one which down-regulates expression of a gene encoding a Type 1 IFN or a Type 1 IFN receptor or a subunit thereof. In an embodiment, the targeted subunit is IFNAR1. The genetic molecule includes an antisense molecule directed to all or part of a gene or mRNA encoding the Type 1 IFN or a subunit portion of its receptor such as IFNAR1. In an embodiment, the antisense is from about 5 nucleotides in length to 15 to 80 nucleotides in length or from about 5 nucleotides in length to full length of the mRNA transcript or 5' or 3' regions thereof. Other genetic molecules include sense molecules such as single- or double-stranded RNAs, RNAi and siRNA molecules, short and long RNA duplexes, ribozymes, DNAzymes, and any DNA or RNA or synthetic DNA or RNA agent which interferes with expression of the expression of the Type 1 IFN gene or of the gene encoding a receptor subunit such as IFNAR1. The genetic molecule may be naked or expressed by a viral or other vector or introduced as part of a formulation.

[0051] A number of viruses may be used as nucleic acid transfer vectors or as the basis for preparing nucleic acid transfer vectors to introduce a genetic agent to the CNS, including papovaviruses (e.g. SV40, Madzak *et al.*, *J Gen Virol* 73:1533-1536, 1992), adenovirus (Berkner, *Curr Top Microbiol Immunol* 158:39-66, 1992; Berkner *et al.*, *BioTechniques* 6:616-629, 1988; Gorziglia and Kapikian, *J Virol* 66:4407-4412, 1992; Quantin *et al.*, *Proc Natl Acad Sci USA* 89:2581-2584, 1992; Rosenfeld *et al.*, *Cell* 68:143-155, 1992; Wilkinson *et al.*, *Nucleic Acids Res* 20:2233-2239, 1992; Stratford-Perricaudet *et al.*, *Hum Gene Ther* 1:241-256, 1990; Schneider *et al.*, *Nat Genetics* 18:180-183, 1998), vaccinia

- virus (Moss, *Curr Top Microbiol Immunol* 158: 5-38, 1992; Moss, *Proc Natl Acad Sci USA* 93:11341-11348, 1996), adeno-associated virus (Muzyczka, *Curr Top Microbiol Immunol* 158:97-129, 1992; Ohi *et al*, *Gene* 89:279-282, 1990; Russell and Hirata, *Nat Genetics* 18:323-328, 1998), herpesviruses including HSV and EBV (Margolske, *Curr Top Microbiol Immunol* 158:67-95, 1992; Johnson *et al*, *J Virol* 66:2952-2965, 1992; Fink *et al*, *Hum Gene Ther* 3:1-19, 1992; Breakefield and Geller, *Mol Neurobiol* 1:339-371, 1987; Freese *et al*, *Biochem Pharmacol* 40:2189-2199, 1990; Fink *et al*, *Ann Rev Neurosci* 19:265-287, 1996), lentiviruses (Naldini *et al*, *Science* 272:263-267, 1996), Sindbis and Semliki Forest virus (Berglund *et al*, *Biotechnology* 11:916-920, 1993) and retroviruses of
- 10 avian (Bandyopadhyay and Temin, *Mol Cell Biol* 4:749-754, 1984; Petropoulos *et al*, *J Virol* 66:3391-3397, 1992), murine (Miller, *Curr Top Microbiol Immunol* 158:1-24, 1992; Miller *et al*, *Mol Cell Biol* 5:431-437, 1985; Sorge *et al*, *Mol Cell Biol* 4:1730-1737, 1984; Mann and Baltimore, *J Virol* 54:401-407, 1985; Miller *et al*, *J Virol* 62:4337-4345, 1988) and human (Shimada *et al*, *J Clin Invest* 88:1043-1047, 1991; Helseth *et al*, *J Virol* 64:2416-2420, 1990; Page *et al*, *J Virol* 64:5270-5276, 1990; Buchschacher and Panganiban, *J Virol* 66:2731-2739, 1992) origin.

- [0052] Non-viral nucleic acid transfer methods include chemical techniques including calcium phosphate co-precipitation, mechanical techniques, for example, microinjection,
- 20 membrane fusion-mediated transfer via liposomes and direct DNA uptake and receptor-mediated DNA transfer. Viral-mediated nucleic acid transfer can be combined with direct *in vivo* nucleic acid transfer using liposome delivery, allowing one to direct the viral vectors to particular cells. Alternatively, the retroviral vector producer cell line can be injected into particular tissue. Injection of producer cells would then provide a continuous
- 25 source of vector particles.

- [0053] An IgNAR (immunoglobulin new antigen receptor) is an antibody isotype found in cartilaginous marine animals (sharks and rays), which has evolved over hundreds of millions of years to be stably expressed in the potent urea environment of the blood stream
- 30 (Greenberg *et al*, *Nature* 374:168-173, 1995; Nuttall *et al*, *Mol Immunol* 38:313-326, 2001). The IgNAR response is antigen-driven in the shark, and both immune and naïve molecular libraries of IgNAR variable domains have been constructed and successfully

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screened for antigen-specific binding reagents (Greenberg *et al.*, 1995 *supra*; Nuttall *et al.*, 2001 *supra*). IgNAR's are bivalent, but target antigen through a single immunoglobulin variable domain (~14kDa) displaying two complementarity determining region (CDR) loops attached to varying numbers of constant domains (Nuttall *et al.*, *Eur J Biochem* 5 270:3543-3554, 2003; Roux *et al.*, *Proc Natl Acad Sci USA* 95:11804-11809, 1998). In contrast, traditional immunoglobulin (Ig) antibodies have a variable heavy (V_H) + variable light (V_L) domain format (~26kDa) and bind antigen through up to six CDRs (Chothia *et al.*, *Nature* 342:877-883, 1989; Padlan, *Mol Immunol* 31:169-217, 1994). The small size, and thermodynamic and chemical stability of IgNAR variable domains (V_{NARS}), offer

10 distinct advantages over conventional antibodies. Furthermore, the small V_{NAR} size enables this unusual antibody domain access to cryptic antigenic epitopes through unusually long and variable CDR3 loops (Greenber *et al.*, 1995 *supra*; Ewert *et al.*, *Biochemistry* 41:3628-2636, 2002; Nuttall *et al.*, *Proteins* 55:187-197, 2004; Stanfield *et al.*, *Science* 305:1770-1773, 2004; Streltsov *et al.*, *Proc Natl Acad Sci USA* 101:12444-12449, 2004; Streltsov *et al.*, *Protein Sci* 14:2901-2909, 2005). IgNAR domains have been identified that recognize

15 a variety of target antigens including: the apical membrane protein 1 (AMA-1) of *P. falciparum* (Nuttall *et al.*, 2004 *supra*); the Kgp protease from *Porphyromonas gingivalis* (Nuttall *et al.*, *FEBS Lett* 516:80-86, 2002); cholera toxin (Goldman *et al.*, *Anal Chem* 78:8245-8255, 2006); the Tom70 mitochondrial membrane spanning protein (Nuttall *et al.*, 20 2003 *supra*), and lysozyme (Streltsov *et al.*, 2004 *supra*).

[0054] The present disclosure teaches analogs and derivatives of a Type 1 IFN or an IFNAR such as IFNAR1 such as which include a modified side chain or which incorporate an unnatural amino acid and/or their derivatives during peptide, polypeptide or protein

25 synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecule or its analogs. This term also does not exclude modification of the glycosylation, acetylation and phosphorylation patterns. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid or polypeptides with substituted linkages. Such polypeptides may need to be

30 able to enter the cell and/or cross the BBB or promote cerebral fluid half-life survival.

[0055] Mimetics of the Type 1 IFN or IFNAR such as IFNAR1 are another useful group of

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agents to test for neuroprotective ability. The term is intended to refer to a substance which has some chemical similarity to the molecule it mimics and which acts as an antagonist. A peptide mimetic of a Type I IFN or IFNAR1, for example, may be a peptide-containing molecule that mimics elements of protein secondary structure (Johnson *et al.*,
5 *Peptide Turn Mimetics in Biotechnology and Pharmacy*, Pezzuto *et al* (Eds), Chapman and Hall, New York, 1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions such as with a receptor or ligand. A peptide mimetic, therefore, is designed to permit molecular interactions similar to the natural
10 molecule but block signalling.

[0056] The designing of mimetics to a pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesize or where it
15 is unsuitable for a particular method of administration, e.g. peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal.

[0057] There are several steps commonly taken in the design of a mimetic from a
20 compound having a given target property. First, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. Alanine scans of peptides, for example, are commonly used to refine such peptide motifs. These parts or residues constituting the
25 active region of the compound are known as its "pharmacophore".

[0058] Once the pharmacophore has been found, its structure is modeled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, x-ray diffraction data and NMR.
30 Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modeling process.

[0059] In a variant of this approach, the three-dimensional structure of a receptor and ligand are modeled. This can be especially useful where the receptor and/or ligand change conformation on binding, allowing the model to take account of this in the design of the mimetic. Modeling can be used to generate agents which interact with the linear sequence or a three-dimensional configuration.

[0060] A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted onto it can conveniently be selected so that the mimetic is easy to synthesize, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. Alternatively, where the mimetic is peptide-based, further stability can be achieved by cyclizing the peptide, increasing its rigidity. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimization or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

[0061] Hence, the present disclosure teaches antagonists of Type 1 IFN-mediated signalling for use in mammals including higher order mammals such as humans. Such antagonists facilitate an attenuation of a neuroinflammatory response following a stroke event. Non-human animals are contemplated such as in veterinary applications as well as for use in animal models. Reference to a mammal includes a mouse, rat, hamster, guinea pig, rabbit, pig, sheep, horse, goat, cow, camel and non-human primate (such as orangutan, gorilla, marmoset and a macaque). In an embodiment, the subject is a human. Reference to a human includes a fetus, *in utero* as well as a human of any age.

[0062] The present disclosure teaches a method for reducing a neuroinflammatory response within the central nervous system (CNS) of a human subject following a stroke event, the method comprising administering to the subject an effective amount of an antagonist of Type 1 interferon (IFN)-mediated signalling, for a time and under conditions sufficient to prevent, reduce or attenuate neuroinflammation.

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[0063] In an embodiment, the present disclosure enables a method for reducing a neuroinflammatory response within the CNS of a subject following a stroke event, the method comprising administering to the subject an antagonist of interferon alpha receptor 1 (IFNAR1)-mediated signalling for a time and under conditions sufficient to prevent or
5 ameliorate the symptoms of neuroinflammation.

[0064] The present disclosure further describes a method for ameliorating the symptoms of a neuroinflammatory response within the CNS of a human subject following a stroke event the method comprising administering to the human subject an antagonist of interferon
10 alpha receptor 1 (IFNAR1)-mediated signalling for a time and under conditions sufficient to prevent or ameliorate the symptoms of neuroinflammation.

[0065] The terms "effective amount" and "therapeutically effective amount" of an agent as used herein mean a sufficient amount of an agent (i.e. a Type 1 IFN-mediated signalling
15 antagonist) to provide the desired therapeutic or physiological effect or outcome as indicated above. Undesirable effects, e.g. side effects, are sometimes manifested along with the desired therapeutic effect; hence, a practitioner balances the potential benefits against the potential risks in determining what is an appropriate "effective amount". The exact amount required will vary from subject to subject, depending on the species, age and
20 general condition of the subject, mode of administration and the like. Thus, it may not be possible to specify an exact "effective amount". However, an appropriate "effective amount" in any individual case may be determined by one of ordinary skill in the art using only routine experimentation. In general, the amount is effective to attenuate a neuroinflammatory response within the CNS or to prevent or reduce the severity of a
25 neuroinflammatory response developing or continuing. The prevention of a neuroinflammatory response developing is also useful in at risk subjects such as those with a genetic disposition or family history of strokes. The present disclosure teaches reducing, preventing or attenuating neuroinflammation anywhere in the CNS, including in the brain. The antagonist may be used as a therapeutic to treat a condition or as a preventative (i.e.
30 prophylactically) to reduce the risk of neuroinflammation in anticipation of a stroke.

[0066] The agent in the form of an antagonist may also be administered with a

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pharmaceutically acceptable carrier, excipient or diluent. By "pharmaceutically acceptable" carrier, excipient or diluent is meant a pharmaceutical vehicle comprised of a material that is not biologically or otherwise undesirable, i.e. the material may be administered to a subject along with the selected active agent without causing any or a
5 substantial adverse reaction. Carriers may include excipients and other additives such as diluents, detergents, coloring agents, wetting or emulsifying agents, pH buffering agents, preservatives, and the like.

[0067] The agent in the form of an antagonist may also be a pharmacologically acceptable
10 form or derivative of a compound. By "pharmacologically acceptable" means a salt, ester, amide, prodrug or derivative that is not biologically or otherwise undesirable.

[0068] The terms "treating" and "treatment" as used herein refer to reduction in severity and/or frequency of symptoms of the condition being treated, elimination of symptoms
15 and/or underlying cause, prevention of the occurrence of symptoms of the condition and/or their underlying cause and improvement or remediation or amelioration of damage following a stroke event leading to or having the potential to lead a neuroinflammatory response in the CNS. In general terms, treatment may involve ameliorating symptoms of a neuroinflammatory response. Such symptoms may arise from neuronal necrosis,
20 apoptosis, senescence or arrest, demyelination and/or axonal or neuronal degeneration. Amelioration of a downstream physiological, psychological or mental condition is also a useful indicator of treatment.

[0069] "Treating" a subject, therefore, may involve prevention or reduction in extent of
25 development of a condition or other adverse physiological or psychological event in a susceptible individual associated with a neuroinflammatory response as well as treatment of a clinically symptomatic individual by ameliorating the symptoms of the neuroinflammatory response.

30 [0070] As indicated above, a "subject" as used herein refers to an animal, such as a mammal including a human who can benefit from the pharmaceutical agents and formulations and methods of the present disclosure. A subject regardless of whether a

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human or non-human animal may be referred to as an individual, patient, animal, host or recipient. The compounds and methods enabled herein have particular applications in human medicine.

- 5 [0071] Taught herein is the use of neuroprotective agents to reduce a neuroinflammatory response or risk of such a response developing or the consequences of degenerative inflammatory processes such as immunodegenerative processes or conditions which induce cell cycle arrest, necrosis and/or apoptosis following a stroke event.
- 10 [0072] The neuroprotective agent enabled herein is an agent which down-regulates the extent or activity of Type 1 IFN-mediated signalling. Such an agent is referred to herein as a "Type 1 IFN signalling antagonist". This includes an antagonist of a Type 1 IFN or a portion of the Type 1 IFN receptor and in particular IFNAR. Reference to an "IFNAR" includes the IFNAR receptor or a subunit thereof such as IFNAR1. The present disclosure
- 15 further teaches combinations of neuroprotective agents or neuroprotective formulations comprising a Type 1 IFN signalling antagonist and another neuroprotective agent such as leukemia inhibitory factor (LIF) or ciliary neurotrophic factor (CNTF) or a homolog, derivative, analog or mimetic thereof.
- 20 [0073] Hence, the present disclosure teaches a method for the treatment or prophylaxis of a stroke event in a subject, the method comprising administering to the subject an effective amount of a Type 1 IFN signalling antagonist for a time and under conditions sufficient to ameliorate adverse neurological inflammation or prevent or reduce its progression.
- 25 [0074] In an embodiment, a method is provided for the treatment or prophylaxis of a stroke event in a subject or at least delaying onset of symptoms thereof, the method comprising administering to the subject an effective amount of a Type 1 IFN alpha receptor (IFNAR1) antagonist for a time and under conditions sufficient to ameliorate adverse neurological inflammation or prevent or reduce its progression in the CNS.
- 30 [0075] As indicated above, the neuroprotective agent includes a Type 1 IFN antagonist or an IFNAR antagonist such as IFNAR1 antagonist. The antagonist may act at the level of

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protein activity or function or gene expression including transcription, translation or processing.

5 [0076] In another aspect, the present disclosure teaches a method for ameliorating the symptoms of a neuroinflammatory response following a stroke event in a subject or delaying development of symptoms thereof, the method comprising administering to the subject an effective amount of a neuroprotective formulation comprising a Type 1 IFN antagonist for a time and under conditions sufficient to reduce neurological inflammation or prevent or reduce its progression.

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[0077] Still another aspect taught herein to a method for ameliorating the symptoms of a neuroinflammatory response following a stroke event or delaying development of symptoms thereof, the method comprising administering to the subject an effective amount of a neuroprotective formulation comprising an IFNAR1 antagonist for a time and under
15 conditions sufficient to reduce neurological inflammation or prevent or reduce its progression.

[0078] As indicated above, the amount or time sufficient to treat the neurodegenerative disease or condition may be the amount or time required to ameliorate one or more
20 symptoms of the stroke event. A symptom includes a psychological or mental symptom. Furthermore, the antagonist may be provided with a pharmaceutically acceptable carrier, excipient or diluent. The antagonist itself is considered to be pharmacologically acceptable.

25 [0079] The Type 1 IFN-mediated signalling antagonist may also be provided in combination with another neuroprotective agent such as LIF and/or CNTF or their homologs, derivatives, analogs or mimetics.

30 [0080] Hence, the present disclosure enables a method for the treatment or prophylaxis of a neuropathological event, disease or condition in a subject selected from a stroke event, the method comprising administering to the subject an effective amount of a neuroprotective formulation comprising a Type 1 IFN-mediated signalling antagonist and

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one or both of LIF and CNTF or a homolog, derivative, analog or mimetic thereof for a time and under conditions sufficient to reduce neurological inflammation or prevent or reduce its progression.

- 5 [0081] Also enabled herein are pharmaceutical compositions and formulations which include one or more of the neuroprotective agents hereinbefore described. The pharmaceutical compositions taught herein may be administered in a number of ways depending upon whether local or systemic treatment as desired including with means for the agent to cross the BBB. Administration includes intravenous, intra-arterial,
10 subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial administration, e.g., intrathecal or intraventricular, administration; or oral administration; or *via* a spinal tap. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Clearly, the formulation needs to enable the agent to cross the BBB. Hence, the agent itself may need to be modified.
15 Alternatively, the formulation may enable retrograde transport. The agents may also be specifically targeted to the brain or other parts of the CNS.

- [0082] The pharmaceutical formulations described herein may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in
20 the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s).

- [0083] The compositions described herein may be formulated into any of many possible dosage forms such as, but not limited to, injectable formulations, and tablets, capsules, gel
25 capsules and liquids.

- [0084] Pharmaceutical compositions herein include, but are not limited to, solutions, emulsions, foams and liposome-containing formulations. The pharmaceutical compositions and formulations herein described may comprise one or more penetration
30 enhancers, carriers, excipients or other active or inactive ingredients.

[0085] Emulsions are typically heterogeneous systems of one liquid dispersed in another in

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the form of droplets usually exceeding 0.1 μm in diameter. Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Microemulsions are included as an embodiment taught herein.

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[0086] Formulations include liposomal formulations. The term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers. Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior that contains the composition to be delivered.

10 Cationic liposomes are positively charged liposomes which are believed to interact with negatively charged DNA molecules to form a stable complex. Liposomes that are pH-sensitive or negatively-charged are believed to entrap DNA rather than complex with it. Both cationic and noncationic liposomes have been used to deliver DNA to cells.

15 [0087] Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome comprises one or more
20 glycolipids or is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety.

[0088] In an embodiment, various penetration enhancers may be employed to effect the efficient delivery of nucleic acids. In addition to aiding the diffusion of non-lipophilic
25 drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs. Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants.

30 [0089] One of skill in the art will recognize that formulations are routinely designed according to their intended use, i.e. route of administration.

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- [0090] The formulation of therapeutic compositions and their subsequent administration (dosing) are within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50} s found to be effective *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μ g to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μ g to 100 g per kg of body weight, once or more daily, to once every 20 years. Alternatively, the antagonists are provided in amounts of 1, 10, 100 or 1000 μ g/ml or amounts in between.
- [0091] The present disclosure teaches a neuroprotective formulation comprising a Type 1 IFN-mediated signalling antagonist and one or more pharmaceutically acceptable carriers and/or diluents. The neuroprotective formulation is provided to patients or at risk subjects of stroke.
- [0092] In another embodiment, the present disclosure teaches a neuroprotective formulation comprising a Type 1 IFN-mediated signalling antagonist and one or both of LIF and/or CNTF and one or more pharmaceutically acceptable carriers and/or diluents. In this aspect, the neuroprotective formulation is provided to patients or at risk subjects of stroke.
- [0093] Diagnostic assays to assess the presence of a neuroinflammatory response following a neuropathological event, disease or condition selected from a stroke event are

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enabled herein. For example, following ordering a stroke or infection, the level of Type 1 IFN-mediated signalling may be determined such as *via* the level of a Type 1 IFN, its corresponding mRNA levels, activity of IFNAR1 or its corresponding mRNA levels or *via* a down-stream effector such as TNF α , IL-6 or a chemokine or other pro-inflammatory effector molecule.

[0094] Hence, a medical protocol is enabled herein to treat a subject which has or may experience a neuropathological event, disease or condition selected from a stroke event, the protocol including:

- 10 (i) assessing physical trauma to the brain or other parts of the CNS;
- (ii) determining parameters of inflammation;
- (iii) administering an antagonist of Type 1 IFN-mediated signalling; and/or
- (iv) monitoring patients including subjecting the patient to behavioral modification procedures. Inflammatory parameters may be detected by any means
- 15 including HPLC, TLC, ELISA, RIA, immuno-fluorescent assay, Southern analysis, Western blot, Northern analysis, gel electrophoresis, nucleic acid expression and the like.

[0095] Another aspect of the present disclosure enables a medical protocol for treating acute neuronal injury in the form of a stroke event in a subject, the protocol comprising

20 administering to the subject, within from 1 to 120 minutes of the injury, a neuroprotective formulation comprising the antagonist of Type 1 interferon-mediated signalling. By "1 to 120" include 1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 69, 70, 71, 72, 73,

25 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119 or 120 minutes. In an embodiment, the time is 30 minutes.

[0096] Still another aspect taught herein the use of a Type I IFN-mediated signalling

30 antagonist in the manufacture of a medicament in the treatment or prophylaxis of neuroinflammation associated with a neurological condition or event selected from a stroke event.

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EXAMPLES

[0097] Aspects taught herein are further described by the following non-limiting Examples. In these Examples, materials and methods as outline below are employed.

5

Animals

[0098] Mice (eight-week-old male mice 23 ± 3 g) used were of a C57BL-6 strain with IFNAR1^{-/-} (knockout or KO) or IFNAR1^{+/+} (wild-type or wt) genotypes (Hwang *et al.*, *Proc Natl Acad Sci USA* 92(24):11284-11288, 1995).

10

Tissue Culture

Mixed hippocampal and cortical neurons

[0099] Pregnant female mice were killed by cervical dislocation when embryos were 14 days old. The embryos were extracted from the female uterus and their heads removed into a petridish of Solution 1 (30ml Krebs stock (36.25g NaCl, 2g KCl, 0.7g NaH₂PO₄·H₂O, 13g d-Glucose, 0.05g Phenol red, 20.7g HEPES (acid form) in 500ml H₂O, pH7.4), 270ml H₂O, 0.9g bovine serum albumin (BSA), 2.4ml 3.4% w/v MgSO₄, pH 7.4). The brains were removed, cerebral cortices were isolated and the meninges removed. The cortices were placed into a fresh petri dish of solution 1 and minced to a fine paste. The paste was transferred to a 50ml Falcon tube containing solution 2 (15ml sol 1, 1.5ml 10x trypsin (25mg trypsin in 10ml)) using a pasture pipette. The tube was incubated for 15-20 minutes at 37°C with occasional mixing by inversion. 12.5ml of Solution 4 (8.5ml Sol 1, 4ml Sol 3) was added to the digested tissue. The tissue was then centrifuged (1000rpm, 45 seconds), supernatant discarded and pellet resuspended in solution 3 (7.5ml Sol 1, 0.75ml 10x DNase/soybean trypsin inhibitor (SBTI) stock (8mg DNase, 52mg SBTI in 10ml), 75μL 3.4% w/v MgSO₄) by passing through a pasture pipette for 2 minutes, the solution was then transferred to a fresh 50ml Falcon tube. Remaining tissue was washed from the old tube with 2 pasture pipettes of solution 5 (6.25ml Sol 1, 50μL MgSO₄ stock, 7.5μL 1.2% w/v CaCl₂). Upon centrifugation (1000rpm, 5 minutes) and supernatant removal, the resulting pellet was resuspended in 10ml plating medium (Neurobasal media (Gibco) containing 10% v/v fetal bovine serum (FBS, Gibco)). Cells were then counted and diluted to achieve a final concentration of 11.2×10^5 cells/6cm dish. All tissue culture dishes were coated with poly-

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l-lysine before neurons were plated. Following 5 hours in plating medium, all medium was exchanged for neuronal culture medium (Neurobasal media containing 10ml B27 supplement (Gibco), 1.25ml 200mM l-glutamine and 0.5ml Gentamycin (Gibco) in 500ml), thereafter half medium changes were performed every 2-3 days with a final concentration of 20 μ M 5-chlorocytosine arabinoside (ARAC) added to prevent proliferation of murine embryonic fibroblasts.

Human neuroblastoma M17 cells (M17)

[0100] M17 cells (ATCC [Registered Trademark] number: CRL-2267 [Trademark]) were cultured in T75 flasks with culture medium (OptiMEM (Gibco), 5% v/v FBS, 0.5% w/v Penicillin-Streptomycin (Gibco)) at 37°C until 90% confluent. Cells were then plated at the densities listed in Table 3. For the Alzheimer's disease studies, M17 cells were housed in an incubator at 37°C and 5% v/v CO₂ in OPTI-MEN (Invitrogen) in 10% v/v FBS and 1% w/v penicillin/streptomycin.

TABLE 3

Densities

Dish	Cell density
24-well plate	5 x 10 ⁴ cells/well
6-well plate	1 x 10 ⁵ cells/well
6cm dish	1 x 10 ⁵ cells/dish

[0101] Plated cultures were then incubated for 24 hours at 37°C. Following incubation cells were serum-starved to halt the cell cycle with culture medium (no FBS) for 24 hours at 37°C, cells were then prepared for treatment.

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Isolation of Murine Cortical Neurons

[0102] A 14 day pregnant mouse was killed by cervical dislocation and uterine horns were removed and placed in a 10cm dish containing working solution 1 (For 300ml: 30ml Krebs 10x stock, 270ml MilliQ, 0.9g BSA, 2.4ml 3.82% w/v MgSO_4). Fetuses were removed from placental sacks, decapitated and heads were placed in a new dish containing solution 1. Under a dissection microscope skull and meninges were removed and cortical tissue was placed in a dish and chopped finely with a scalpel. Flamed Pasteur pipettes were then used to separate tissue into tubes using 20ml solution 1 with 10% w/v trypsin added. Cells were then incubated for 15 minutes at 37°C with occasional shaking. 25ml of solution 1 with 3% w/v added 10X DNase (Sigma D-5025)/SBTI (Sigma T-9003) was then added and gently inverted until DNA strings appeared. Cell suspension was then spun for 1 minute (1000rpm, RT) and supernatant removed. Cell pellet was then resuspended in 8ml of solution 1 with 10% w/v DNase/STBI by 60 pasteur pipette passages and spun for 5 minutes at 1000rpm at RT. Following discarding of supernatant, cell pellet was resuspended in 10ml of Dulbecco's Modified Eagle Medium (Invitrogen) with 10% v/v FBS. The suspension was placed in a T-75 flask and allowed to incubate at 37°C for 1 hour to allow fibroblast adhesion. Cell media containing only neurons was then removed from the flask, cell density determined, and plated into appropriate poly-L-lysine coated plates and kept in an incubator (37°C and 5% v/v CO_2). 4 hours following plating Neurons are given a full media change to DMEM with 10% FBS and 0.5% w/v penicillin/streptomycin.

Tissue culture treatments***Oxygen Glucose Deprivation (OGD)***

[0103] To engender OGD, culture media was replaced with no glucose and FBS DMEM (0.5 standard culture media volume) and cells were introduced into an hypoxic chamber (1% v/v O_2 , 5% v/v CO_2 , 94% v/v N_2 and 60% humidity) for 3 hours (M17 cells) or 4 hours (WT or $\text{IFNAR1}^{-/-}$ neurons). Cells were then removed from the chamber and reperused with DMEM (high glucose, 0.5 standard culture media volume) for 0, 0.5, 2 or 24 hours. After this reperfusion event, cells were immediately utilized for MTT assay, RT-PCR or Western blot analysis.

IFN α /IFN β cytokine treatment

[0104] 6cm dishes of M17 cells were treated with human (hu) IFN α (huIFN α) and human IFN β (huIFN β) at 1, 10, 100 or 1000U/ml for a time course of 5, 10 or 30 minutes whilst incubated at 37°C. Post-treatment harvested for Western blot analysis.

5 SDS PAGE gel protein separation and western blotting

[0105] Cells (6cm dish) were scraped in 1ml PBS/plate and centrifuged at 5000x g for 5 minutes. The resultant cell pellet was resuspended in 100 μ L of lysis buffer (50mM Tris HCl, 150mM NaCl, 1% v/v Triton x-100, 1% v/v SDS, 1 tablet PhosphoSTOP (Registered Trademark) and protease inhibitor (Roche), pH 7.4) and lysed *via* sonication. 60 μ g of protein was added to 1:1 v/v reducing buffer (2.5ml 0.5M Tris, 2ml Glycerol, 4ml 10% w/v SDS, 1ml (3-mecaptoethanol, bromophenol blue, in 10ml) and heated to 99°C for 5 minutes. 10% w/v acrylamide SDS PAGE (17.85ml dH₂O, 11.25ml 1.5M Tris, 450 μ L 10% w/v SDS, 15ml 30% w/v Acrylamide/BIS (Bio-rad), 15 μ L N,N,N',N'tetramethylethylenediamine (TEMED, Bio-rad), 450 μ L 10% w/v ammonium persulphate (APS), per 5 gels) in running buffer (0.1%w/v SDS, 1.44% v/v glycine, 0.3% w/v Tris, pH 8.4) was used to separate the denatured protein. Samples were loaded on a stacking gel (3ml dH₂O, 1.25ml 0.5M Tris, 100 μ L 10% w/v SDS, 650 μ L Acrylamide/BIS, 7.5 μ L TEMED, 100 μ L APS) and run at 80-120V for 1.5 hours. Polyvinylidene fluoride (PVDF) membrane was presoaked in 100% v/v methanol for 5 minutes and washed in transfer buffer (20% v/v methanol in running buffer). Separated proteins were transferred from PAGE gel to PVDF membranes utilizing a semi-dry transfer unit (60mA/gel for 75 minutes). Membranes were blocked with 5% w/v skim milk powder in TBS-T (10mM Tris, 15mM NaCl, 0.01% v/v Tween-20) for 1 hour. Phospho-STAT-1, Phospho-STAT-3 (Cell Signalling) and α -tubulin (Millipore) antibodies were diluted 1:1000 in 5% w/v BSA in TBS-T and incubated on membranes at 4°C overnight. Membranes were then washed 3 times for 5 minutes with TBS-T and incubated with goat anti-rabbit or goat anti-mouse (for (3-tubulin) horseradish peroxidase (HRP) conjugated secondary antibodies, diluted 1:1000 in 5%w/v BSA in TBS-T, for 60 minutes at 25°C. HRP product was detected using an ECL advance western blotting detection kit (Amersham) After 4 washes of 5 minutes in TBS-T the ECL solution was applied to the membrane and visualized using the IQ350 imaging machine (GE Healthcare).

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Real-Time Polymerase Chain Reaction (RT-PCR)***TRIzol (Registered Trademark) mRNA extraction***

[0106] Cells were plated at 2×10^5 cells/6cm dish and incubated overnight in OptiMEM (5% w/v FBS, 0.5% w/v Penicillin-Streptomycin). Post 3 hours OGD and 0, 0.5, 2 or 24 hours reperfusion media was removed and cells were washed in 1ml/6cm dish PBS. Cells were lysed in 1ml/6cm dish TRIzol (Registered Trademark) [Invitrogen], transferred to a 1.7ml microcentrifuge tube and incubated for 10 minutes at 25°C. 0.2ml/sample chloroform was added and after vigorous shaking was incubated at 25°C for 3 minutes. Samples were centrifuged (12,000x g, 15 minutes, 4°C) and resultant aqueous layer containing RNA was removed to fresh 1.7ml microcentrifuge tube. RNA was precipitated by adding 0.5ml/sample isopropanol and incubating at 25°C for 10 minutes. Following centrifugation (12,000x g, 10 minutes, 4°C), the supernatant was removed and resultant pellet was washed with 1ml/sample 75% v/v EtOH in DEPC treated dH₂O and centrifuged again (7,500x g, 5 minutes, 4°C). The RNA pellet was air-dried and redissolved in 30μL/sample RNAase free water. Sample RNA concentration was then analyzed by the nanodrop 1000 spectrophotometer (Thermo-scientific).

Reverse transcription of RNA to cDNA

[0107] RNA was reversed transcribed into cDNA using SuperScript (Registered Trademark) III kit (Invitrogen) according to manufacturer guidelines. Briefly, 1μg of sample RNA was converted to cDNA using reverse transcriptase under the following conditions: 25°C for 10 minutes, 50°C for 30 minutes then 85° for 5 minutes. Residual RNA was digested by the addition of RNase H to ensure purification of cDNA which was 1:10 in dH₂O for use in RT-PCR.

25

RT-PCR using Taqman (Registered Trademark) probes

[0108] Taqman probes (all reagents from Applied Biosystems) were used as RT-PCR primers, these included: Tumor Necrosis Factor α (TNFα, Hs00174128_m1), Interleukin-1β (IL-1β, Hs00174097_m1), Interleukin-6 (IL-6, Hs00985639_m1), Interferon-α1 (IFNα1, Hs00256882_sl), Interferon-β (IFNβ1, Hs01077958_sl) and 18s ribosomal RNA (18s rRNA, 4352930E). RT-PCR was performed in a 384-well plate with 4μL diluted cDNA, 0.5μL DEPC dH₂O, 0.5μL Taqman primer, 5μL Taqman Fast Universal PCR master

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mix (2x) per well. Each sample was repeated in triplicate and RT-PCR performed using the 7900ht Fast Real-Time PCR system (Applied Biosystems) under the following conditions: 50°C for 2 minutes, 94.5°C for 10 minutes, (97°C for 30 seconds, 59.7°C for 1 minute)x 40 repeats. Data were collated and fold change calculated using the AACt method.

5

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

[0109] Brains were dissected into ipsilateral and contralateral hemispheres 2, 4 and 24 hours after injury, or sham operation. Hemispheres were homogenized in 2 ml Trizol (Invitrogen), and placed at room temperature for 10 minutes. 0.2ml Chloroform (Chem Supply) per 1ml Trizol was added to the samples, and samples were centrifuged at 12000g for 15 minutes at 4°C to separate samples into phases. The colorless, aqueous phase of each sample, which contained RNA, was transferred into a new 1.7ml microcentrifuge tube. RNA was precipitated by adding 0.5ml Propan-2-ol (Chem Supply) per 1ml Trizol, and samples were again centrifuged at 12000g for 10 minutes at 4°C. The supernatant from the tubes was discarded, and the RNA pellet was washed with 75% Ethanol (Chem Supply) in Diethyl pyrocarbonate (DEPC)-treated water (Sigma), vortexed and centrifuged at 7500g for 5 minutes at 4°C. The RNA pellet was air-dried and redissolved in RNase-free H₂O (Invitrogen). Concentration of the RNA samples was assessed using the NanoDrop 1000 Spectrophotometer (ThermoScientific).

20

[0110] 1 µg of RNA was transcribed per cDNA reaction using the SuperScript (Registered Trade Mark) III kit (Invitrogen) according to these conditions: 10 minute incubation at 30°C, 30 minute incubation at 50°C, 5 minute incubation at 85°C, and finally, a 20 minutes incubation at 37°C with 1µl *E. coli* RNase H (from the same kit).

25

[0111] Samples were diluted 1:10 in RNase free H₂O. The following Taqman primers were obtained from Applied Biosciences: TNFα (ID: Mm00443258_m1), IFNβ (ID: Mm00439552_s1), IL-1β (ID: Mm01336189_m1) and 18S rRNA (ID: 4352930E). Taqman primers were incubated along with sample in a 384 well plate (MicroAmp, Singapore) in the following ratios: 0.5µl primer, 0.5µl H₂O, 5µl 2x Taqman Mix (Invitrogen) and 4µl diluted cDNA. SybrGreen primers were obtained from GeneWorks (sequences are listed in Figure 8): Allalpha forward, Allalpha Reverse 1, Allalpha Reverse

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2, GADPH forward and GADPH reverse. SybrGreen primers were incubated in the following ratios: 2µl diluted cDNA, 5µl Fast SybrGreen Master Mix, 1µl Forward GADPH primer, 1µl Reverse GADPH primer and 1µl H₂O. The plate was incubated according to the following conditions and read using the 7900ht spectrophotometer
5 (Applied Biosciences): 95°C for 20 minutes, 95°C for 3 minutes (40 repeats), 60°C for 30 minutes, 95°C for 15 minutes, 60°C for 15 minutes and 95°C for 15 minutes. Ct values were obtained for each sample, and relative transcript levels for each gene were calculated using the SSCT method.

10 MTT cell viability assay

[0112] M17 cells in 24-well plates were subjected to OGD and subsequent reperfusion (24 hours). Post-reperfusion 50µL/well MTT reagent (2mg/ml, Sigma) was added to media and incubated for 1 hour at 37°C. Culture medium was then removed and cells were solubilized with 200µL/well of dimethyl sulfoxide (DMSO) of which 100µL was
15 transferred to a clean 96-well plate and absorbance read at 595nm.

cDNA Preparation

[0113] Conversion of RNA to cDNA involved the use of a High Capacity RNA to cDNA kit (Applied Biosystems) as per manufacturers instructions. Briefly this involved loading
20 1µg of RNA with 10µl 2X reaction mix with 1 µl RT enzyme mix and DEPC H₂O to 20µl. Tube was then heated at 35°C for 30 minutes, and 95°C for 5 minutes (termination step) and chilled on ice.

RT PCR

25 [0114] Relative expression levels of RNA were measured using the prepared cDNA in accordance with the TaqMan (Registered Trademark) [Applied Biosystems] primer preparation. This involves adding in triplicate 4µl cDNA with 5µl 2X TaqMan Mix, 0.5µl DECP H₂O, and 0.5µl of primer of mRNA in question. PCR plate was then read on a 7900ht Fast Real-Time PCR System. Fold increase was determined by using the delta-delta
30 method.

Gateway (Registered Trademark) cloning system

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Ligation of subclone AttB1 and subclone AttB2 primers to mIFNAR1/mIFNAR2 genes

[0115] cDNA encoding mouse interferon receptor α 1 (mIFNAR1) and mouse interferon receptor α 2 (IFNAR2) were custom synthesized and inserted into vector puc57 by GeneScript (U.S.A.). Subclone forward primers containing AttB1 motif and subclone reverse primers containing AttB2 motif were designed and synthesized (Geneworks) for both mIFNAR1 and mIFNAR2 genes to use with the Gateway (Registered Trademark) cloning system (Invitrogen). These primers were ligated to the mIFNAR1 and mIFNAR2 genes via PCR using GoTaq (Registered Trademark) polymerase (Promega). Nucleotide sequences of IFNAR1 & 2 and AttB1 and AttB2 sequences are shown in Figures 6 and 7.

10

Polymerase chain reaction conditions

[0116] Briefly a 20 μ L sample reaction consisted of 4 μ L 5x GoTaq buffer, 1 μ L MgCl₂ (25mM), 0.4 μ L dNTPs (2mM), 0.2 μ L Subclone AttB1 primer (20 μ M), 0.2 μ L Subclone AttB2 primer (20 μ M), 0.1 μ L GoTaq polymerase (5U/ μ L), DEPC H₂O (to 20 μ L). The ligation reaction was performed under the conditions given in Table 4.

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TABLE 4*Ligation conditions*

Step	Temperature	Duration
Initial Denaturation	95°C	2 minutes
Denaturation	95°C	40 seconds
Anncaling	60°C	40 seconds
Extension	72°C	110 seconds
Final Extension	72°C	5 minutes

Purification of AttB1-mIFNAR1/2 -AttB2 PCR product

- [0117] Samples were then electrophoresized on a 1% w/v agarose gel (1g agarose, 2μL Ethidium Bromide per 100ml TAE buffer (242g Tris Base, 57.1ml glacial acetic acid, 100ml 0.5M Ethylenediaminetetra-acetic acid (EDTA) per Liter)) and a 1.77kb
- 5 (mIFNAR1) or 1.54kb (mIFNAR2) was selectively cut from the entire gel. The gel bound AttB1-mIFNAR1/2-AttB2 ligation product was then purified from the PCR reaction using the QIAquick (Registered Trademark) PCR Purification Kit (QIAGEN) according to manufacturer's protocol. Briefly, the gel containing AttB1-mIFNAR1/2-AttB2 was redissolved and DNA was retained on a silicon filter whilst impurities were discarded.
- 10 Post-washing with ethanol buffer the purified DNA was then dissolved in elution buffer (10mM Tris-HCL, pH8.5).

The BP reaction

- [0118] The Invitrogen Gateway (Registered Trademark) cloning system was used. The
- 15 purified AttB-mIFNAR1 and AttB-mIFNAR2 PCR products were translocated into vector pDONR201 (Invitrogen) using the BP Clonase (Trademark) II enzyme kit (Invitrogen), according to manufacturers guidelines. A 10μL reaction comprised of 0.6μL pDONR201 (289ng/μL), 1.8μL AttB-mIFNAR1 PCR product (33ng/μL) or 1.5μL AttB-mIFNAR2 PCR product (37ng/μL), 2μL BP Clonase (Trademark) II enzyme mix, TE buffer to 10μL.
- 20 The reaction was incubated for 24 hours at 25°C and subsequently terminated upon addition of Proteinase K (1μL, 2μg/μL).

DH5α bacterial transformation

- [0119] The BP reaction product was transformed into DH5α competent *E. coli* cells
- 25 (Invitrogen) by adding 1μL BP reaction product to each DH5α aliquot (50μL/tube). Cells were then incubated on ice for 30 minutes and heat-shocked by incubation at 42°C for 30 seconds. 250μL/tube of S.O.C. medium (Invitrogen) was added and cells were shaken (37°C, 225rpm, 1 hour) before 20μL was spread evenly on a kanamycin (50μg/ml) selective agar plate (1% w/v Tryptone, 1% w/v NaCl, 0.5% w/v Yeast extract, 0.75% w/v
- 30 agar) and incubated for 16 hours at 37°C. A single bacterial colony was picked for colonies PCR to verify transformation success. The selected colony was grown in 10μL Luria Broth (LB, 1% w/v Tryptone, 1% w/v NaCl, 0.5% w/v Yeast Extract) of which 5μL was lysed at

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99°C for 5 minutes. PCR was performed using the conditions above to confirm successful mIFNAR1/2 insertion into pDONR201 and the subsequent DH5 α colony. The PCR products were then electrophoresized on a 1% w/v agarose gel and 1.77kb (for mIFNAR1) or 1.54kb (for mIFNAR2) bands were visualized using the IQ-350.

5

Isolation of pDONR201-mIFNAR1/2 plasmid

- [0120] Once the selected colony was deemed positive for pDONR201-mIFNAR1/2 insertion, the remaining 5 μ L of culture (not used in PCR) was supplemented with a further 5ml kanamycin (50 μ g/ml) selective LB media and shaken for 16 hours (225rpm, 37°C).
- 10 Plasmid DNA was then isolated from the bacterial culture using QIAprep (Registered Trademark) Spin Miniprep kit (QIAGEN) as per manufacturer's protocol. Briefly, bacteria were lysed, excess protein precipitated and DNA retained in the filter of a spin column. After subsequent washing with an ethanol buffer, DNA was eluted from the filter column giving isolated pDONR201-mIFNAR1/2 plasmid. DNA yields were then measured using
- 15 the nanodrop 1000.

The LR reaction

- [0121] The mIFNAR1/2 genes were removed from the pDONR201-mIFNAR1/2 plasmid and translocated into a pcDNA6.2/cEM-GFP destination vector using the LR Clonase
- 20 (Trademark) II kit (Invitrogen) as per manufacturers protocol. A 10 μ L reaction consisted of 1 μ L pDONR- mIFNAR1 (122.6ng/ μ L) or 1 μ L pDONR-mIFNAR2 (156.9ng/ μ L) entry clones, 2 μ L pcDNA6.2/cEM-GFP (75ng/ μ L) destination vector, 2 μ L LR Clonase (Trademark), TE buffer to 10 μ L. The reaction was incubated for 24 hours at 25°C and subsequently terminated upon addition of Proteinase K (1 μ L, 2 μ g/ μ L). Reaction samples
- 25 were then transformed into DH5 α competent *E. coli* cells as per above. A colony was selected from an ampicillin (50mg/ml) agar plate and grown in 10 μ L LB media of which 5 μ L was lysed by heating (99°C, 5 minutes). PCR was then performed under the conditions described above and samples run on a 1% w/v agarose gel. Imaging the gel using the IQ-350 showed a 1.77kb mIFNAR1 specific or a 1.54kb mIFNAR2 specific band for
- 30 successful LR reaction and transformation samples. Positive colonies were then selected and shaken in 250ml ampicillin (50 μ g/ml) selective LB media for 16 hours (225rpm, 37°C).

Isolation of final pcDNA6.2/cEM-GFP-mIFNAR1/2 plasmids

[0122] Bacterial plasmid DNA was isolated using EndoFree (Registered Trademark) plasmid Maxi Kit (QIAGEN) following manufacturers protocol. Briefly, bacteria were
5 lysed, excess protein precipitated and DNA retained in the filter of a gravity flow column. After subsequent washing with an ethanol buffer DNA was eluted from the gravity flow column. The DNA was then precipitated using isopropanol, washed with endotoxin-free ethanol and resuspended in supplied TE buffer. DNA yields of pcDNA6.2/cEM-GFP-mIFNAR1/2 plasmids were then determined using the nanodrop 1000.

10

Statistical analysis

[0123] All numerical data are stated as mean $\pm$ SEM and analyzed using GraphPad Prism 5.0. RT-PCR and MTT assay data were analyzed using Student's t-test or one-way ANOVA and subsequent Dunnett's Post-hoc test where applicable. For all statistical tests
15 $P < 0.05$ was considered significant. For qRT-PCR data, a one way Analysis of Variance (ANOVA) was also performed followed by Bonferroni's post-hoc analysis, with a value of $P < 0.05$ considered statistically significant. Infarct volume values were analyzed using an unpaired Student's t-test, with a value of $P < 0.05$ considered statistically significant.

20 Preparation of serial sections for staining

[0124] Mice were transcardially perfused at various time points after injury (or sham surgery) with 0.1% v/v heparinized Phosphate-Buffered Saline (Pfizer), followed by 4% v/v paraformaldehyde (Scharlab S.L.), and their brains dissected. Brains were cut by the Histology facility, University of Melbourne. Brain sections were cut into 10 μ m coronal
25 sections starting at the rostral end, paraffin-embedded and mounted onto glass slides. Every 10th slide was stained with Haematoxylin and Eosin (H&E).

Infarct volume analysis

[0125] Infarct volume analysis was conducted on slides, which had been stained with
30 H&E. Analysis was performed using Image J. Area of infarct was calculated by measuring around regions appearing less intensely stained for viable cells. The average area of infarct per brain was then multiplied by the number of sections per slide by the thickness of each

section (10µm) and the number of slides in the sequence to get the volume of infarct.

Immunohistochemistry

[0126] Paraffin-embedded sections were put in a 60°C oven for 20 minutes and taken
5 through a series of Histolene (Lomb Scientific) incubations, followed by 100, 95 and 70%
ethanol. Sections were incubated in Phosphate-Buffered Saline for a further 5 minutes.
Sections were blocked firstly for endogenous peroxidase activity with a Peroxidase
blocking solution (DAKO), followed by a protein-blocking buffer (5% v/v whole Goat
serum [Invitrogen] and Triton X-100 solution [Sigma] in 1x Phosphate Buffered Saline)
10 for another hour. Where sections were used for immunofluorescence, only protein-
blocking buffer was used. The primary antibody was diluted in an appropriate dilution
buffer (1% w/v BSA [Bovogen] in 1 x Phosphate Buffered Saline), and slides were
incubated with the antibody overnight at 4°C. Primary antibodies used were: NeuN
(Millipore) and Mac-1 (Monash University), [Flentjar *et al.*, *Exp Neurol* 177(1):9-20,
15 2002]). Sections were washed in PBS, and incubated in an appropriate secondary antibody.
Fluorescent secondary antibodies (Alexa Fluor 594 anti-mouse and rabbit, Alexa Fluor 488
anti-mouse, rabbit and rat) were obtained from Invitrogen and the biotinylated secondary
antibody (Anti-Mouse IgG, horse biotinylated) was obtained from Vector Laboratories.
Biotinylated primary antibodies were visualized using the Vector Vecstain ABC kit
20 (Vector Laboratories) using Diaminobenzidine (DAB [DAKO]) as a substrate.

Magnetic Resonance Imaging

[0127] Wild-type mice were intra-venously injected with IgG Isotype control (25mg/kg) or
MAR1 antibody (25mg/kg) prior to and subsequently to subjecting the mice to a stroke
25 event. Mice were initially anaesthetized with approximately 3% v/v Isoflurane in a 1:1
mixture of medical grade air and oxygen. Anesthesia was maintained throughout scanning
with 0.25-1.5% v/v Isoflurane through a nosecone placed over the animal's snout and
respiration was continuously monitored throughout the experiment with a pressure
sensitive probe positioned over the animal's diaphragm. Anaesthetized animals were laid
30 supinely on a purpose built animal holder and their head fixed into position with ear and
bite bars. A surface receive coil was placed over the animals head and the cradle was
inserted into a transmit coil fixed inside a BGA12S gradient set for imaging with a 4.7

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Tesla Bruker Biospec 47/30 scanner. The scanning protocol consisted of a 3-plane localizer sequence followed by multi-slice axial, coronal and sagittal scout images to ascertain the orientation and position of the brain. To calculate a T2 map, three T2-weighted images were acquired using a rapid acquisition, relaxation enhanced (RARE) sequence with the following imaging parameters: recovery time (TR) = 3000 ms, RARE factor = 8, field of view (FOV) = 12.8 x 12.8 mm², matrix size = 128 x 128, in-plane resolution = 100 x 100 µm², number of slices = 15, slice thickness = 0.6 mm, averages (NEX) = 16 and total scan time of 12 minutes. The effective echo times (TE_{eff}) were: 45 ms, 60 ms and 80 ms. Diffusion weighted images (DWI) were also acquired with the following parameters: TR = 3000 ms, TE = 60 ms, FOV = 12.8 x 12.8 mm², matrix size = 128 x 128, in-plane resolution = 100 x 100 µm², number of slices = 6, slice thickness = 0.6 mm, number of repetitions (NR) = 4 and total scan time of 12 minutes 48 seconds per repetition. A single b₀ and diffusion image were acquired with the following diffusion parameters: δ = 7 ms, Δ = 14 ms and b-value = 1500 s/mm². Images were analyzed for infarct volume using Matlab R2010b (Mathworks).

MTS Viability Assay

[0128] CellTiter 96 (Registered Trademark) Aqueous MTS Reagent (Promega) was prepared according to manufacturer's instructions. Briefly, this involved 42mg of MTS Reagent powder dissolved in 21ml Dulbecco's PBS solution, pH to 6.0-6.5 and filter sterilized. For viability assays, 20 parts MTS solution was combined with 1 part phenazine methosulfate (PMS, Sigma) and 1/5 final well volume was added to cell wells in triplicate (i.e. 80µl MTS/PMS in 400µl well). MTS well preparation was then allowed to incubate for 2 hours at 37°C and absorbance was measured at 490nm. A Students T-Test was used to test statistical significance using Graphpad Prism 5 Software.

Western Blot Analysis

[0129] Protein was extracted by removal of media and scrapping cells into a tube using PBS. Cells were spun to a pellet at 5000g (5mins, 4°C). Supernatant was removed and cells were re-suspended in 150 µl Lysis Buffer (10uM Tris-HCl, 2% w/v SDS, Protease inhibitor, PMSI) and machinated using a probe sonicator.

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[0130] Protein levels were detected using Western Blot Analysis. The 12% w/v resolving gel (8ml 30% w/v Acrylamide, 5ml 1.5M Tris, 200 μ l 10% w/v SDS, 6.68ml mQH₂O, 200 μ l 10% APS, 20 μ l TEMED) was prepared days in advance with the 5% w/v stacking gel (1.67ml 30% w/v Acrylamide, 2.5ml 0.5M Tris, 100 μ l 10% w/v SDS, 5.67ml mQH₂O, 100 μ l 10% w/v APS, 10 μ l TEMED) poured the day the gel was run. 50 μ g of protein was taken with an equal volume of 2XTris-glycine sample buffer with 5% v/v β -mercaptoethanol, boiled for 5 minutes at 95 degrees and loaded into the well. A ladder consisting of 2 μ l SeeBlue (Registered Trademark) Marker 2 μ l MagicMark (Trademark) [Invitrogen] and 6 μ l PBS was used. Gels were run at a voltage of 80mV until protein passed the stacking gel, when voltage was kept constant at 120mV. Protein was transferred from gel to membrane using a semi-dry apparatus at a constant current of 60mA per gel for 1 hour. Membranes were then blocked in 5% w/v milk powder (In TBST) for 1 hour. Primary antibodies (1/1000) were incubated at 4°C overnight on a roller. Membranes were rinsed three times with TBST and secondary antibodies (1/1000) were then incubated at RT in 5% Milk for 1 hour. Membrane were developed using ECL (Amersham) and imaged on an IQ350.

Immunohistochemistry

[0131] Cells plated on glass cover slips were harvested for immunohistochemistry by removing cell media, rinsing three times with ice-cold PBS and fixing with 4% v/v para-formaldehyde for 15 minutes at RT. Cells were then permeabilized using 0.2% v/v Triton X-100 in PBS at RT for 20 minutes and rinsed three times in PBS. CAS-Block (Trademark) [Invitrogen] was then used to block at RT for 1 hour. Cells were then incubated overnight with primary antibody (1/100) in CAS-Block (Trademark). Following primary antibody binding, cells were rinsed in PBS three times and incubated 2 hours with the secondary antibody in CAS-Block (Trademark). Cells were then rinsed three times in PBS and mounted onto slides using Vectashield (Registered Trademark) mounting media with DAPI.

EXAMPLE 1***IFNAR1^{-/-} neurons demonstrate increased viability in response to OGD***

[0132] Previous data had demonstrated that Middle Cerebral Artery Occlusion (MCAO) surgery performed on IFNAR1^{-/-} and IFNAR2^{-/-} mice showed a decreased infarct size of IFNAR1^{-/-} ($24.9 \pm 7.1 \text{ mm}^3$), but not IFNAR2^{-/-} ($52.3 \pm 4.9 \text{ mm}^3$), mice compared to wild-type ($65.1 \pm 4.8 \text{ mm}^3$) ($n=6-8$, $P<0.05$, One-way ANOVA, Dunnett's Post-hoc test, Figure 1). To support these findings primary cultures of IFNAR1^{-/-} and wild-type neurons were subjected to 4 hours OGD with 24 hours reperfusion and an MTT assay was performed. IFNAR1^{-/-} neurons showed a significant increase in cell viability ($91.0 \pm 4.9\%$) compared to wild-type ($49.8 \pm 0.8\%$) in response to OGD ($n=6$, $*P<0.05$, students t-test, Figure 1).

EXAMPLE 2***IFN-dependent STAT phosphorylation***

[0133] IFN is known to induce STAT phosphorylation governed by the JAK-STAT pathway. To identify the optimal concentration of IFN required to induce STAT phosphorylation, M17 cells were treated with increasing concentrations of IFN α and IFN β (1, 10, 100 and 1000U/ml) for 15 minutes and harvested for Western blot analysis. 1000U/ml of IFN α induced the most prominent phosphorylation of STAT-1 in contrast to IFN β treatment which induced similar levels of STAT-1 phosphorylation across all concentrations.

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EXAMPLE 3***IFN α preferentially induces P-STAT-1 not P-STAT-3***

[0134] To investigate the STAT phosphorylation profile induced from IFN-dependant signalling, M17 cells were treated with 1000U/ml IFN α or IFN β (concentration selected from previous concentration-response Western blots) for a time-course of 5, 10 or 30 minutes. Western blot analysis of STAT-1 and STAT-3 phosphorylation was then conducted. IFN α treatment induced P-STAT-1 across all time-points with 10 minutes treatment showing the most prominent phosphorylation. IFN β treatment also induced P-STAT-1 across all time-points with noticeable phosphorylation at 10 and 30 minutes treatment. IFN β also stimulated STAT-3 phosphorylation at all time-points but the more robust responses came earlier than that of P-STAT-1 induction (5 and 10 minutes treatment). Interestingly, IFN α treatment was causative of only minimal STAT-3 phosphorylation at 10 minutes compared to the robust P-STAT-1 induction (IFN α , 10 minutes).

EXAMPLE 4***M17 cells subjected to OGD show induction of P-STAT-1 but not P-STAT-3***

[0135] STATs initiate pro-inflammatory gene transcription leading to increased cytokine release. In the OGD environment, cells are exposed to an inflammation state which contributes to the persistent cell death. To analyze if STATs play a role in the signalling events responsible for OGD-dependant cell death, cells were exposed to 3 hours OGD and a time-course reperfusion period (0, 2 and 24 hours). OGD treatment induces P-STAT-1 across all reperfusion time-points (0, 2 and 24 hours) but is most prominent at 2 hours reperfusion. Interestingly, OGD treatment did not cause STAT-3 phosphorylation across any reperfusion time-points.

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EXAMPLE 5***IFN α mRNA levels are elevated earlier in OGD reperfusion than IFN β mRNA levels***

[0136] Given the STAT-1 phosphorylation in the OGD response, the time-course of IFN mRNA transcript production was investigated. M17 cells were subjected to 3 hours OGD with a time-course reperfusion (0, 0.5, 2 and 24 hours) and cellular RNA was isolated. RNA was reverse transcribed to give cDNA and RT-PCR was performed. IFN α mRNA levels were significantly increased at 2 hours reperfusion (15.1 ± 2.3 fold change) before returning to basal levels by 24 hours. In contrast, IFN β mRNA levels were only increased after 24 hours reperfusion (2.4 ± 0.1 fold change) ($n=3$, $*P<0.05$, One-way ANOVA, Dunnett's Post-hoc test, Figure 2).

EXAMPLE 6***IL-6, TNF- α and IL-1 β cytokine mRNA levels show elevation post-OGD***

[0137] Apart from IFN α and IFN β mRNA common pro-inflammatory cytokines IL-6, TNF- α and IL-10 were also analyzed *via* RT-PCR to confirm the inflammatory state created by OGD treatment. IL-6 mRNA levels were significantly increased at 0.5 and 2 hours reperfusion (266.9 ± 10.9 and 194.6 ± 39.4 fold change, respectively) before returning to basal levels by 24 hours reperfusion. TNF- α mRNA levels were significantly elevated at an earlier time-course of 0 and 0.5 hours reperfusion (10.0 ± 3.1 and 10.1 ± 2.8 fold change respectively) and returned to control levels by 2 hours reperfusion. IL-10 mRNA levels showed a later onset significant increase at 24 hours reperfusion (4.6 ± 1.2 fold change). ($n=3$, $*P<0.05$, One-way ANOVA, Dunnett's Post-hoc test, Figure 3). Figure 4 shows a summary of collated RT-PCR data.

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EXAMPLE 7***Verification of mIFNAR1/2 gene insert within pcDNA6.2/cEM-GFP plasmid***

[0138] In order to investigate the effects of IFNAR1 and IFNAR2 subunit over-expression during OGD and subsequent reperfusion, the genes were inserted into the pcDNA6.2/cEM-GFP plasmid for subsequent cellular transfection. To verify both the IFNAR1 and IFNAR2 gene inserts in the plasmid, PCR using a forward plasmid-specific T7 and reverse gene-specific attB2 primers was used to amplify the gene product. A 1.77kb genomic band specific for the IFNAR1 gene insert was identified. A 1.54kb genomic band specific for the IFNAR2 gene insert was also observed.

EXAMPLE 8***Confirmation of mIFNAR1/2-GFP plasmid cellular transfection***

[0139] To evaluate the efficiency of M17 cell-line transfection with pcDNA6.2/cEM-GFP-MIFNAR1/MIFNAR2 constructs, fluorescence microscopy was used to detect GFP expression in the transient transfected M17 cell-line. Results show >50% transfection efficiency, with IFNAR1-GFP located in the cytoplasm and bound to the cell membrane of M17 cells.

EXAMPLE 9***Over-expression of IFNAR1 in OGD treated M17 cells is detrimental***

[0140] In order to evaluate the *in vitro* effect of IFNAR1 and IFNAR2 over-expression, M17 cells were subjected to 3 hours OGD and 24 hours reperfusion. MTT assay was then used to assess cell viability. Interestingly, M17-IFNAR1 cells showed decreased viability ($21.6 \pm 5.3\%$) in comparison to wild-type ($44.9 \pm 2.1\%$). Furthermore, M17-IFNAR2 cells showed no difference in cell viability ($39.8 \pm 5.7\%$) when compared to wild-type ($n=4$, $*P<0.05$, One-way ANOVA, Dunnett's Post-hoc test, Figure 5).

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EXAMPLE 10***IFNAR1 is both membrane-bound and intra-nuclear in WT neurons***

5 [0141] Considering the difference in IFNAR1 and IFNAR2 over-expression in terms of cell viability IFNAR1 cellular trafficking was briefly investigated. Isolated CBL57/6 WT neurons were used for immunohistochemistry probed with mAb-IFNAR1. Fluorescence microscopy shows membrane-bound, but more interestingly, intra-nuclear localization of the IFNAR1 subunit.

10

EXAMPLE 11***H&E Staining and NeuN immunohistochemistry and
Magnetic Resonance Imaging***

15 [0142] Both procedures represent traditional methods used to assess neuronal damage following stroke. Magnetic Resonance Imaging (MRI) is a particularly powerful approach used to analyze brain damage, as it allows an *in vivo* assessment of the progression of oedema and injury, done in real time. MRI scans were done on mice, 2 and then 24 hours after a stroke event. T2 images show the diffusion of water inside tissue, and are thus ideal
20 for visualization of tissue oedema. The development of the infarct and penumbra (surrounding area of damage) is shown, with the size of the infarct and penumbra increasing drastically after 24 hours. Another imaging sequence used, Diffusion Weighted Imaging (DWI) allowed an assessment of the movement of water molecules within injured and uninjured tissue. This generated an Apparent Diffusion Coefficient (ADC) map, and
25 using this sequence, brains were again measured for infarct volume. An ADC map of two sequential slices is taken of an injured brain, showing both infarct and penumbra size, in voxels. A T2 map is generated by T2-weighted imaging of the same brain, showing again infarct and penumbra size. The two types of sequence give different values for infarct and penumbra size. To determine whether this difference is also observed through a more
30 conventional way of measuring infarct volume (H&E sections measured through ImageJ), the infarct sizes, in voxels, of the three wild-type brains, which had been previously imaged at 24 h, are converted into mm³ (one voxel in this scanning sequence is equivalent to

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100x100x600 μm^3). A comparison of all the methods is used to obtain infarct volumes (T2-weighted imaging, DWI and H&E infarct analysis). The mean infarct volumes obtained by DWI ($1.50\text{mm}^3 \pm 0.22$) and H&E analysis ($2.56\text{mm}^3 \pm 0.13$) are not statistically different ($P>0.05$, $n=3$), and both differ to the infarct volume obtained using T2-weighted imaging
5 ($5.65\text{mm}^3 \pm 0.48$, $P<0.05$, $n=3$).

EXAMPLE 12

Role of Type-1 interferon signalling in stroke outcomes

10 [0143] To examine the role of Type-1 interferon signalling in stroke, IFNAR1^{-/-} mice underwent mid cerebral artery occlusion (MCAO) surgery. The mice demonstrated a decreased infarct size ($24.9 \pm 7.1\text{mm}^3$ $n=8$) compared to wild-type controls ($65.1 \pm 4.8\text{mm}^3$ $n=8$). Western blot and immunohistochemistry showed alterations in the Stat-1 and 3 phosphorylation profiles in the IFNAR1^{-/-}. Neuroprotection conferred by the absence of
15 IFN signalling was confirmed in IFNAR-deficient primary cultures that were protected from cell death when exposed to oxygen glucose deprivation (OGD).

[0144] Co-culture experiments using IFNAR1^{-/-} glia and WT neurons and WT glia and IFNAR1^{-/-} neurons were carried out in the OGD model. IFNAR1^{-/-} neurons in the presence
20 of WT glia no longer displayed a neuroprotective phenotype suggesting the glia are a major driver of the neuroinflammatory response. In an attempt to block IFNAR signalling *in vivo* a blocking monoclonal antibody targeting the IFNAR1 receptor (IFNAR1 mAb) was injected into WT mice *via* the tail vein (0.5mg) 30 minutes prior to MCAO. This resulted in a 60% decrease in infarct size when compared to the IgG control.

25

[0145] Collectively these results indicate signalling through the IFNAR1 subunit is deleterious in stroke. Furthermore, data indicate that therapeutic agents targeting the IFNAR1 subunit are beneficial in reducing the severity of a neuro-inflammatory event following stroke and in doing so limit infarct size.

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EXAMPLE 13

IFNAR1 localization

- 5 [0146] Figures 9A through C show by fluorescent immunohistochemistry that IFNAR1 is localized on primary cultured mouse neurons.

EXAMPLE 14

10 *Effects on IFNAR1 mAb in a stroke model*

[0147] IFNAR^{-/-} (KO) C57 BL6 mice exhibited reduced infarct size in this stroke model. The infarct size was assessed 24 hours post injury in wild-type (IFNAR^{+/+}) and IFNAR^{-/-} C57 BL6 mice. The results are shown in Figure 10. Figure 11 shows that a mAb specific
15 for IFNAR1 (IFNAR1 mAb) reduces infarct size when 0.5mg mAb is given iv 1 hour prior to the stroke. The infarct volume is assessed at 24 hours post-injury. This further shows the utility of an IFNAR1 antagonist (e.g. on IFNAR1 mAb) to reduce an infarct prophylactically.

- 20 [0148] Those skilled in the art will appreciate that aspects described herein are susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure includes all such variations and modifications to these aspects. The disclosure also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and
25 any and all combinations of any two or more of the steps or features.

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CLAIMS:

1. A method for reducing a neuroinflammatory response within the central nervous system (CNS) of a subject following a stroke event, said method comprising administering to said subject an effective amount of an antagonist of Type 1 interferon-mediated signalling for a time and under conditions sufficient to prevent or attenuate neuroinflammation.
2. The method of Claim 1 wherein the neuroinflammatory response is in the brain.
3. The method of Claims 1 or 2 wherein the antagonist targets a Type 1 interferon.
4. The method of Claim 3 wherein the Type 1 interferon is IFN α or IFN β .
5. The method of Claims 1 to 2 wherein the antagonist targets the Type 1 interferon receptor or a component thereof.
6. The method of Claim 5 wherein the receptor component is Type 1 interferon alpha receptor 1 (IFNAR1).
7. The method of Claim 1 wherein the subject is a human.
8. The method of Claim 1 or 3 or 6 wherein the antagonist is selected from a protein, small molecule, antibody including an immunoglobulin new antigen receptor (IgNAR) specific, for a Type 1 interferon or IFNAR1, a genetic molecule which down-regulates expression of a Type 1 interferon or IFNAR1, a modified Type 1 interferon and a soluble IFNAR1.
9. A method for reducing a neuroinflammatory response within the central nervous system (CNS) of a subject following a stroke event, said method comprising administering to said subject an antagonist of interferon alpha receptor 1 (IFNAR1)-mediated signalling

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for a time and under conditions sufficient to prevent or ameliorate the symptoms of neuroinflammation.

10. The method of Claim 9 wherein the neuroinflammatory response is in the brain.

11. The method of Claim 9 wherein the subject is a human.

12. The method of Claim 9 wherein the antagonist is selected from a protein, small molecule, antibody including an immunoglobulin new antigen receptor (IgNAR) specific for IFNAR1, a genetic molecule which down-regulates IFNAR1, a modified form of a Type 1 interferon and a soluble IFNAR1.

13. A neuroprotective formulation comprising an antagonist of Type 1 interferon-mediated signalling and one or more pharmaceutically acceptable carriers, diluents and/or excipients.

14. The neuroprotective formulation of Claim 13 wherein the antagonist is selected from a protein, small molecule, antibody including an immunoglobulin new antigen receptor (IgNAR) specific, for a Type 1 interferon or IFNAR1, a genetic molecule which down-regulates expression of a Type 1 interferon or IFNAR1, a modified Type 1 interferon and a soluble IFNAR1.

15. A medical protocol for treating acute neuronal injury following a stroke in a subject, said protocol comprising administering to said subject, from 1 to 120 minutes of the injury, a neuroprotective formulation of Claim 13 or 14.

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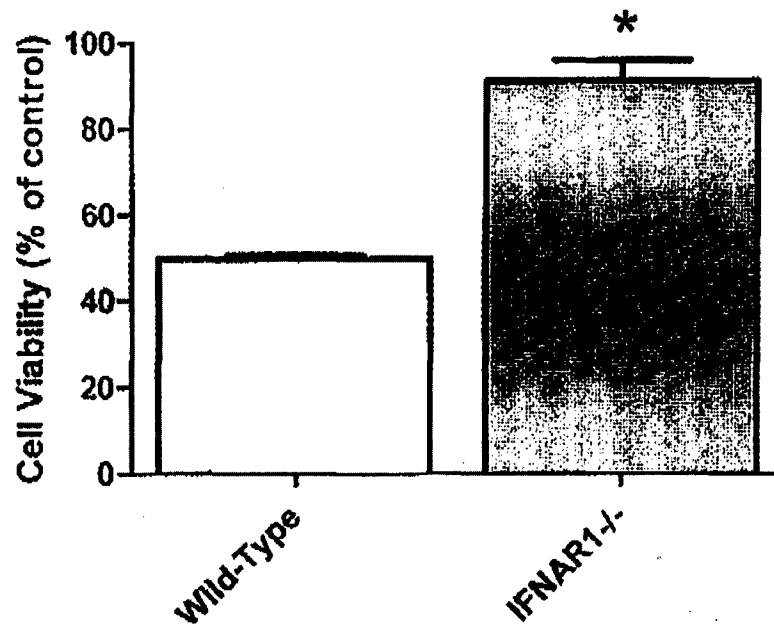


Figure 1

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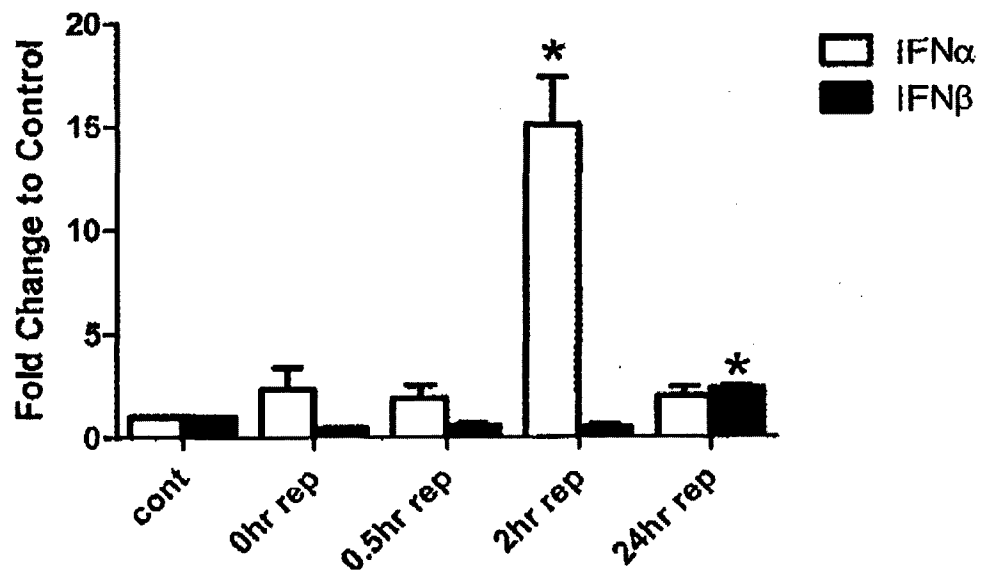
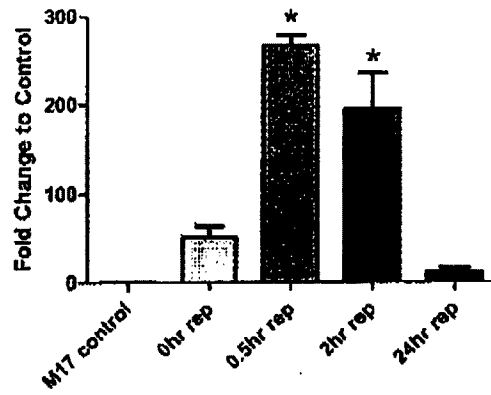
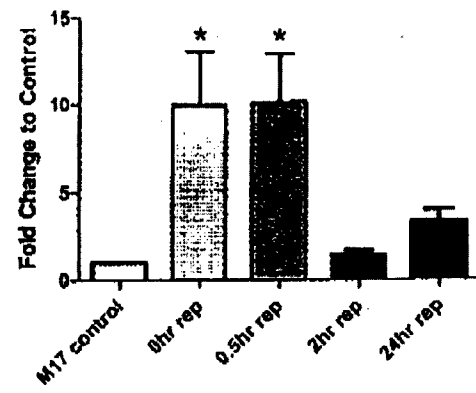
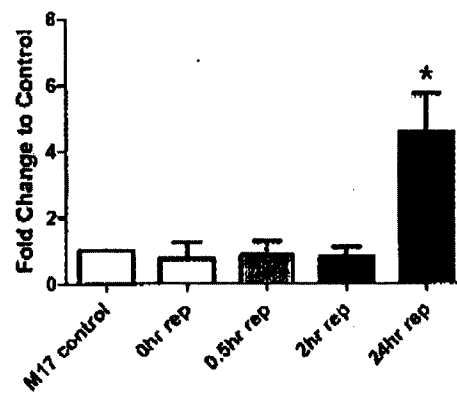


Figure 2

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A**B****C****Figures 3A to C**

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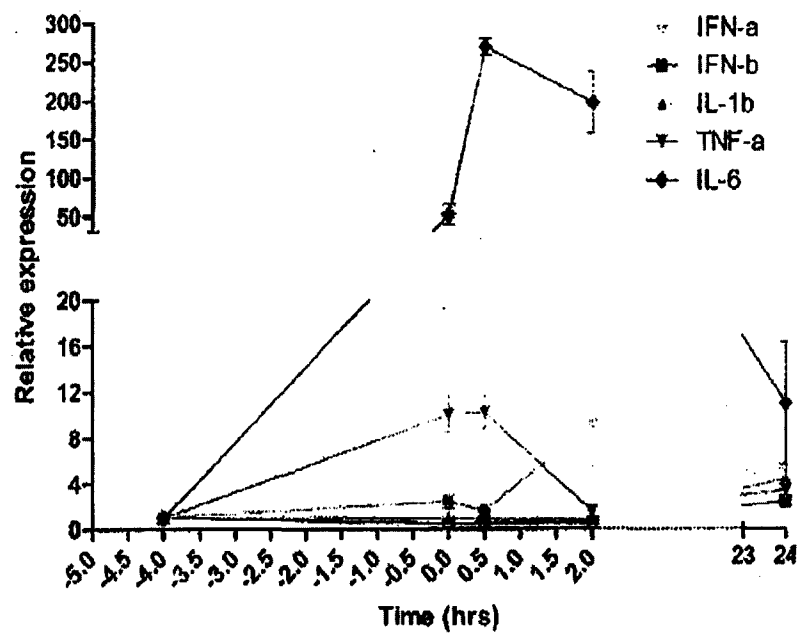


Figure 4

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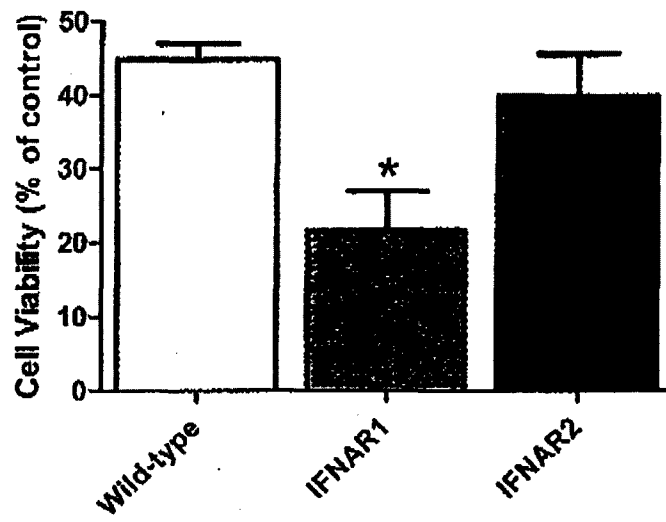


Figure 5

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Gene/Primer	Sequence 5' - 3'
IFNAR1 gene	ATGCTGGCCGTGCTGGGAGCTGCTGCCCTGGTGGCTGGTGGCTGGAGCTCCCTGGGTGC TGCCTTCTGCTGCCGGCGGAGAAAAACCTGAAGCCCCCTGAGAATATCGACGTGTACAT CATTGACGATAACTATACCTGAAAAGGAGCTCCACGCGGAGTCTATGGGCAGTGTGA CCTTCAGCGCCGAATACAGGACTAAGGACGAGGCCAAGTGGCTGAAAAGTCCCCGAATG CCAGCATACCACTACCAAGTGTGAGTTCAAGCTGGTGGATACTAACGTGTACATC AAAAACCCAGTTTAGGGTGAGAGCTGAGGAAAGGCAACTCCACCTCTAGTTGGAATGAAG TGGACCCCTTTCATTCCCTTCTACACAGCTCACATGTCCCCACCCGAGGTGGGGCTGGAG GCTGAAGACAAAGGCCATCCTGGTGCATATTTCTCCTCCAGGCCAAGATGGAACATGT GGCCCCCTGGAGAAGCCAAGCTTCTCCTATACCATCCGCATTGGCAGAAAAGCTCCTC TGATAAGAAAAACCATCAATTCACATACTATGTGAGAAAGATTCCAGAACTGCTGCC GAGACAACTTACTGCTGGAGGTGAAAAGCCATCCACCCCTCTCTGAAAGAAACATAAGCA ACTATTCACAGTGCAGTGTATTAGTACCACAGTGGCTAACAAAGATGCCCTGGCTGG GAATCTGCAGGTGGACGCCAGGCCAAGTCTTACGTGCTGAAATGGGACTATATCGCT AGTGGCGATGTGCTGTTTAGGGCTCAGTGGCTGGCTGGCTACTCTAAGAGTAGCTCCGG ATCTAGAAAGTGACAAGTGGAAACCAATTCCCACTTGGGCCAACGTGCAGACTACCCAC TGGCTGTTTCAGGCCAGGATACCGTGTATACAGGCACCTTCTTCTGACAGTGCAGGCTTC CGAGGGAAATCATACCTCTTCTGGAGTGAAGAAAGTTTATCGACTCTCAGAAACAT ATTCTGCCCCCTCCACCCGTGATCACCCTGACAGCCATGAGCGATACCTGCTGTGTGA CGTGAACCTGCCAGGACTCCACCTGTGATGGGCTGAATTATGAAATCATTTTCTGGGAG AACACAAAGCAATACTAAGATCTCCATGGAAAAAGATGGCCCCGAGTTTACCTGAAGA ACCTGCAGCTCTGACAGTGTACTGCGTGCAAGCTCGGGTGGCTGTTCCGCGCCCTGCTG AACAAAAACAAGCAATTTTCCGAAAAAGCTGTGCGAAGAAAACTAGGCCTGGAAGCTTCT CCACTATCTGGATCATTACCGGACTGGGGGTGGTGTCTTTAGCGTGATGGTGGCTGTAC GCCCTGCGGTCCGTGTGGAAAGTATCTGTGCCAGTGTGCTTTCTCCACTGAAACCCCC TGGCTCCATCGACGAATTCTTTTCTGAGCCACCCAGTAAGAACCCTGGTGGCTGCTGACAG CTGAGGAACACACTGAAAGGTGCTTCACTATTGAGAATACTGATACCGTGGCTGTGGA GGTGAAGCATGCCCCAGAGGAAGACCTGAGAAAAACTCTAGTCAGACATCTCAGGAT AGTGGCAACTATAGTAATGAGGAAAGAAATCTGTGGAACTGAGAGTGGGCAAGCT GTGCTGAGCAAGGCTCCTTGGGGCGGACCATGTAGCGTCCCATCCCTCCAGGCACCC TGGAAAGACGGAACATGCTTCTGGGGAACGAGAAATACCTGCAGAGCCCTGCTCTGAG AACAGAGCCAGCCCTGCTGTCTGA
IFNAR1 AttB1 subclone	GGGGACAAAGTTGTACAAAAAGCAGGCTTCACCATGCTGGCCGTGGTGGGAGCTGCT
IFNAR1 AttB2 Subclone	GGGGACCACTTTGTACAAGAAAGCTGGGTTGCACAGCAGGGCTGGCTCTGTTCTCAG

Figure 6

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Gene/ Primer	Sequence 5'-3'
IFNAR2 gene	ATGCCTTCTCGTTGTACCGTGAAGTCCCGTTGCCCTGCTGTGCTGTGTCTGGTGGT TAOTGCCCTCCCTGGAAACCATTACCCCGTCAGCCTTTGACGGTTATCCGGATGAAC CGTGCAACCATTAACATCACGATTCTGTAATAGCCGCCTGATTCTGTCTTGGGAACGTG GAAAACAAAAGCGGTCCGCCGGCGAATTATACCCCTGTGGTACACGGTCATGTCAA AAGACGAAAACCTGACCAAAAGTGAAGAAATTCCTCGGATACCACGAAAAGCTCTTG TGACGTGACGGATAAATGGCTGGAGGGTATGGAAAGTTATGTGGTTGCGATCGTT ATTGTCCATCGCGCGGACCTGACCGTTTGGCGTTGTTCCGATTACATTGTCCCGGC CAATGCCCCGCTGGAACCGCCAGAATTGAAATCGTGGGTTTACCAGTCACATT AATGTCACGATGGAATTTCCCGCGGTGACCTCAAAAATCATCCAAGAAAAAATGA AAACCACCGCCCTTCGTGATCAAGAACAATTTGGTGATAGCCTTCGCAAAAAACA TGAACCGAAAAGTCAACAAATGTGACCGGCAACTTTACGTTCTGTGCGTGATCTGC TGCCGAAAACCAATTATTGCGTCAGTCTGTACTTTGATGACGATCCCGCAATCAA ATCCCCCGCTGAAATGTATTGTGCTGACGCGCGGTCAAGAAAGTGGCGTGAGCGAA TCTGCTATCGTTGGTATTACCACGAGCTGCCCTGGTCTGTGATGGTGTTCGTTTCTAC CATCGTGATGCTGAAAACGCATCGGCTATATTGTCTGAAAGATAATCTGCCGAAC GTTCTGAATTTTCGTCACTTCTGACCTGGATTATCCCGGAACGCAGTCCGTCCGA AGCAATTGATCGTCTGGAATTATCCCGACCAACAAGAAAAACGTCTGTGGAAT TATGACTACGAAGATGGTTCAAGCTCGGATGAAGAAGTGCCGACCGCTAGCGTTA CCCGCTATACGATGCATGGTCTGACGGCGCAAACCGCTGCAGCAAAACCTCTGATAC CAGCCCTCTCCGGAAGACCCGCTGCACGAAGAAGATAGTGGCGCCGAAGGAATCC GATGAAGCAAGTGTCTGGTGGCGGTGCAGAACCGGAACCTCCGACCGGAAGCCGGT GCCGGTCCGAAGCAAGATCCGACCGGTCCGTACGAACGTGCAAAATCAGTGCTGG AAGACTCGTTTCCGCGCGAAGATAACAGTAGCATGGATGAACCGGGCGATAACAT CATCTTCAACGTTAACCTGAACCTCTGTTTCTGCGTGTCTGCAAGGATG CGTCAGAAACCTGTGCGTGGAAAGACACGATCCGTGCTGGATGAAGGTCCGCA GCGTACCGAAAAGCGACCTGCGCATTGCCGGCGGTGATCGTACCCAGCCGCCGCTG CCGAGTCTGCCGTCCCAAGACCTGTGACCGAAGATGGCTCATCGGAAAAAAGTG ACACCAAGTGACAGCGATGCCGACGTTGGCGATGGCTACATTATGCGTTGA
IFNAR2 AttB1 subclone	GGGGACAAATTTGTACAAAAAAGCAGGCTTCACCATGCGTTCTCGTTGTACCGTG
IFNAR2 AttB2 subclone	GGGGACCACTTTGTACAAAGAAAGCTGGGTCACGCATAATGTAGCCATCACC

Figure 7

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SYBRGreen Primer	Sequence (5'...3')
GADPH Forward Primer	ATCTTCTTGTGCAGTGCCAGC
GADPH Reverse Primer	ACTCCACGACATACTCAGCACC
All Alpha Forward Primer	GCAATCCTCCTAGACTCACTTCTGCA
All Alpha Reverse Primer (1)	TATAGTTCCTCACAGCCAGCAG
All Alpha Reverse Primer (2)	TATTTCTTCATAGCCAGCTG

Figure 8

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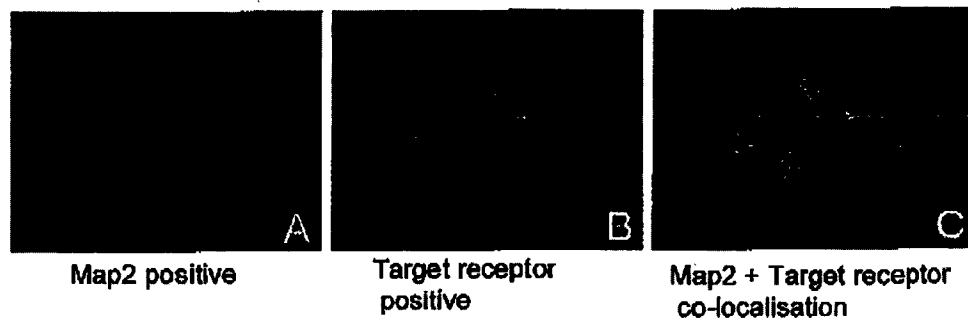


Figure 9

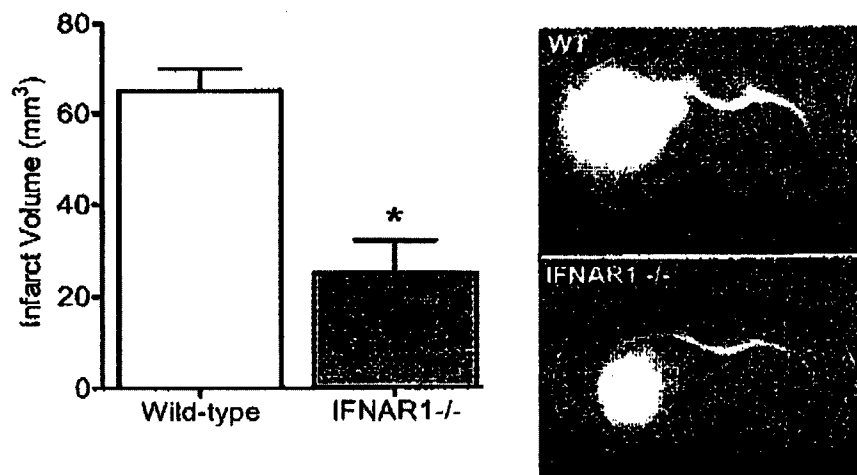


Figure 10

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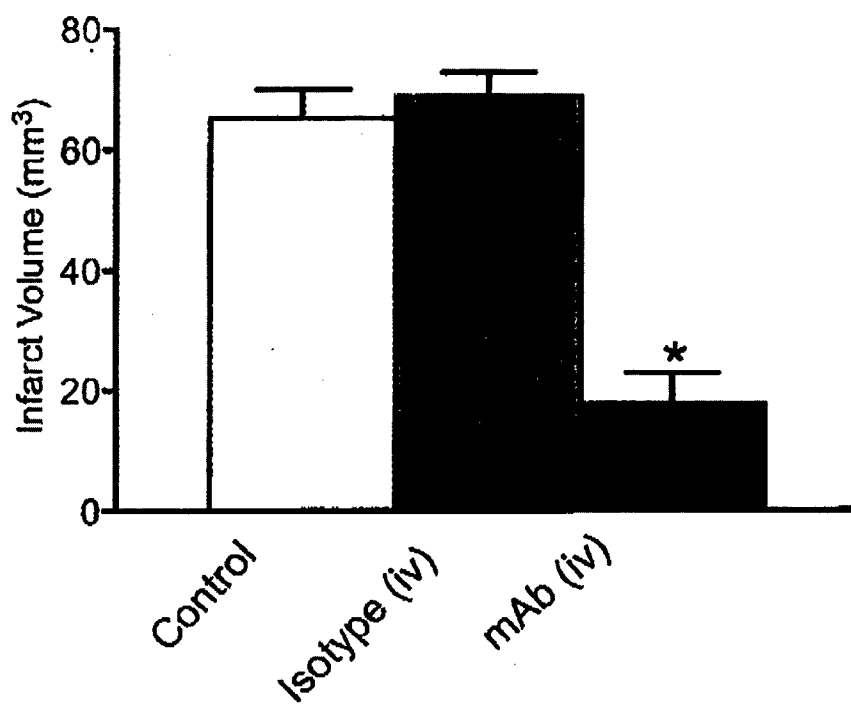


Figure 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2011/001522**A. CLASSIFICATION OF SUBJECT MATTER**

Int. Cl.

A61P 25/00 (2006.01)**A61K 39/395** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DWPI, EPODOC, BIOSIS, MEDLINE: type 1 interferon, type 1 interferon mediated signalling, interferon 1, interferon alpha, interferon beta, IFN, Roferon A, SCH 30500, RO 22-8181, Wellferon, Fiblaferon, interferon fibroblast, interferon leukocyte, interferon lymphoblast, IFNAR, antagonist, block, inhibit, neutralise, disrupt, reduce, attenuate, prevent, competitor, stroke, ischemia, CVA, cerebrovascular accident, cerebral ischemia, ischemia reperfusion injury, cerebral infarction, neuroinflammation, IgNAR, blood brain barrier, neural, CNS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/030671 A1 (UNIVERSITY OF MEDICINE AND DENTISTRY OF NEW JERSEY ET AL) 18 March 2010 Abstract; Page 21, lines 5-12; Claims 1, 14, 43, 44	13-14
X	US 2003/0018174 A1 (KIM, KJ ET AL) 23 January 2003 Abstract; Page 1, [0008-0010]; Page 20, [192-199]	13
X	WO 2001/054721 A1 (PHARMA PACIFIC PTY LTD ET AL) 2 August 2001 Abstract; Claims 1-9	13-14

☒ Further documents are listed in the continuation of Box C☒ See patent family annex

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"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
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Date of the actual completion of the international search
19 December 2011Date of mailing of the international search report
21/12/2011Name and mailing address of the ISA/AU
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PO BOX 200, WODEN ACT 2606, AUSTRALIA
E-mail address: pct@ipaustalia.gov.au
Facsimile No. +61 2 6283 7999Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2011/001522

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Veldhuis, WB <i>et al</i> (2003) Interferon-Beta Blocks Infiltration of Inflammatory Cells and Reduces Infarct Volume After Ischemic Stroke in the Rat. <i>Journal of Cerebral Blood Flow and Metabolism</i> 23 : 1029-1039 Abstract	13-14
X	Sas, AR <i>et al</i> (2009) Interferon- α Causes Neuronal Dysfunction in Encephalitis. <i>The Journal of Neuroscience</i> 29 : 3948-3955 Abstract	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2011/001522

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	2010030671	US	2011224407		
US	2003018174	NONE			
WO	200154721	AU	28656/01	CA	2401086
		JP	2003531822	US	2003147889
				EP	1251873
				US	2006275298
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.					
END OF ANNEX					