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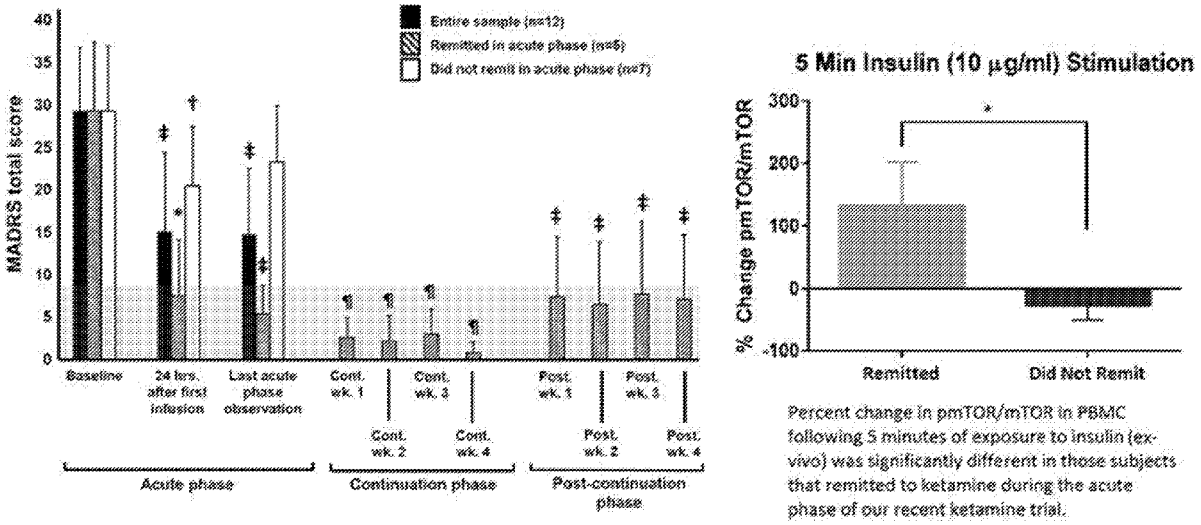


FIG. 9

(57) **Abstract:** Provided herein is a method of predicting the responsiveness of a neurological disease, disorder or condition in a subject to an agent, which includes determining a level of insulin sensitivity in blood-derived cells of the subject that have been treated with the agent, in which the level of insulin sensitivity of the one or plurality of blood-derived cells is indicative of responsiveness of the neurological disease, disorder or condition to the agent. Further provided herein is a method of treating a neurological disease, disorder or condition in a subject, which includes determining a level of insulin sensitivity of blood-derived cells of the subject that have been treated with an agent and based on the determination made, initiating, continuing, modifying or discontinuing treatment with the agent. A kit for use in the aforementioned methods is also provided.

WO 2021/179031 A1

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TITLE

DETERMINING RESPONSIVENESS TO TREATMENT OF NEUROLOGICAL DISEASES BASED ON INSULIN SENSITIVITY

FIELD

THIS INVENTION relates to methods of utilising levels of cellular metabolism, including insulin signalling, glucose uptake and mitochondrial function, in peripheral blood mononuclear cells (PBMCs) for determining the responsiveness of neurological diseases, disorders and conditions to treatment. Additionally, this invention relates to the use of insulin sensitizing agents to enhance the responsiveness of neurological diseases, disorders and conditions to treatment or overcome resistance thereto.

BACKGROUND

Depression is a leading cause of global disability (Whiteford et al. 2013; Friedrich 2017) and a key risk factor for suicide. Depression often emerges for the first time during adolescence (Kessler et al., 2005), a time notable for rapid changes in brain development and synaptic remodelling (Giedd et al., 1999; Paus et al., 2008; Raznahan et al., 2011). Although effective treatments are available, a significant number of adolescents with depression do not respond to standard treatments (March et al., 2006), allowing the disease course to progress (Amital et al., 2008; Maalouf et al., 2011). Such treatment-resistant depression (TRD) is associated with poor outcomes including academic failure, loss of relationships, and exacerbation of depression symptoms such as poor self-esteem, hopelessness and suicide attempts (Asarnow et al., 2011; Crown et al., 2002; Greden, 2001). TRD negatively impacts development and impedes the successful transition to adulthood (Aalto-Setälä et al., 2002; Fergusson and Woodward, 2002; McLeod et al., 2016). Hence, novel assays are urgently needed for predicting the response of various neurological diseases, such as TRD, to therapeutic agents, with the goal of restoring healthy neurobehavioral development early in the disease course and preventing long-term negative outcomes.

SUMMARY

Surprisingly, the present inventors have discovered that levels of insulin sensitivity and cellular metabolism in PBMCs following their sequential treatment with a therapeutic agent and insulin may be utilised as a biomarker of treatment response to the therapeutic agent in subjects. Accordingly, the present invention broadly relates to determining levels of cellular metabolism in PBMCs as a predictive marker of the

response of neurological diseases, disorders and conditions to treatment with a therapeutic agent, such as a glutamatergic receptor antagonist or lithium. In further aspects, the invention also broadly relates to the treatment of neurological diseases, disorders or conditions using therapeutic agents that increase or enhance levels of insulin sensitivity and cellular metabolism in the PBMCs of subjects in need thereof.

In a first aspect, the invention provides a method of predicting the responsiveness of a neurological disease, disorder or condition in a subject to an agent, said method including the step of determining a level of insulin sensitivity of one or a plurality of blood-derived cells of the subject that have been treated with the agent, wherein the level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased or decreased responsiveness of the neurological disease, disorder or condition to the agent.

In certain embodiments, a relatively increased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent; and/or a relatively decreased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively decreased responsiveness of the neurological disease, disorder or condition to the agent.

In some embodiments, the method of the present aspect further includes the step of treating the neurological disease, disorder or condition in the subject.

In a second aspect, the invention resides in a method of treating a neurological disease, disorder or condition in a subject, the method including the step of determining a level of insulin sensitivity of one or a plurality of blood-derived cells of the subject treated with an agent and based on the determination made, initiating, continuing, modifying or discontinuing treatment with the agent.

In embodiments of the aforementioned aspects, the method includes the step of administering to the subject a therapeutically effective amount of the agent when the level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent.

In particular embodiments of the first and second aspects, the method includes the step of administering to the subject a therapeutically effective amount of an insulin sensitizing agent.

In certain embodiments of the above aspects, the method includes the step of administering to the subject therapeutically effective amounts of: (a) the agent; and (b) an insulin sensitizing agent when the level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively decreased responsiveness of the neurological disease, disorder or condition to the agent.

In certain embodiments, a relatively increased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent; and/or a relatively decreased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively decreased responsiveness of the neurological disease, disorder or condition to the agent.

Suitably the method of the first and second aspects includes the initial steps of:

(a) treating the one or plurality of blood-derived cells with an effective amount of the agent; and/or

(b) treating the one or plurality of blood-derived cells with an effective amount of an insulin agonist.

In particular embodiments, the blood-derived cells are treated sequentially with the agent and the insulin agonist.

With respect to the above aspects, determining the level of insulin sensitivity suitably comprises determining one or more of a level of insulin signalling, a level of glucose uptake and a level of mitochondrial activity of the one or plurality of blood-derived cells of the subject. To this end, determining the level of insulin signalling may comprise determining an activity and/or expression level of one or more proteins selected from the group consisting of mTOR, Akt, AMPK, CaMKII, GSK3α, GSK3β and phosphorylated forms thereof. By way of example, determining the level of mitochondrial activity may comprise determining levels of adenosine triphosphate (ATP) and/or glycerol phosphorylation in the one or plurality of blood-derived cells.

Suitably, the method of the first and second aspects, and in particular the steps of treating the blood-derived cells with the agent and/or the insulin agonist, is performed *in vitro*. In this regard, the present methods may further include the initial step of isolating the one or plurality of blood-derived cells from the subject.

In a third aspect, the invention relates to a kit for predicting the responsiveness of a neurological disease, disorder or condition in a subject to an agent, the kit comprising at least one reagent capable of determining a level of insulin sensitivity of

one or a plurality of blood-derived cells of the subject that have been treated with the agent, wherein the level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased or decreased responsiveness of the neurological disease, disorder or condition to the agent.

Suitably, a relatively increased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent; and/or a relatively decreased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively decreased responsiveness of the neurological disease, disorder or condition to the agent.

In some embodiments, the kit of the present aspect further comprises reference data for correlating the level of insulin sensitivity of the one or plurality of blood-derived cells with responsiveness of the neurological disease, disorder or condition to the agent. In particular embodiments, the reference data is on a computer-readable medium.

Suitably, the present kit is for use in the method of the first and second aspects. In this regard, the kit may be or comprise a companion diagnostic.

Referring to the aforementioned aspects, the agent suitably is or comprises a glutamatergic modulator, such as a glutamate receptor antagonist or inhibitor. In certain embodiments, the glutamatergic modulator is or comprises an N-methyl-D-aspartate (NMDA) receptor antagonist, an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, a metabotropic glutamate receptor antagonist, a kainate receptor antagonist, a glycine transportor-1 antagonist, a dopamine modulator, an immune modulator, an anti-inflammatory agent and/or a lithium compound or salt.

In a fourth aspect, the invention provides a method of screening, designing, engineering or otherwise producing an agent for treating a neurological disease, disorder or condition in a subject, said method including the step of determining whether a candidate molecule is capable of at least partly increasing or enhancing a level of insulin sensitivity in one or a plurality of blood-derived cells treated with effective amounts of the candidate molecule.

Suitably, determining whether the candidate molecule is capable of at least partly increasing or enhancing the level of insulin sensitivity in the one or plurality of blood-derived cells comprises determining a level of cellular metabolism of the one or plurality of blood-derived cells, such as by determining one or more of a level of insulin

signalling, a level of glucose uptake and a level of mitochondrial activity of the one or plurality of blood-derived cells.

Suitably, the present method includes the initial steps of:

(a) treating the one or plurality of blood-derived cells with an effective amount of the candidate molecule; and/or

(b) treating the one or plurality of blood-derived cells with an effective amount of an insulin agonist.

Referring to the aforementioned aspects, the blood-derived cells suitably are or comprise PBMCs.

For the methods and kit of the above aspects, the subject is suitably a mammal, and preferably a human.

With respect to the invention of the first, second, third and fourth aspects, the neurological disease, disorder or condition is or comprises: a mood disorder, including depressive disorders and bipolar disorders; post-traumatic stress disorder; Alzheimer's disease; an anxiety disorder, including panic disorders, phobias, obsessive-compulsive disorders, stress disorders and generalized anxiety disorders; suicidality; and any combination thereof.

In a fifth aspect, the invention resides in agent screened, designed, engineered or otherwise produced according to the method of the fourth aspect.

Suitably, the present agent is for use according to the method of the first and second aspects.

Unless the context requires otherwise, the terms "*comprise*", "*comprises*" and "*comprising*", or similar terms are intended to mean a non-exclusive inclusion, such that a recited list of elements or features does not include those stated or listed elements solely, but may include other elements or features that are not listed or stated.

The indefinite articles '*a*' and '*an*' are used here to refer to or encompass singular or plural elements or features and should not be taken as meaning or defining "one" or a "single" element or feature. For example, "a" cell includes one cell, one or more cells and a plurality of cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Simplified schematic diagram of the effects of lithium on insulin signaling. Lithium inhibits GSK3β both directly and indirectly (via Akt facilitation), which can have subsequent downstream effects on mTOR activity.

Figure 2: Schematic diagram depicting of the timeline for behavioural experiments.

Figure 3: Lithium restores antidepressant-like effect of imipramine in FST.

Treatment group means for (**a**) distance traveled (**b**) velocity of travel, and (**c**) time spent in center in the OFT; as well as (**d**) immobility duration, (**e**) climbing time and (**f**) swimming time in the FST ($n = 10–12$). Data are expressed as mean ± S.E.M; $^{\dagger}p < 0.08$; $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

Figure 4: Lithium treatment upregulates insulin signaling in the ILPFC of ACTH animals.

Mean levels of total protein, phosphorylated protein, and ratio of phosphorylated:total for Akt (**a**–**c**), mTOR (**d**–**f**), and GSK3β (**g**–**i**) in the ILPFC by treatment group, as measured by Western blot ($n = 7–12$ per group). Data are expressed as mean ± S.E.M; $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$

Figure 5: mTOR signaling following insulin challenge differ with treatment, immobility correlated with mTOR activation. The effects of treatment group on the change in (**a**) mTOR and (**b**) *p*mTOR between baseline (t_0) and after 5 min of insulin stimulation (t_5) in PBMCs ($n = 7–12$ per group). Figure also shows the relationship between immobility duration and change in PBMC (**c**) mTOR and (**d**) *p*mTOR following 5 min of insulin stimulation in ACTH pre-treated animals co-administered imipramine (10 mg/kg) and lithium (100 mg/kg), ($n = 10$). Linear regression data are expressed as r^2 values, where significance was assessed via slope regression F-tests; $^{\dagger}p < 0.08$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

Figure 6. Treatment effect on rodent coping-like behaviors in the forced swim test.

Treatment modifies animal behaviors in response to acute stress via exposure to the forced swim test. Behaviors of interest include (a) immobility time, (b) latency to immobility, (c) swimming time, and (d) climbing time.

Abbreviations: FST, forced swim test; SAL, saline; VEH, vehicle saline; KET, ketamine; R, responder; N, non-responder; ACTH, adrenocorticotropic hormone. †p<0.07; *p<0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001

Figure 7. treatment-moderated changes in mTOR and GSK3 activation (insulin-dependent pathway activation) versus TrkB activation (insulin-independent pathway activation) in the prefrontal cortex and PBMC buffy coat.

Treatments moderate expression of total mTOR, pmTOR, total GSK3α, pGSK3ß, total GSK3ß, and pGSK3ß in the (A) Infralimbic prefrontal cortex, (B) Prelimbic prefrontal cortex, and (C) PBMC buffy coat.

Abbreviations: p, phosphorylated; mTOR, mammalian target of rapamycin; GSK3α, glycogen synthase kinase-3 α; GSK3ß, glycogen synthase kinase-3 ß; PBMC, peripheral blood mononuclear cell; SAL, saline; VEH, vehicle saline; ACTH, adrenocorticotropic hormone; KET, ketamine; R, responder; N, non-responder. tp<0.08; *p<0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001

Figure 8. Behavioral and biochemical impacts of ketamine and metformin administration in rodents. Modulations of behavioral and biochemical measures following drug treatments were examined to determine impacts of treatment on measures associated with antidepressant-like effect, including (A) time spent immobile in the FST, (B) time spent swimming in the FST, (C) time spent climbing in the FST, (D) time spent in the center of the OFT apparatus, (E) total distance traveled in the OFT, (F) change in glucose levels before initial treatment and following final treatment, (G) insulin levels in peripheral plasma, and (H) change in mTOR expression following 5 minutes of insulin stimulation of PBMCs.

Abbreviations: FST, forced swim test; OFT, open field test; mTOR, mammalian target of rapamycin, ACTH, adrenocorticotropic hormone; VEH, vehicle saline; KET, ketamine; MET, metformin. †p<0.06; *p<0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001

Figure 9. Antidepressive response to i.v. ketamine during acute and continuation phase treatment and post-continuation follow-up. Subjects who remitted after any acute phase treatment received continuation phase treatment and post-continuation phase follow up. All 5 acute phase remitters retained remission status during continuation phase treatment. Although mean MÅDRS total scores were ≤9, only one of 5 acute phase remitters retained remission status during post-continuation phase follow up. MADRS data was previously published in Vande Voort et al., 2016.

Key: †p<0.06; *p<0.05; ‡p≤0.01; p≤0.001. Ex vivo insulin-induced changes in mTOR activation (pmTOR/mTOR) in human peripheral blood mononuclear cells (PBMC), which significantly differed between ketamine responsive and nonresponsive individuals.

Figure 10. Individual changes in right NAc log(-entropy) vs. individual percent changes in CDRS-R ratings. Entropy changes were assessed for the 0.08-0.12 Hz range. All responders (n=5, blue) showed increased post-pre ketamine right NAc log(-entropy), while all nonresponders (n=6, red) showed reduced post-pre ketamine right NAc log(-entropy). Increased post-pre ketamine right NAc log(-entropy) was strongly correlated with depression response.

Figure 11. Group level entropy changes (0.08-0.12 Hz) for depression-related regions of interest. Post-pre ketamine entropy changes were assessed in the following ROIs: Hipp=hippocampus, Amyg=amygdala, NAc=nucleus accumbens, SubCal=subcallosal cortex, insula, ACC=anterior cingulate cortex, PCC=posterior cingulate cortex, PreC=precuneus, and Thal=thalamus. A significant group difference in right NAc log(-entropy) change was observed between responders (n=5, blue) and non-responders (n=6, red).

Figure 12. Post-ketamine percent changes in *p*mTOR/mTOR vs. post-pre ketamine changes in CDRS-R ratings and right NAc log(-entropy). Responders (n=5, blue) had a significantly greater percent change in post-ketamine *p*mTOR/mTOR compared with non-responders (n=5, red) *(A)*. Post-ketamine percent changes in *p*mTOR/mTOR were strongly correlated with postpre ketamine changes in CDRS-R ratings *(B)* and post-pre ketamine changes in right NAc log(-entropy) *(C)*, respectively.

Figure 13. Post-ketamine percent changes in *p*GSK3β/GSK3β vs. post-pre ketamine changes in CDRS-R ratings and right NAc log(-entropy). Responders (n=5, blue) had a significantly greater percent change in post-ketamine *p*GSK3β/GSK3β compared with non-responders (n=5, red) *(A)*. Post-ketamine percent changes in *p*GSK3β/GSK3β were strongly correlated with postpre ketamine changes in CDRS-R ratings *(B)* and post-pre ketamine changes in right NAc log(- entropy) *(C)*, respectively.

Figure 14. Schematic of in-cell western assay for PBMCs.

Figure 15. Protein expression results for treated PBMCs from in-cell western assay.

Figure 16. Changes in glucose uptake in PBMCs treated with ketamine.

Figure 17. Changes in mitochondrial function of PBMCs treated with ketamine.

Figure 18. Changes in Akt, mTOR, GSK3α/β protein expression in PBMCs with ketamine treatment. Akt: ($p = 0.0187$, number of pairs = 8), mTOR: (0.0002, number of pairs = 8), GSK3α/β: ($p = 0.0056$, number of pairs = 8).

DETAILED DESCRIPTION

The present invention is at least partly predicated on the surprising discovery that insulin challenge in PBMCs from subjects with a depressive disorder is a useful probe for predicting antidepressant response or resistance to therapeutic agents that act to modulate glutamatergic neurotransmission, such as lithium and ketamine. To this end, it was surprisingly found the enhanced levels of cellular metabolism in PBMCs

derived from subjects previously treated with these therapeutic agents in response to insulin challenge was a positive indicator of therapeutic response to such agents. It was further demonstrated that treatment with an insulin sensitizing agent like metformin was capable of enhancing levels of cellular metabolism in PBMCs and so may be used to improve therapeutic responses in subjects whose neurological disease, disorder or condition is resistant or refractory to treatment, such as treatment-resistance depression.

In one aspect, the invention provides a method of predicting the responsiveness of a neurological disease, disorder or condition in a subject to an agent, said method including the step of determining a level of insulin sensitivity of one or a plurality of blood-derived cells of the subject that have been treated with the agent, wherein the level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased or decreased responsiveness of the neurological disease, disorder or condition to the agent.

The term "*predicting the responsiveness*" to a treatment or agent against a neurological disease, disorder or condition, as used herein, refers to an ability to assess the likelihood that treatment of the subject with a particular agent potentially effective against a neurological disease, disorder or condition will or will not be clinically or therapeutically effective (*e.g.*, provide a measurable benefit to) in the subject. Subjects whose blood-derived cells treated with a particular agent demonstrate, for instance, an increased level of insulin sensitivity and cellular metabolism in response to insulin treatment or challenge as described herein can then be selected for treatment with that particular agent useful against a neurological disease, disorder or condition. The ability to assess the likelihood that treatment with a particular agent will or will not be clinically or therapeutically effective typically can be exercised before treatment with that agent useful against a neurological disease, disorder or condition is initiated (*e.g.*, blood-derived cells are isolated from the subject and treated with the agent *in vitro*). However, it is also possible that the ability to assess the likelihood that treatment with a particular agent will or will not be clinically effective in a subject can be exercised after treatment with that agent has begun to aid in optimizing treatment protocols.

The term "*neurological disease, disorder or condition*" refers to any undesirable condition of a mammalian central or peripheral nervous system. The term includes neurodegenerative (*e.g.*, Alzheimer's disease, Parkinson's disease and amyotropic lateral sclerosis) and neuropsychiatric (*e.g.*, schizophrenia, depression and anxieties such as general anxiety disorder) diseases, disorders and conditions. In the context of

the present invention, by "*a neurodegenerative or neuropsychiatric disease, disorder or condition*" is meant any disease, disorder and/or condition that comprises a progressive decline and/or deterioration in the structure, function, signalling and/or population of the neurons or neural tissue in an animal. Suitably, the neurological disease, disorder or condition is associated with altered glutamatergic signalling and/or can be modulated (*e.g.*, treated at least in part) by alteration of glutamate levels or signalling.

Examples of neurological diseases, disorders and conditions include a mood disorder, including depressive disorders and bipolar disorders; post-traumatic stress disorder; dementia; Alzheimer's disease; an anxiety disorder, including panic disorders, phobias, obsessive-compulsive disorders, stress disorders; generalized anxiety disorders; and suicidality.

In particular embodiments, the neurological disease, disorder or condition is or comprises a depressive disorder. As used herein the term "*depressive disorder*" includes major depressive disorder, persistent depressive disorder, bipolar disorder, treatment resistant depression and bipolar depression, albeit without limitation thereto.

The term "*insulin sensitivity*" refers to the capacity of a cell, such as a blood-derived cell or PBMC, tissue or organism to sense and/or respond to stimulation by insulin, an insulin agonist or to insulin signalling. The preferred response to insulin, an insulin agonist or insulin signalling is glucose uptake and/or activation of cellular metabolism. The term "*insulin resistance*" refers to a condition or disorder in which the tissues of the body fail to respond normally to insulin or an insulin agonist. With respect to the present invention, insulin sensitivity or conversely insulin resistance of the blood-derived cells of the subject may be determined by any means known in the art.

In particular embodiments, however, a level of insulin sensitivity may be assessed or determined by determining a level of cellular metabolism in the blood-derived cells of the subject. It will be appreciated by the skilled artisan that cellular metabolism refers to a complex network of biochemical reactions necessary for the production of energy in the form of adenosine triphosphate (ATP) and the biosynthesis of macromolecules. These reactions include a vast number of different chemical and enzymatic reactions, better known as metabolic pathways, relating to the growth and maintenance of the cell.

In some embodiments, determining the level of cellular metabolism, and hence insulin sensitivity, at least partly includes determining a level of insulin signalling of the one or plurality of blood-derived cells of the subject.

Generally, insulin (or an insulin agonist) binding to its receptor results in receptor autophosphorylation on tyrosine residues and the tyrosine phosphorylation of insulin receptor substrates (IRS-1, IRS-2 and IRS-3) by the insulin receptor tyrosine kinase. This allows association of IRSs with the regulatory subunit of phosphoinositide 3-kinase (PI3K) through its SRC homology 2 (SH2) domains. PI3K may then activate PtdIns(3,4)P_2 / PtdIns(3,4,5)P_3-dependent kinase 1 (PDK1), which activates protein kinase B (PKB)/Akt, a serine kinase. PKB in turn deactivates glycogen synthase kinase 3 (GSK-3), leading to activation of glycogen synthase and thus glycogen synthesis. Activation of PKB also results in the translocation of GLUT-4 vesicles from their intracellular pool to the plasma membrane, where they allow uptake of glucose into the cell. PKB also leads to mTOR-mediated activation of protein synthesis by PHAS/elf4 and $p70^{s6k}$.

In view of the above, the level of insulin signalling may be at least partly determined by determining an activity (*e.g.*, kinase activity) and/or expression level of one or more proteins associated with insulin signalling, such as mTOR, Akt, AMPK, CaMKII, GSK3α, GSK3β, BDNF, TrkB, inclusive of unphosphorylated and phosphorylated forms (*e.g.*, phospho-$mTOR^{Ser2448}$, phospo-Akt^{Ser473}, phospho-$AMPK^{Thr172}$, phospho-$CaMKII^{Thr286}$, phospho-$GSK3\alpha^{Ser21}$, phospho-$GSK3\beta^{Ser9}$, phospho-$TrkB^{Tyr516}$, phospho-$TrkB^{Tyr702}$, phospho-$TrkB^{Tyr706}$, phospho-$TrkB^{Tyr707}$, phospho-$TrkB^{Tyr817}$) thereof. In some embodiments, the level of insulin signalling is determined by determining an activity and/or expression level of AMPK, CaMKII, BDNF and TrkB, including phosphorylated forms thereof and any combination thereof.

In particular embodiments, an amount or expression level of a phosphorylated form of an insulin signalling protein (*e.g.*, phospho-mTOR, phospo-Akt, phospho-AMPK, phospho-CaMKII, phospho-GSK3α, phospho-GSK3β and phospho-TrkB) may be compared to a total amount or expression level of the respective insulin signalling protein (*i.e.*, inclusive of both unphosphorylated and phosphorylated forms of the protein) so as to determine a ratio thereof.

It will be appreciated that increased or elevated levels of insulin signalling, such as indicated by increased levels of one or more of the aforementioned proteins or phosphoproteins, suitably indicate an increased level of insulin sensitivity and hence

may be correlated with an increased responsiveness to the agent. Conversely, decreased or lowered levels of insulin signalling suitably indicate a reduced level of insulin sensitivity and hence may be correlated with a decreased responsiveness to the agent.

In certain embodiments, determining the level of cellular metabolism, and hence insulin sensitivity, at least in part comprises determining a level of glucose uptake of the one or plurality of blood-derived cells of the subject.

As used herein, the term "*glucose uptake*" refers to the process of glucose being taken into cells. The method of glucose uptake can differ throughout tissues depending on two factors; the metabolic needs of the tissue and availability of glucose. The two ways in which glucose uptake can take place are facilitated diffusion (a passive process) and secondary active transport (an active process which indirectly requires the hydrolysis of ATP).

Glucose uptake may be measured or assessed by any means in the art, such as a glucose uptake assay as are known in the art. In methods of determining glucose uptake, glucose or any appropriate glucose analogue may be used. Suitably, the glucose or glucose analogue may be labelled. By way of example, glucose uptake assays may utilise 2-NBD Glucose, a fluorescently labelled deoxyglucose analogue, to assess glucose uptake by the blood-derived cells following exposure of the cells to the agent and/or the insulin agonist. The level of glucose or glucose analogue in the blood-derived cells may be determined by techniques known in the art, such as enzyme linked immunosorbent assays (ELISAs), immunoprecipitation, immunofluorescence, enzyme immunoassay (EIA), radioimmunoassay (RIA), Western blot analysis, nuclear magnetic resonance, NMR and MRI, Mass spectrometry, in vivo glucose sensor proteins based on fluorescence (for instance FRET, fluorescence resonance energy transfer, probes). Levels of glucose uptake may also be assessed indirectly by measuring an activity and/or expression level of one or more glucose transporters (*e.g.*, GLUT1, GLUT3 and GLUT4) in the blood-derived cells, such as before and after exposure of the cells to the agent and/or the insulin agonist.

As will be understood, increased or elevated levels of glucose uptake suitably indicate an increased level of insulin sensitivity and hence may be correlated with an increased responsiveness to the agent. Conversely, decreased or lowered levels of glucose uptake suitably indicate a reduced level of insulin sensitivity and hence may be correlated with a decreased responsiveness to the agent.

In some embodiments, determining the level of cellular metabolism, and hence insulin sensitivity, at least in part comprises determining a level of mitochondrial activity of the one or plurality of blood-derived cells of the subject. In particular embodiments, determining a level of insulin sensitivity comprises determining levels of mitochondrial activity and/or glucose uptake of the one or plurality of blood-derived cells of the subject.

It is envisaged that mitochondrial activity of the blood-derived cells may be determined by any means known in the art. By way of example, mitochondrial activity or function has been assessed with Clark-type electrode probes for measuring oxygen consumption, luminescent ATP assays for quantification of total energy metabolism, glycerol phosphorylation assay (*i.e.*, glycerol kinase catalyses the transfer of a phosphate from ATP to glycerol forming glycerol 3-phosphate) and MTT or Alamar Blue assays for determination of metabolic activity.

In this regard, increased or elevated levels of mitochondrial activity, such as indicated by increased levels of one or more of cellular ATP and glycerol phosphorylation, suitably indicate an increased level of insulin sensitivity and hence may be correlated with an increased responsiveness to the agent. Conversely, decreased or lowered levels of mitochondrial activity suitably indicate a reduced level of insulin sensitivity and hence may be correlated with a decreased responsiveness to the agent.

The term "*determining*" includes any form of measurement, and includes determining if an element is present or not. As used herein, the terms "*determining*", "*measuring*", "*evaluating*", "*assessing*" and "*assaying*" are used interchangeably and include quantitative and qualitative determinations. Determining may be relative or absolute. "*Determining the presence of*" includes determining the amount of something present (*e.g.*, a protein biomarker), and/or determining whether it is present or absent.

As will be understood by the skilled person, the level of insulin sensitivity, such as a level of insulin signalling, a level of glucose uptake and/or a level of mitochondrial activity, of the one or plurality of blood-derived cells of the subject may be relatively (i) higher, increased or greater; or (ii) lower, decreased or reduced when compared to a level of insulin sensitivity in a control or reference sample, such as in blood-derived cells of the subject prior to treatment with the agent and/or the insulin agonist, or to a threshold level. In one embodiment, a level of insulin sensitivity of the blood-derived cells, inclusive of insulin signalling, mitochondrial activity and/or glucose uptake, may be classified as higher, increased or greater if it exceeds a mean and/or median level of

a reference population. In one embodiment a level of insulin sensitivity may be classified as lower, decreased or reduced if it is less than the mean and/or median level of the reference population. In this regard, a reference population may be a group of subjects who have the same neurological disease, disorder or condition as said mammal for which the level of insulin sensitivity of the blood-derived cells is determined.

In certain embodiments, a relatively increased level of insulin sensitivity of the one or plurality of blood-derived cells, which may be indicated, for example, by increased levels of insulin signalling, glucose uptake and/or mitochondrial activity, indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent; and/or a relatively decreased level of insulin sensitivity of the one or plurality of blood-derived cells, which may be indicated, for example, by decreased levels of insulin signalling, glucose uptake and/or mitochondrial activity, indicates or correlates with relatively decreased responsiveness of the neurological disease, disorder or condition to the agent.

In this regard, the level of insulin sensitivity in the blood-derived cells after treatment with the insulin agonist can be useful in the prediction of sensitivity and/or resistance of the subject's neurological disease, disorder or condition to the agent, and in particular a glutamatergic modulator, as well as indicating if additional insulin sensitization may be required to overcome resistance to the agent.

Accordingly, in particular embodiments, the subject's neurological disease, disorder or condition demonstrates a reduced responsiveness or resistance to the agent, such as in subjects whose blood-derived cells demonstrate a relatively reduced level of insulin sensitivity after treatment with the agent. In such embodiments, and without being bound by any theory, it is believed that activation of cellular metabolic pathways, particularly insulin-sensitive metabolic pathways, facilitate neural and synaptic plasticity, generation and neurotransmission. Hence, targeted promotion of insulin signalling by an insulin sensitizer may be able to assist in overcoming, at least in part, resistance to such agents.

Terms such as "*higher*", "*increased*" and "*greater*" as used herein refer to an elevated level of insulin sensitivity, such as elevated levels of insulin signalling, glucose uptake and/or mitochondrial activity, in a blood-derived cell, when compared to a control or reference level thereof. The level of insulin sensitivity may be relative or absolute. In some embodiments, a level of insulin sensitivity is higher, increased or greater if it is more than about 0.5%, 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%,

30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 150%, 200%, 300%, 400% or at least about 500% above the level of insulin sensitivity in a control or reference level or amount.

The terms, "*lower*", "*reduced*" and "*decreased*", as used herein refer to a lower level of insulin sensitivity, such as lowered levels of insulin signalling, glucose uptake and/or mitochondrial activity, in a blood-derived cell, when compared to a control or reference level thereof. The level of insulin sensitivity may be relative or absolute. In some embodiments, a level of insulin sensitivity is lower, reduced or decreased if it is less than about 95%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20% or 10%, or even less than about 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.01%, 0.001% or 0.0001% of the level of insulin sensitivity in a control or reference level or amount.

The term "*control sample*" typically refers to a biological sample from a (healthy) non-diseased individual not having a neurological disease, disorder or condition. In one embodiment, the control sample may be from a subject known to be free of a neurological disease, disorder or condition. Alternatively, the control sample, such as control or untreated PBMCs, may be from the same subject prior to treatment with the agent and/or the insulin agonist. The control sample may be a pooled, average or an individual sample. An internal control is a marker from the same biological sample being tested.

As used herein, an expression level may be an absolute or relative amount of an expressed nucleic acid or protein. Accordingly, in some embodiments, the activity and/or expression level of a gene and/or a product thereof (*e.g.*, mTOR, Akt, AMPK, CaMKII, GSK3α, GSK3β, inclusive of unphosphorylated and phosphorylated forms thereof) is compared to a control level of activity and/or expression, such as the level of gene and/or protein expression of one or a plurality of "housekeeping" genes and/or proteins in the blood-derived cells.

As used herein a "*gene*" is a nucleic acid which is a structural, genetic unit of a genome that may include one or more amino acid-encoding nucleotide sequences and one or more non-coding nucleotide sequences inclusive of promoters and other 5' untranslated sequences, introns, polyadenylation sequences and other 3' untranslated sequences, although without limitation thereto. In most cellular organisms a gene is a nucleic acid that comprises double-stranded DNA.

The term "*nucleic acid*" as used herein designates single- or double-stranded DNA and RNA. DNA includes genomic DNA and cDNA. RNA includes mRNA, RNA,

RNAi, siRNA, cRNA and autocatalytic RNA. Nucleic acids may also be DNA-RNA hybrids. A nucleic acid comprises a nucleotide sequence which typically includes nucleotides that comprise an A, G, C, T or U base. However, nucleotide sequences may include other bases such as inosine, methylcytosine, methylinosine, methyladenosine and/or thiouridine, although without limitation thereto.

By "*protein*" is meant an amino acid polymer. The amino acids may be natural or non-natural amino acids, D- or L- amino acids as are well understood in the art. As would be appreciated by the skilled person, the term "*protein*" also includes within its scope phosphorylated forms of a protein (*i.e.*, a phosphoprotein), such as those described herein, and/or glycosylated forms of a protein (*i.e.* a glycoprotein). A "*peptide*" is a protein having no more than fifty (50) amino acids. A "*polypeptide*" is a protein having more than fifty (50) amino acids.

Determining, assessing, evaluating, assaying or measuring nucleic acids described herein, such as RNA, mRNA and cDNA, may be performed by any technique known in the art. These may be techniques that include nucleic acid sequence amplification, nucleic acid hybridization, nucleotide sequencing, mass spectroscopy and combinations of any these.

Nucleic acid amplification techniques typically include repeated cycles of annealing one or more primers to a "template" nucleotide sequence under appropriate conditions and using a polymerase to synthesize a nucleotide sequence complementary to the target, thereby "amplifying" the target nucleotide sequence. Nucleic acid amplification techniques are well known to the skilled addressee, and include but are not limited to polymerase chain reaction (PCR); strand displacement amplification (SDA); rolling circle replication (RCR); nucleic acid sequence-based amplification (NASBA), Q-β replicase amplification; helicase-dependent amplification (HAD); loop-mediated isothermal amplification (LAMP); nicking enzyme amplification reaction (NEAR) and recombinase polymerase amplification (RPA), although without limitation thereto. As generally used herein, an *"amplification product"* refers to a nucleic acid product generated by a nucleic acid amplification technique.

PCR includes quantitative and semi-quantitative PCR, real-time PCR, allele-specific PCR, methylation-specific PCR, asymmetric PCR, nested PCR, multiplex PCR, touch-down PCR, digital PCR and other variations and modifications to "basic" PCR amplification.

Nucleic acid amplification techniques may be performed using DNA or RNA extracted, isolated or otherwise obtained from a cell or tissue source. In other embodiments, nucleic acid amplification may be performed directly on appropriately treated cell or tissue samples.

Nucleic acid hybridization typically includes hybridizing a nucleotide sequence, typically in the form of a probe, to a target nucleotide sequence under appropriate conditions, whereby the hybridized probe-target nucleotide sequence is subsequently detected. Non-limiting examples include Northern blotting, slot-blotting, *in situ* hybridization and fluorescence resonance energy transfer (FRET) detection, although without limitation thereto. Nucleic acid hybridization may be performed using DNA or RNA extracted, isolated, amplified or otherwise obtained from a cell or tissue source or directly on appropriately treated cell or tissue samples.

It will also be appreciated that a combination of nucleic acid amplification and nucleic acid hybridization may be utilized.

Determining, assessing, evaluating, assaying or measuring levels of one or more proteins described herein (*e.g.*, mTOR, Akt, AMPK, BDNF, CaMKII, GSK3α, GSK3β, inclusive of unphosphorylated and phosphorylated forms thereof) may be performed by any technique known in the art that is capable of detecting cell- or tissue-expressed proteins whether on the cell surface or intracellularly expressed, or proteins that are isolated, extracted or otherwise obtained from the cell of tissue source. These techniques include antibody-based detection that uses one or more antibodies which bind the protein, electrophoresis, isoelectric focussing, protein sequencing, chromatographic techniques and mass spectroscopy and combinations of these, although without limitation thereto. Antibody-based detection may include flow cytometry using fluorescently-labelled antibodies that bind a protein of interest, ELISA, immunoblotting, immunoprecipitation, *in situ* hybridization, immunohistochemistry and immunocytochemistry, although without limitation thereto. Suitable techniques may be adapted for high throughput and/or rapid analysis such as using protein arrays such as a TissueMicroArray™ (TMA), MSD MultiArrays™, multiwell ELISA, In-Cell Western™ assay (see, *e.g.*, Figure 14), although without limitation thereto.

It will be appreciated that determining the expression of one or more protein markers of insulin sensitivity may include determining both the nucleic acid levels thereof, such as by nucleic acid amplification and/or nucleic acid hybridization, and/or the protein levels thereof.

In further embodiments, the level of insulin sensitivity of the blood-derived cells is compared to a threshold level thereof, such as a level of cellular metabolism in blood-derived cells from subjects having or not having a neurological disease, disorder or condition. Typically, a level of insulin sensitivity or cellular metabolism in the blood-derived cells that exceeds or falls below the threshold level of activity and/or expression is predictive of a particular disease state or outcome, such as resistance or responsiveness of the subject's neurological disease, disorder or condition to agent (*e.g.*, a glutamatergic modulator). The nature and numerical value (if any) of the threshold level of insulin sensitivity or cellular metabolism will typically vary based on the method chosen to determine the level of insulin sensitivity or cellular metabolism in the blood-derived cells, used in determining, for example, a response to the agent (*e.g.*, a glutamatergic modulator), in the subject.

A person of skill in the art would be capable of determining the threshold level of insulin sensitivity in a blood-derived cell that may be used in determining, for example, responsiveness to the agent and/or the insulin agonist, using any method of measuring insulin sensitivity known in the art, such as those described herein. In one embodiment, the threshold level is a level (mean, median or absolute) of insulin sensitivity in a reference or control sample of blood-derived cells derived from the subject, that, for example, have not been treated with the agent and/or the insulin agonist. Additionally, the concept of a threshold level should not be limited to a single value or result. In this regard, a threshold level may encompass multiple threshold levels that could signify, for example, a high, medium, or low probability of, for example, response to the agent, as described herein.

The "*blood cells*" or "*blood-derived cells*" of the present invention refers to cells or cell groups which may be present in the bloodstream *in vivo*, but not necessarily limited to peripheral blood cells. In this regard, the blood-derived cells may also be found or be able to be isolated from a cell population from, for example, bone marrow or umbilical cord blood, as well as pleural, peritoneal, cerebrospinal or synovial fluids or from various tissues, such as spleen and lymph node. In specific embodiments, the blood-derived cells express one or more marker molecules of blood cells, such as CD45, CD11a.

Suitably, the blood-derived cells are or comprise blood-derived mononuclear cells, such as peripheral blood mononuclear cells (PBMCs).

The term "*peripheral blood mononuclear cell*" or "*PBMC*" relates to a peripheral blood cell having a round nucleus. These cells typically include lymphocytes (T cells, B cells, NK cells) and monocytes, whereas erythrocytes and platelets have no nuclei, and granulocytes (neutrophils, basophils, and eosinophils) have multi-lobed nuclei. These cells can be extracted from whole blood using ficoll and gradient centrifugation, which will separate the blood into a top layer of plasma, followed by a layer of PBMCs and a bottom fraction of polymorphonuclear cells (such as neutrophils and eosinophils) and erythrocytes, as hereinafter described.

In an alternative embodiment, the blood-derived cells are bone marrow-derived mononuclear cells.

In certain embodiments, the present method includes the initial steps of:

(a) treating the one or plurality of blood-derived cells with an effective amount of the agent; and/or

(b) treating the one or plurality of blood-derived cells with an effective amount of an insulin agonist.

In this regard, the blood-derived cells are suitably treated sequentially with the agent and the insulin agonist.

To this end, the blood-derived cells may be treated for any time period with the agent, such as a glutamatergic modulator described herein, and prior to treatment with the insulin agonist. In embodiments, the blood-derived cells may be treated with the agent for about at least 1 hour (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 etc hours), about at least 6 hours, about at least 12 hours, about at least 1 day (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 etc days), about at least 2 days, about at least 7 days, about at least 14 days, about at least 21 days or about at least 28 days.

It is envisaged that the blood-derived cells may be treated *in vivo* by direct administration of a therapeutically effective amount of the agent to the subject and then subsequently isolated therefrom prior to treatment with the insulin agonist *in vitro*. Alternatively, the blood-derived cells may first be isolated from the subject prior to treatment with the agent and the insulin agonist *in vitro*. Accordingly, the methods described herein may further include the initial step of isolating the one or plurality of blood-derived cells from the subject prior to or after treatment with the agent.

For the purposes of this invention, by "*isolated*" is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that

normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native, chemical synthetic or recombinant form.

Suitably, a level of insulin sensitivity of the blood-derived cells may be measured at any time period after the step of treating with the insulin agonist. In embodiments, insulin sensitivity may be assessed by those means described herein after about at least 1 minute (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 etc minutes), about at least 5 minutes, about at least 10 minutes, about at least 15 minutes, about at least 30 minutes, about at least 1 hour, about at least 2 hours, about at least 3 hours or about at least 4 hours of incubation of the blood-derived cells in a suitable cell culture medium.

By "*effective amount*" is meant an amount of a compound, such as the agent and insulin agonist, that has the desired effect or activity *in vivo* or *in vitro*, such as on the blood-derived cells. As will be appreciated, the effective amount will depend at least in part on the agent in question, the neurological, disease, disorder or condition of the subject and whether the blood-derived cells are to be treated with the agent *in vivo* or *ex vivo*/*in vitro*. To this end, the blood-derived cells may be treated with any effective amount of the agent and/or the insulin agonist as required. Further, and with respect to *ex vivo* treatment of the blood-derived cells with the agent, it will be appreciated that standard or routine therapeutic doses of the agent known in the art suitably qualify as effective amounts thereof.

In particular embodiments, the blood-derived cells may be treated *in vivo* and/or *ex vivo* with the agent at concentrations between about 1 nM to about 1mM (*e.g.*, about 1 nM, 10 nM, 50 nM, 100 nM, 200 nM, 300 nM, 400 nM, 500 nM, 600 nM, 700 nM, 800 nM, 900 nM, 1 μM, 10 μM, 50 μM, 100 μM, 200 μM, 300 μM, 400 μM, 500 μM, 600 μM, 700 μM, 800 μM, 900 μM, 1 mM and any range therein), more particularly between about 100 nM to about 100 μM, and even more particularly about 500 nM to about 10 μM.

In certain embodiments, the blood-derived cells may be treated *in vitro* or *ex vivo* with the insulin agonist at concentrations between about 1 nM to about 1 mM (*e.g.*, about 1 nM, 10 nM, 50 nM, 100 nM, 200 nM, 300 nM, 400 nM, 500 nM, 600 nM, 700 nM, 800 nM, 900 nM, 1 μM, 10 μM, 50 μM, 100 μM, 200 μM, 300 μM, 400 μM, 500 μM, 600 μM, 700 μM, 800 μM, 900 μM, 1 mM and any range therein), more

particularly between about 100 nM to about 100 μM, and even more particularly about 500 nM to about 50 μM.

The term "*insulin agonist*" refers to a compound or molecule that binds to the insulin receptor and exhibits the same or similar biological activity as insulin, regardless of the insulin structure. The insulin agonist may include native insulin and analogues, fragments, variants or derivatives thereof, as are known in the art.

The term "*insulin*" refers to a polypeptide hormone (molecular weight of approximately 5700) naturally produced by the pancreas (secreted by beta cells in the islets of Langerhans) of a mammal which controls the amounts of glucose present in the blood by stimulating the uptake of glucose by muscle and adipose tissue. Insulin can exist in various states, such as preproinsulin and proinsulin. The term "*insulin*" also refers to synthetic versions, such as Humulin™ (available commercially from Eli Lilly).

In view of the above, the present method may further include the step of treating the neurological disease, disorder or condition in the subject. By way of example, this can include administering to the subject a therapeutically effective amount of the agent, such as a glutamatergic modulator, when the level of insulin sensitivity indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent. Alternatively, if the level of insulin sensitivity indicates or correlates with relatively decreased responsiveness of the neurological disease, disorder or condition to the agent, the current method may further include the step of administering to the subject therapeutically effective amounts of: (a) an insulin sensitising agent; and (b) the agent (*e.g.*, a glutamatergic modulator).

As used herein, the term *"therapeutically effective amount"* describes a quantity of a specified agent, such as a glutamatergic modulator and/or an insulin sensitizer, sufficient to achieve a desired effect in a subject being treated with that agent(s). For example, this can be the amount of a composition comprising one or more agents that are necessary to reduce, alleviate and/or prevent a neurological disease, disorder or condition. In some embodiments, a *"therapeutically effective amount"* is sufficient to reduce or eliminate a symptom of a neurological disease, disorder or condition, such as depression. In other embodiments, a *"therapeutically effective amount"* is an amount sufficient to achieve a desired biological effect, for example an amount that is effective to decrease or prevent progression of neurological disease, disorder or condition or overcome resistance to and/or enhance the activity of a therapeutic agent.

Ideally, a therapeutically effective amount of an agent is an amount sufficient to induce the desired result without causing a substantial cytotoxic effect in the subject. The effective amount of an agent useful for reducing, alleviating and/or preventing a a neurological disease, disorder or condition will be dependent on the subject being treated, the type and severity of any associated disease, disorder and/or condition, and the manner of administration of the therapeutic composition.

By "*administering*" or "*administration*" is meant the introduction of an agent and/or an insulin sensitizing agent disclosed herein into an animal subject by a particular chosen route.

Methods of treating neurological diseases, disorders or conditions may be prophylactic, preventative or therapeutic and suitable for treatment of neurological diseases, disorders or conditions in mammals, particularly humans. As used herein, "*treating*", "*treat*" or "*treatment*" refers to a therapeutic intervention, course of action or protocol that at least ameliorates a symptom of a neurological disease, disorder or condition after it and/or its symptoms have at least started to develop. As used herein, "*preventing*", "*prevent*" or "*prevention*" refers to therapeutic intervention, course of action or protocol initiated prior to the onset of a neurological disease, disorder or condition and/or a symptom thereof so as to prevent, inhibit or delay or development or progression of the neurological disease, disorder or condition or the symptom.

It is envisaged that the agent may be any type of compound. For example, the compound may be a small organic molecule or a biological compound such as an antibody or an enzyme.

In particular embodiments, the agent is or comprises a glutamatergic modulator.

The term "*glutamatergic modulator*" broadly refers to an agent which affects or modulates the amount, function or signalling of glutamate in a cell. Thus, the glutamatergic modulator may, for example, act to alter the synthesis of glutamate in the cell, the metabolism of glutamate in the cell, the binding of glutamate to its target receptors on the cell and/or the rate of transport of glutamate out of the cell.

In certain embodiments, the glutamatergic modulator is or comprises an N-methyl-D-aspartate (NMDA) receptor antagonist (*e.g.*, Ketamine, Esketamine, Dextromethorphan, AVP-786, Nitrous oxide (N_2O), AZD6765 (lanicemine), CP-101,606/traxoprodil, MK-0657 (CERC-301)), an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, a metabotropic glutamate receptor antagonist (e.g., AZD2066, RO4917523/basimglurant

JNJ40411813/ADX71149, R04995819 (RG1578)), a kainate receptor antagonist, a glycine transportor-1 antagonist (*e.g.*, D-cycloserine, GLYX-13, Sarcosine, AV-101), a dopamine modulator, including a stimulant, a dopamine reuptake inhibitor, a norepinephrine-dopamine reuptake inhibitor and a dopamine receptor agonist and/or antagonist (*e.g.*, levodopa, levodopalisuride, pergolide, a DOPA decarboxylase inhibitor, a catechol-O-methyl transferase inhibitor), an immune modulator, an anti-inflammatory agent and/or a lithium compound or salt (*e.g.* LiCl). In some embodiments, the glutamatergic modulator is or comprises an NMDA receptor antagonist, an AMPA receptor antagonist, a metabotropic glutamate receptor antagonist, a kainate receptor antagonist, a glycine transportor-1 antagonist, a dopamine modulator, an immune modulator and/or an anti-inflammatory agent. As such, in embodiments, the glutamatergic modulator provided herein does not comprise a lithium compound or salt.

In particular embodiments, the immune modulator or anti-inflammatory agent is or comprises a tumour necrosis factor (TNF) inhibitor. As used herein, the term "*TNF inhibitor*" refers to any molecule that suppresses a physiologic response to TNF, such as TNFα. Any suitable TNF inhibitor known in the art can be used with the presently disclosed methods. In certain embodiments, the TNF inhibitor is a monoclonal antibody, a small molecule inhibitor or a protein biologic. In particular embodiments, the TNF inhibitor is a selective inhibitor of soluble TNF, including soluble TNFα. Non-limiting examples of TNF inhibitors include XPro1595 (INmune Bio), adalimumab (Humira), adalimumab-adbm (Cyltezo), adalimumab-adaz (Hyrimoz), adalimumab-atto (Amjevita), certolizumab pegol (Cimzia), etanercept (Enbrel), eanercept-szzs (Ereizi), golimumab (Simponi, Simponi Aria), infliximab (Remicade), infliximab-abda (Renflexis), infliximab-dyyb (Inflectra), infliximab-qbtx (Ixifi), thalidomide (Immunoprin), lenalidomide (Revlimid), pomalidomide (Pomalyst, Imnovid), xanthine derivatives, pentoxifylline, bupropion, hallucinogens (e.g., (R)-DOI, TCB-2, LSD and LA-SS-Az), biosimilars thereof, and combinations thereof.

In certain embodiments, the agent is or comprises XPro1595 or a variant or derivative (*e.g.*, an analogue or prodrug) thereof. As will be appreciated, XPro1595 is a therapeutic agent that acts primarily as an anti-inflammatory agent by selectively blocking tumor necrosis factor (TNF) activity in the cell . TNF typically exists in two functional forms: soluble TNF (sTNF) and transmembrane TNF (tmTNF), each having distinct functional impacts. sTNF signaling occurs via TNF receptor 1 (TNFR1), while

tmTNF signaling occurs via TNF receptor 2 (TNFR2). sTNF has far-reaching pro-inflammatory and bioenergetic impacts: sTNF promotes blood-brain barrier permeability, allowing immune cell recruitment to the central nervous system while promoting n-methyl-d-aspartate (NMDA) receptor activity and glutamate excitotoxicity. This cytokine has also been shown to produce upregulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). It also inhibits the activity of the insulin receptor, thereby depriving the mitochondria of bioenergetic resources. This deprivation of resources exposes the mitochondria to redox dysregulation, increases energy (ATP) demand, promotes reactive oxygen species, and magnifies the potential for mitochondrial DNA defects. Within the context of a proinflammatory immune response, sTNF can dampen engagement of TNFR2 by tmTNF. Generally, tmTNF is neuroprotective against glutamate excitotoxicity.

The terms "*insulin sensitizer*" and "*insulin sensitizing agent*" can be used interchangeably and refer to any compound capable of increasing the sensitivity of a cell or tissue to insulin or an insulin agonist. Examples of insulin sensitizers include tyrosine phosphatase inhibitors (PTP inhibitors), GSK-3 inhibitors, retinoid X receptor agonists (RXR agonists), glitazones (TZD), non-TZD PPARγ agonists, PPARα/PPARγ dual agonists, agonists based on compounds containing vanadium, and biguanides, such as metformin. Insulin sensitizers also include pharmaceutically acceptable salt forms, such as hydrochloride, hydrobromide, hydroiodide, sulfate, nitrate, phosphate, citrate and methanesulfonic acid, thereof. Additionally, it may be in the form of a salt, trifluoroacetate or acetate, sodium ion, potassium ion, calcium ion or magnesium ion, but is not limited thereto.

Further aspects of the invention relate to treatment of a neurological disease, disorder or condition in a subject.

In one particular aspect, the treatment is performed in conjunction with determining a level of insulin sensitivity of one or a plurality of blood-derived cells of the subject treated with effective amounts of an agent and based on the determination made, initiating, continuing, modifying or discontinuing treatment with the agent. In this regard, it would be appreciated that those methods described herein for predicting the responsiveness of a neurological disease, disorder or condition to an agent, such as a glutamatergic modulator, may further include the step of administering to the subject a therapeutically effective amount of the agent and optionally an insulin sensitizing agent.

In particular embodiments, the method of treatment of the present aspect comprises administration of an agent, such as a glutamatergic modulator and optionally an insulin sensitizing agent, such as those hereinbefore described. In particular embodiments, the agent is or comprises a glutamatergic modulator, such as those provided herein. In some embodiments, the insulin sensitizing agent is or comprises metformin.

In one specific embodiment, the agent or treatment is administered when the level of insulin sensitivity in the blood-derived cells treated with the agent indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent. In this regard, the present method may further include the step of administering to the subject a therapeutically effective amount of the agent, such as glutamatergic modulator, when a relatively increased or high level of insulin sensitivity is determined. Alternatively, the present method may further include the step of administering to the subject therapeutically effective amounts of: (a) the agent; and (b) the insulin sensitizer when a relatively decreased or low level of insulin sensitivity is determined.

In certain embodiments, a relatively increased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent; and/or a relatively decreased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively decreased responsiveness of the neurological disease, disorder or condition to the agent.

In particular embodiments, the agent (*e.g.*, the glutamatergic agent) is administered (i) prior to; (ii) after; or (iii) simultaneously with, administration of the insulin sensitizing agent. In one embodiment, administration of the agent and administration of the insulin sensitizing agent (either sequentially or concurrently) results in treatment or prevention of the neurological disease, disorder or condition that is greater than such treatment or prevention from administration of either the said agent or the insulin sensitizing agent in the absence of the other.

Suitably, the various therapeutic agents and treatments described herein are administered to a subject as a pharmaceutical composition comprising a pharmaceutically-acceptable carrier, diluent or excipient. In this regard, any dosage form and route of administration, such as those provided therein, may be employed for providing a subject with the composition of the invention.

By "*pharmaceutically-acceptable carrier, diluent or excipient*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, liposomes and other lipid-based carriers, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates and pyrogen-free water.

A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991), which is incorporated herein by reference.

Any safe route of administration may be employed for providing a patient with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the like may be employed. Intra-muscular and subcutaneous injection is appropriate, for example, for administration of immunotherapeutic compositions, proteinaceous vaccines and nucleic acid vaccines.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

The therapeutic agents and compositions described herein may be administered in a manner compatible with the dosage formulation, and in such amount as is pharmaceutically-effective. The dose administered to a patient, in the context of the present invention, should be sufficient to effect a beneficial response in a patient over an appropriate period of time. The quantity of agent(s) to be administered may depend

on the subject to be treated inclusive of the age, sex, weight and general health condition thereof, factors that will depend on the judgement of the practitioner.

In particular embodiments, the methods and kit described herein provide a "*companion diagnostic*" with respect to the treatment of a neurological disease, disorder or condition, whereby the level of insulin sensitivity in the blood-derived cells treated with the agent provides information to a clinician or the like that is used for the safe and/or effective administration of such a treatment.

Suitably, the neurological disease, disorder or condition is of a type hereinbefore described, albeit without limitation thereto. More particularly, the neurological disease, disorder or condition suitably is or comprises a mood disorder, including depressive disorders and bipolar disorders; post-traumatic stress disorder; Alzheimer's disease; an anxiety disorder, including panic disorders, phobias, obsessive-compulsive disorders, stress disorders and generalized anxiety disorders; and any combination thereof.

Suitably, the blood-derived cells are or comprise blood-derived mononuclear cells, such as peripheral blood mononuclear cells (PBMCs).

In yet another aspect, the invention provides a kit for predicting the responsiveness of a neurological disease, disorder or condition in a subject to an agent, the kit comprising at least one reagent capable of determining a level of insulin sensitivity of one or a plurality of blood-derived cells of the subject that have been treated with the agent, wherein the level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased or decreased responsiveness of the neurological disease, disorder or condition to the agent.

Suitably, a relatively increased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent; and/or a relatively decreased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively decreased responsiveness of the neurological disease, disorder or condition to the agent.

The agent suitably is or comprises a glutamatergic modulator, such those hereinbefore described. In certain embodiments, the glutamatergic modulator is or comprises an N-methyl-D-aspartate (NMDA) receptor antagonist, an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor agonist or antagonist, a metabotropic glutamate receptor antagonist, a kainate receptor antagonist, a glycine transportor-1 antagonist, a dopamine modulator, an immune modulator, an anti-

inflammatory agent and/or a lithium compound or salt. In some embodiments, the glutamatergic modulator is or comprises an NMDA receptor antagonist, an AMPA receptor antagonist, a metabotropic glutamate receptor antagonist, a kainate receptor antagonist, a glycine transportor-1 antagonist, a dopamine modulator, an immune modulator and/or an anti-inflammatory agent. As such, in embodiments, the glutamatergic modulator does not comprise a lithium compound or salt.

In some embodiments, the kit of the present aspect further comprises reference data for correlating the level of insulin sensitivity of the one or plurality of blood-derived cells with responsiveness of the neurological disease, disorder or condition to the agent.

In particular embodiments, the reference data is on a computer-readable medium (e.g., software embodying or utilised by any one or more of the methodologies or functions described herein). The computer-readable medium can be included on a storage device, such as a computer memory (*e.g.*, hard disk drives or solid state drives) and preferably comprises computer readable code components that when selectively executed by a processor implements one or more aspects of the present invention.

Suitably, the blood-derived cells are or comprise blood-derived mononuclear cells, such as those previously described (*e.g.*, PBMCs).

Suitably, the neurological disease, disorder or condition is that hereinbefore described. In embodiments, the neurological disease, disorder or condition is or comprises: a mood disorder, including depressive disorders and bipolar disorders; post-traumatic stress disorder; Alzheimer's disease; an anxiety disorder, including panic disorders, phobias, obsessive-compulsive disorders, stress disorders and generalized anxiety disorders; suicidality; and any combination thereof.

Suitably, the present kit is for use in the methods described herein. In this regard, the kit may be or comprise a companion diagnostic.

A further aspect of the invention provides a method of screening, designing, engineering or otherwise producing an agent for treating a neurological disease, disorder or condition in a subject, said method including the step of determining whether a candidate molecule is capable of at least partly increasing or enhancing a level of insulin sensitivity in one or a plurality of blood-derived cells treated with effective amounts of the candidate molecule.

The invention also provides an agent screened, designed, engineered or otherwise produced according to the aforementioned aspect. It will be appreciated that the agent may be suitable for use in the method of the first two mentioned aspects.

The candidate molecule may be a protein (inclusive of peptides, antibodies and antibody fragments), a nucleic acid (inclusive of inhibitory RNA molecules such as ribozymes, RNAi, miRNA and siRNA, although without limitation thereto), a lipid, a carbohydrate, a small organic molecule or any combination of these (*e.g* a glycoprotein, a lipoprotein, a peptide-nucleic acid *etc*).

In some embodiments, the candidate modulator may be rationally designed or engineered *de novo* based on desired or predicted structural characteristics or features that indicate the candidate modulator could increase or promote insulin sensitivity in blood-derived cells, such as being able to modulate glutamate signalling. In other embodiments, the candidate modulator may be identified by screening a library of molecules without initial selection based on desired or predicted structural characteristics or features that indicate the candidate modulator could increase or promote insulin sensitivity in blood-derived cells. Such libraries may comprise randomly generated or directed libraries of proteins, peptides, nucleic acids, recombinant antibodies or antibody fragments (*e.g.* phage display libraries), carbohydrates and/or lipids, libraries of naturally-occurring molecules and/or combinatorial libraries of synthetic organic molecules.

Non-limiting examples of techniques applicable to the design and/or screening of candidate modulators may employ X-ray crystallography, NMR spectroscopy, computer assisted screening of structural databases, computer-assisted modelling or biochemical or biophysical techniques which detect molecular binding interactions, as are well known in the art.

Biophysical and biochemical techniques which identify molecular interactions include competitive radioligand binding assays, co-immunoprecipitation, fluorescence-based assays including fluorescence resonance energy transfer (FRET) binding assays, electrophysiology, analytical ultracentrifugation, label transfer, chemical cross-linking, mass spectroscopy, microcalorimetry, surface plasmon resonance and optical biosensor-based methods, such as provided in Chapter 20 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997) Biochemical techniques such as two-hybrid and phage display screening methods are provided in

Chapter 19 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, 1997).

Accordingly, an initial step of the method may include identifying a plurality of candidate molecules that are selected according to broad structural and/or functional attributes, such as an ability to increase or promote insulin sensitivity.

Suitably, the insulin sensitizing effect of a candidate molecule may be determined using an *in vivo* or *in vitro* insulin challenge model, such as those known in the art and described herein.

Accordingly, in particular embodiments, the method of the present aspect further includes the initial steps of:

(a) contacting or treating the one or plurality of blood-derived cells, such as PBMCs, with an effective amount of a candidate agent; and/or

(b) contacting or treating the one or plurality of the blood-derived cells of (a) with an effective amount of an insulin agonist.

In some embodiments, the method of the present aspect includes contacting or treating the blood-derived cells of one or more subjects, such as those derived from a broad population of subjects with the neurological disease, disorder or condition, with the candidate compound or molecule for a sufficient period; contacting or treating the blood-derived cells with an amount of insulin sufficient to activate cellular metabolism (*e.g.*, insulin signalling, glucose uptake and/or mitochondrial activity) thereof; evaluating activation or levels of insulin sensitivity or cellular metabolism in the blood-derived cells, such as those hereinbefore described; comparing activation or levels of insulin sensitivity or cellular metabolism in the blood derived cells to a reference or threshold level of insulin sensitivity or cellular metabolism in the blood-derived cells, such as control blood-derived cells not previously treated with the test compound or insulin, and selecting the candidate compound as a candidate therapeutic agent for treating a neurological disease, disorder or condition in a subject if activation or levels of insulin sensitivity or cellular metabolism are greater with prior treatment of the test compound as compared to activation or levels of insulin sensitivity or cellular metabolism without prior treatment of the test compound.

In some embodiments, the level of insulin sensitivity, and hence activation of cellular metabolism pathways, in the blood-derived cells is determined by one or more of a level of insulin signalling, a level of glucose uptake and a level of mitochondrial activity thereof, such as by those methods previously described.

Suitably, the blood-derived cells are or comprise blood-derived mononuclear cells, such as those previously described (e.g., PBMCs). In particular embodiments, the blood-derived cells are harvested or isolated from one or more subjects having a particular neurological disease, disorder or condition, such as those described herein.

Suitably, the agent is or comprises a glutamatergic modulator, such as those hereinbefore described (*e.g.*, a glutamate receptor antagonist or inhibitor).

It will be appreciated that for the present aspect, the neurological disease, disorder or condition may be any as are known in the art, such as those provided herein. In embodiments, the neurological disease, disorder or condition is or comprises: a mood disorder, including depressive disorders and bipolar disorders; post-traumatic stress disorder; Alzheimer's disease; an anxiety disorder, including panic disorders, phobias, obsessive-compulsive disorders, stress disorders and generalized anxiety disorders; suicidality; and any combination thereof.

It will be understood that the method of this aspect may be performed iteratively, whereby multiple rounds of screening, design, and biological testing are performed. This may include where a candidate molecule is structurally modified before each round, thereby enabling "fine-tuning" of the candidate molecule.

It will also be appreciated that the method may be performed in a "high throughput", "automated" or "semi-automated" manner, particularly during early stages of candidate molecule identification and selection.

An agent screened, designed, engineered or otherwise produced according to the aforementioned aspect may be used according to the methods of the aforementioned aspects, preferably in the form of a pharmaceutical composition as hereinbefore described.

With respect to the aforementioned aspects, the term "*subject*" includes but is not limited to mammals inclusive of humans, performance animals (such as horses, camels, greyhounds), livestock (such as cows, sheep, horses) and companion animals (such as cats and dogs). Preferably, the subject is a human.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated in its entirety herein by reference.

The following non-limiting examples illustrate the methods and kit of the invention. These examples should not be construed as limiting: the examples are included for the purposes of illustration only.

EXAMPLE 1

Introduction

Suboptimal response to antidepressant treatments remains a major challenge in psychiatry. Indeed few (if any) useful biomarkers exist to predict early treatment efficacy[1]. The identification of acute physiological correlates for early antidepressant response could potentially lead to their utilization as biomarkers. Such biomarkers could provide insight into discreet pathophysiological processes contributing to the antidepressant response. Such processes, in turn, have potential to curtail the extended misapplication of ineffective treatments currently necessitating prolonged periods of observation. Although many preclinical models of depression exist, few models focus on improving our understanding of behavioral resistance to antidepressants.

Aberrant activation of the hypothalamic-pituitary adrenal (HPA) stress axis is commonly reported in both unipolar and bipolar depressed patients[2,3] and is associated with sub-optimal treatment responses, increased symptom severity, and poorer remission rates[3,4]. Chronic stimulation of the HPA axis via administration of adrenocorticotropic hormone-(1–24) (ACTH) blocks the immobility-reducing effects of imipramine in the forced swim test (FST)[5–9], a well-established behavioral screen for antidepressant efficacy in rodents. Kitamura and colleagues[6] previously reported that lithium co-administration (100 mg/kg p.o.) in this model could rescue the antidepressant response to imipramine (10 mg/kg) in the FST.

Lithium is effective in both manic and prophylactic treatment, and remains the gold standard mood stabilizing medication for bipolar patients[10,11]. This mood stabilizer has also been shown to improve therapeutic efficacy and extend remission when applied as an adjunctive treatment for intractable forms of major depression (reviewed in refs. [11,12]); however, there remains a proportion of patients that do not receive therapeutic benefit from lithium[13,14]. Our understanding of lithium's therapeutic mechanism, and consequently, our ability to rationally identify biomarkers predictive of lithium's therapeutic efficacy, is still limited. One mechanism believed to contribute to lithium's therapeutic action is the inhibition of glycogen synthase kinase-3β (GSK3β), a key mediator of energy metabolism within the insulin signaling pathway[15]. Importantly, in the context of insulin signaling, GSK3β is known to interact with mammalian target of rapamycin (mTOR). mTOR serves an integral regulatory role in cellular energy, growth and plasticity; responding to various environmental stressors and modulating gene

transcription/translation, apoptotic processes, and synapse formation in accordance with energy availability[16–18]. Via these actions, mTOR activation has been implicated in the novel antidepressant-like effects of ketamine and other antidepressants (for review, see ref. [19]). Together with protein kinase B (Akt), mTOR and GSK3β form an integral portion of the insulin signaling pathway, and are important for cellular energy regulation and metabolism (see Fig. 1[20,21]).

Post-mortem human brain studies comparing unipolar MDD patients to healthy controls have demonstrated abnormalities in both mTOR and GSK3β levels in the prefrontal cortex (PFC)[22,23]. Reduced levels of phosphorylated GSK3β have also been previously reported in the peripheral blood mononuclear cells (PBMCs) of bipolar patients, correlating with symptom severity[24]. Additionally, Machado-Vieira and colleagues[25] reported lowered *Akt1* and *mTOR* mRNA expression in the blood of bipolar patients during depressive episodes, where changes in *Akt1* expression following lithium treatment were associated with clinical improvement.

Given that a direct and interdependent relationship exists between the neuroendocrine response to stress and energy regulation and metabolism, we propose that long-term aberrant HPA activity may impair metabolic functions under stress, contributing to poor antidepressant efficacy at the molecular and behavioral level. Such metabolic consequences may be particularly pertinent for antidepressant action in regions of the depression network known to be hypermetabolic, such as the infralimbic cortex (ILPFC). The ILPFC region of the medial PFC plays a critical role in regulating behavioral responses to stress, particularly in the FST. The ILPFC is considered to be the rodent homolog of Brodmann's area 25 (BA25) in humans[26,27]. Notably, BA25 is metabolically overactive in patients with depression; and normalizing this metabolic disturbance has been associated with antidepressant responses across multiple modalities[28,29]. Failure to normalize this metabolic hyperactivity through antidepressant treatment is, by contrast, associated with poor clinical outcomes in treatment-resistant depression.

In this Example, we sought to verify that lithium augmentation restores the antidepressant-like effects of imipramine in ACTH-pretreated animals as measured by the FST and to determine if such effects were associated with the expression of insulin signaling pathway proteins. ACTH pre-treatment is expected to block the typical immobility-reducing effects of imipramine in the FST, while a reduction in immobility

duration and corresponding increase in active coping strategies, such as climbing is expected following co-administration of lithium and imipramine in ACTH animals.

Materials and Methods

Animal treatments

Male albino Wistar rats ($n = 60$) were used in this study, weighing 250–350 g at the time of testing. Animals were housed individually in a room with controlled temperature (20–22 °C) on a 12 h light–dark cycle (lights: on 07:00; off 19:00). Food and water were available ad libitum. Animals entered the study at 5 weeks of age following a 3-day acclimatization period, and completed testing in their seventh week, at which point they were sacrificed. All procedures were carried out in accordance with institutional guidelines for ethical animal care and use.

Drugs

The drugs used in this study included: ACTH-(1–24) (AnaSpec, San Jose, CA, USA), 100 μg/day dissolved in distilled water; imipramine hydrochloride 10 mg/kg (Sigma-Aldrich, St. Louis, MO, USA); lithium chloride 100 mg/kg (Sigma-Aldrich); control vehicle 0.9% saline (Fisher Healthcare, Hanover Park, IL, USA); and FatalPlus® (Vortech Pharmaceuticals, Dearborn, MI, USA), (constituents: pentobarbital sodium 390 mg/mL; propylene glycol 0.01 mg/mL; ethyl alcohol 0.29 mg/mL; benzyl alcohol (preservative) 0.20 mg/mL) 0.70 cc. Drugs were all delivered via intraperitoneal (i.p.) injection.

Experimental procedure

This study implemented a treatment protocol described previously with minor modifications (see ref. [9]). Briefly, following acclimatization, male Wistar rats were randomly assigned to receive daily injections of either ACTH-(1–24) 100 μg/day or saline (0.9%) for 14 days. On treatment day 14 the open field test (OFT) was administered and 2 h post-test animals received their initial 15 min forced swim stress exposure. A final 6 min FST was conducted on day 15. Behavioral data was recorded for analyses. Animals were administered either imipramine hydrochloride 10 mg/kg, imipramine hydrochloride 10 mg/kg with lithium chloride 100 mg/kg, or control vehicle saline (0.9%) on day 14 and 15, 30 min prior to the OFT and FST, respectively. Animals were sacrificed 30 min after FST on day 15 via anesthetic overdose of pentobarbital sodium (0.7 cc Fatal-Plus®). Brains were harvested and cardiac blood

samples were also collected. Samples were frozen on dry ice and stored at −80 °C until use. A schematic diagram of the experimental timeline is depicted in Fig. 2.

Behavioral testing

Open field test

The OFT was implemented to examine ambulatory/locomotor behaviors in response to treatments. Animals were each placed in the central zone of an open field arena (60 cm length × 60 cm width × 60 cm height), and allowed to move freely for 6 min. Behaviors were recorded by video camera. Data were analyzed using the behavioral analysis package TopScan (CleverSys Inc., Reston, VA, USA). Behaviors of interest included: *distance traveled*, *mean velocity of travel*, and *time spent in central region of arena*.

Forced swim test

The FST is a well-established screening tool for evaluating antidepressant efficacy, with robust predictive validity[30]. The forced swim apparatus consisted of clear plexiglass cylindrical tanks (45 cm height × 20 cm diameter) filled with tap water (23 °C) to a depth of 30 cm. Animals were first exposed to 15 min learning trial, conducted 2 h after the completion of the OFT on day 14. A 6 min test session was then conducted on the subsequent day. Sessions were recorded, and analyzed using the behavioral analysis package ForcedSwimScan (CleverSys Inc.). Following ForcedSwimScan analysis footage was then deidentified and reanalyzed by hand in 1 s intervals for verification. As measured previously[9], behaviors of interest included *immobility* (passive behavior), *swimming*, and *climbing* (active behaviors). The final 4 min of the FST were used for this analysis.

Tissue collection

Following behavioral testing on day 15, animals were humanely euthanized by anesthetic overdose 30 min after completion of the FST. Cardiac blood samples were collected for PBMC isolation. The brain of each animal was extracted, frozen on dry ice, and stored at −80 °C until dissection.

Western blotting

Brains were manually dissected on a ThermalTray™ LP (BioCision, Mill Valley, CA, USA) maintained at −20 °C using dry ice. Brain regions were identified using a rodent brain atlas (Figs. 9–13 in Paxinos and Watson[31]). Following dissection, ILPFC tissue was lysed in radioimmunoprecipitation assay (RiPA) lysis buffer for

Western blotting. Protein concentration was determined by bicinchoninic acid (BCA) protein assay.

Equal amounts of ILPFC protein lysate were loaded and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), before transfer to polyvinylidene fluoride (PVDF) membrane (Immobilon-P). Membranes were blocked for 2 h with tris-buffered saline solution with the detergent Tween® 20 (TBST) containing 5% milk (or 5% bovine serum albumin (BSA) for phosphorylated antibodies), before being incubated at 4 °C with primary antibodies overnight. Antibodies used included: total Akt (pan), phospho-Akt (Ser^{473}), GSK-3β, phospho-GSK-3β (Ser^{9}), total mTOR, phospho-mTOR (Ser^{2448}) (Cell Signaling Technology, Danvers, MA, USA); total β-actin (Sigma-Aldrich). Herein phosphorylated proteins will be prefixed with '*p*'.

The following day, blots were washed with TBST three times and incubated with anti-rabbit HRP-linked secondary antibody (Cell Signaling) for 45 min. Blots were then washed three more times with TBST and exposed to enhanced chemiluminescence (ECL) substrate. Band detection and densitometric analysis was conducted using Bio-Rad ChemiDoc™ imaging system. Readings were normalized to β-actin, and expressed as a ratio to cerebellar tissue (positive control).

PBMC isolation and insulin challenge

Cell isolation and preparation

Dulbecco's phosphate-buffered saline (DPBS) (1–4 mL) (Gibco Life Technologies, Rockvile, MD, USA) was added to a 10 mL heparinized tube (Monoject®: Kendall Healthcare, Mansfield, MA, USA) containing blood sample; added according to sample volume (2–8 mL), and mixed well with a pipette until homogenous. The blood/DPBS mixture was slowly added to a 15 mL conical polypropylene tube (BD Falcon™: BD Biosciences, Bedford, MA, USA) prefilled with 2 mL of Histopaque® medium (solution containing polysucrose and sodium diatrizoate adjusted to a density of 1.077 g/mL) (Sigma-Aldrich), forming a layer atop it. Tubes were immediately centrifuged at 400 × *g* for 30 min facilitating the separation of the PBMCs from the plasma and erythrocytes. Excess plasma was then removed from the tube before the PBMC layer was collected with a 5 mL pipette.

The PBMCs were then deposited into T25 tissue culture flasks (BD Biosciences) filled with 5 mL Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco Life Technologies) containing 10% fetal bovine serum (FBS), l-glutamine, 4-

(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and penicillin/streptomycin solution. Flasks were incubated overnight at 37 °C 5% CO_2 in a humidified tissue culture incubator.

The following day, the cells were harvested and centrifuged at 1200 rpm for 5 min at 4 °C. Media was then aspirated from the tube, leaving the cells in a pellet at the base of the tube. Cells were then resuspended in 2 mL media containing 5% dimethyl sulfoxide (DMSO) (ATTC, Manassas, VA, USA), and dispensed into 2 mL cryogenic vials (Corning Inc., Corning, NY, USA) in 1 mL aliquots, placed in a styrofoam holder and slow frozen in a −80 °C freezer.

Insulin challenge and enzyme-linked immunosorbent assays (ELISA)

Frozen PBMCs were thawed and left to recover in a humidified tissue culture incubator (37 °C; 5% CO_2) overnight. The following morning, cells were centrifuged and then re-suspended in media without FBS. Re-suspended cells were then divided into two wells of a six-well plate and left to incubate, devoid of growth factors for 4 h at 37 °C to deplete any residual insulin.

The cells were then challenged with 10 mg/mL of insulin for 5 min (37 °C; 5% CO_2). Following challenge, cells were then centrifuged and lysed using 50 mL of RIPA lysis buffer. Cellular debris was removed by centrifugation (14,000 rpm; 4 °C; 10 min) and the lysate was then dispensed into a new tube. Commercially available competitive ELISA kits were used to test concentrations of mTOR (Total) and *p*mTOR (Ser^{2448}) (Cell Signaling). A BCA protein assay was not possible because of the limited measurable protein levels; therefore results were reported in relative arbitrary units and relative differences pre-versus post-insulin quantified.

Statistical analyses

In all cases, the Shapiro–Wilk test of normality and Brown–Forsythe test for homogeneity of variance were utilized; these assumptions were met unless otherwise specified. Separate one-way analysis of variance (ANOVA) with subsequent Tukey's honest significant difference (HSD) tests were used to analyze both behavioral and molecular data. One-way ANOVA is considered a robust test against violations of the normality assumption, in this case minor violations were noted. Where the assumption of homogeneity of variance was violated, data were analyzed using non-parametric Kruskal–Wallis one-way tests followed by Dunn's multiple comparison tests. Linear regression analysis was used to evaluate the relationship between the protein levels and behavioral results. Calculations were performed using GraphPad Prism 6.0.

Results

Behavioral data

Open field test

Brown–Forsythe tests for homogeneity of variance were significant for *distance*: $F(4,50) = 3.071$, $p = 0.0244$; *velocity*: $F(4,50) = 3.195$, $p = 0.0206$; and *centre duration*: $F(4, 50) = 32.88$, $p = 0.0319$. As such, non-parametric tests were used.

A one-way Kruskal–Wallis test unveiled a significant main effect of treatment on *distance* traveled in the OFT, $H(4) = 19.06$, $p = 0.0008$. Post-hoc Dunn's tests indicated that the addition of lithium to imipramine treatment in ACTH pre-treated animals significantly reduced distance traveled compared to saline-control, and ACTH-control treatment groups (shown in Fig. 3a).

A one-way Kruskal–Wallis test also revealed a significant main effect of treatment on ambulatory *velocity*, $H(4) = 19.13$, $p = 0.0007$. Post-hoc Dunn's tests suggested that ACTH animals treated with imipramine and lithium showed significantly lower velocity of travel when compared to saline-control, and ACTH-control animals (see Fig. 3b).

A one-way Kruskal–Wallis test also revealed a significant main effect of treatment on ambulatory *velocity*, $H(4) = 19.05$, $p = 0.0019$. Dunn's post-tests identified that ACTH animals administered both imipramine and lithium spent significantly less time in the center region than animals in the saline-control, saline-imipramine, and ACTH-imipramine groups (shown in Fig. 3c).

Five animals were omitted from further analysis following their identification as outliers in the OFT, with ambulatory behavioral scores exceeding two standard deviations from the mean.

Forced swim test

One-way ANOVA exposed a significant main effect of treatment on *immobility* duration, $F(4,50) = 14.954$, $p < 0.0001$. Shapiro–Wilk normality test for ACTH-imipramine group was significant, $W = 0.8213$, $p = 0.0179$. Post-hoc Tukey's HSD tests multiple comparisons revealed no significant difference between control saline-control and ACTH-control groups. Saline animals administered imipramine showed a significant reduction in immobility time relative to saline-control animals. ACTH animals treated with imipramine, on the other hand, were found to have significantly higher immobility time relative to ACTH-vehicle animals, and saline-imipramine

animals. ACTH animals co-administered lithium with imipramine had significantly lower immobility duration relative to saline-vehicle, ACTH-vehicle and ACTH-imipramine groups (see Fig. 3d).

One-way ANOVA also revealed a significant main effect of treatment on *climbing* duration, $F(4,50) = 17.42$, $p < 0.0001$. Shapiro–Wilk normality test was significant for ACTH-imipramine group, $W = 0.8229$, $p = 0.0188$. Tukey's HSD tests suggest that lithium-treated animals showed significantly longer climbing duration than control-vehicle, ACTH-vehicle, and ACTH-imipramine-treated groups. Saline control animals administered imipramine simlarly showed longer climbing duration than animals in saline-control and ACTH-imipramine groups. ACTH animals administered imipramine alone displayed significantly lower levels of climbing behavior relative to ACTH-control animals (Fig. 3e). No statistically significant main effect of treatment on *swimming* time was observed, one-way ANOVA, $F(4,50) = 2.221$, $p = 0.0799$ (Fig. 3f). As such no post-hoc tests for swimming were conducted. Refer to tables 1 and 2 for behavioural test descriptives.

Western blot

The amount of total and phosphorylated Akt, mTOR, and GSK3β in the ILPFC at time of euthanasia (30 min following the FST) was quantified using western blotting, corrected for β-actin, and expressed as a ratio to positive control tissue. Descriptives for Western blot ILPFC proteins are provided in supplementary table 3. The Shapiro–Wilk test was significant for the ACTH group co-administered imipramine and lithium for measures of *p*Akt: $W = 0.8204$, $p = 0.0471$; and *p*GSK3β/GSK3β: $W = 0.8215$, $p = 0.0358$, reflecting minor violations of the assumption of normality.

One-way ANOVA demonstrated a significant main effect of treatment for phosphorylated and total Akt and mTOR (Akt: $F(4,47) = 8.275$, $p < 0.0001$; pAkt: $F(4,47) = 7.105$, $p = 0.0001$; mTOR: $F(4,43) = 5.116$, $p = 0.0018$; *p*mTOR: $F(4,47) = 7.187$, $p = 0.0001$). Post-hoc Tukey's HSD tests multiple comparisons revealed several significant group differences as illustrated in Fig. 4. Some variation in group *n* for protein assays occurred as a result of unviable tissue samples (undetectable protein levels). As a result, these group protein data are depicted as scatter plots.

No significant main effect of treatment was observed for *p*GSK3β: $F(4,50) = 0.3817$, $p = 0.82$; or GSK3β: $F(4,47) = 2.535$, $p = 0.0524$. Closer inspection of GSK3β data however, indicated post-tests were appropriate. Post-tests unveiled a

significant reduction in GSK3β for ACTH animals co-administered lithium compared to those that received imipramine alone ($p = 0.034$) (see Fig. 4g).

The Brown–Forsythe test for homogeneity of variance was significant for *p*Akt/Akt: $F(4,44) = 5.841$, $p < 0.001$; *p*mTOR/mTOR, $F(4,48) = 5.442$, $p = 0.001$; and *p*GSK3β/GSK3β: $F(4,42) = 4.1$, $p = 0.007$. Kruskal–Wallis one-way tests indicated significant main effects for pAkt/Akt: $H(4) = 10.94$, $p = 0.027$; *p*mTOR/mTOR: $H(4) = 16.75$, $p = 0.002$; but not *p*GSK3β/GSK3β: $H(4) = 5.055$, $p = 0.282$. Dunn's post-tests indicated significant group differences for *p*Akt/Akt and *p*mTOR/mTOR, shown in Fig. 4c and f, respectively.

PBMC insulin challenge

One-way tests were used to evaluate the effects of treatment on change (Δ) in levels of mTOR, and *p*mTOR in PBMCs between baseline (t_0) and after 5 min of insulin stimulation (t_5). One-way ANOVA unveiled a statistically significant main effect of treatment for ΔmTOR, $F(4,39) = 7.863$, $p < .0001$. The Brown–Forsythe test was found to be significant for Δ*p*mTOR, $F(4, 41) = 3.019$, $p = 0.028$. A Kruskal–Wallis one-way test indicated a significant main effect of treatment on Δ*p*mTOR, $H(4) = 14.46$, $p = 0.006$. Subsequent group comparisons utilizing Tukey's HSD tests and Dunn's tests unveiled several significant group differences shown in Fig. 5a, b).

Linear regression was subsequently performed on PBMC data to assess the relationship between immobility duration and the reported protein differences and ratios following 5 min of insulin stimulation. For lithium-treated ACTH animals, slope regression tests for both ΔmTOR and Δ*p*mTOR vs. immobility duration were significantly non-zero, $F(1,8) = 30.52$, $p = 0.0006$ and $F(1,8) = 5.521$, $p = 0.0467$, respectively. Immobility duration shared a strong correlation with both ΔmTOR ($r^2 = 0.792$) and Δ*p*mTOR ($r^2 = 0.408$). Graphical representation of this relationship is shown in Fig. 5c, d. For mean PBMC protein levels, and slope regression data for all treatment groups, see tables 3 and 4.

Linear regression was also performed on PBMC data to assess the relationship between protein levels in the ILPFC following exposure to the FST and in PBMCs post-insulin challenge across all animals. Significant positive correlations were observed between *p*mTOR/mTOR levels in the ILPFC and ΔmTOR ($r^2 = 0.351$) and Δ*p*mTOR ($r^2 = -0.314$) in PBMCs.

Discussion

Lithium can improve or augment antidepressant response in some individuals who are otherwise resistant; reportedly reducing recurrence of depressive episodes, lowering suicidality and improving rates of remission[32,33]. Adjunctive lithium treatment is a typical "next-step" after switching to a non-SSRI antidepressant to cater for non-response[4,32]; however, it is only effective for about half of patients with treatment-resistant depression[13,14]. To date, no clear predictive markers for resistance or response to lithium treatment have been uncovered. In this study, we reaffirmed that animals pretreated with ACTH for 14 days are resistant to the acute effects of imipramine (10 mg/kg) in the FST. We also affirmed that acute co-administration of lithium (100 mg/kg) alongside imipramine (10 mg/kg) in these animals rescues the typical antidepressant-like effects of imipramine in the FST. Given the impact of lithium on cellular metabolic processes mediated, in part, by the insulin signalling pathway, we further explored the direct impact of lithium augmentation on insulin signalling in brain and blood tissue.

Behavioural findings

Locomotor effects associated with lithium co-administration

As previously reported, neither ACTH pre-treatment nor imipramine antidepressant administration significantly altered locomotor behavior[6,9]. However, an effect of lithium on locomotor behaviour in the OFT was observed. ACTH animals treated with the imipramine–lithium combination showed an overall reduction in distance and mean velocity of travel when compared to both vehicle control-treated saline and ACTH animals. This is less surprising when considering lithium's anti-manic and mood stabilizing clinical outcomes. Within this context, lithium has been previously described to attenuate exploratory and locomotor-associated behaviours in rodents[34,35]. Notably, previous studies did not directly report on locomotor effects for lithium treatment in their original study undertaken in ACTH pretreated rats[6].

ACTH animals that received imipramine in conjunction with lithium also exhibited lower time spent in the central region of the OFT, relative to other groups. Typically, low center duration is considered indicative of an anxious phenotype[36]. We propose that this somewhat unexpected anxiety-like effect results instead from the overall impact of lithium on mobility. That is, the reduced time spent in the central region duration may simply reflect a reduction in exploratory behaviour corresponding to the aforementioned reduction in locomotor behaviour. As well, it is possible that the

acute lithium injection may have caused some discomfort through an adverse side-effect, affecting these behaviours (for example, a negative gastrointestinal effect)[37]; however, this is unlikely given the observed increased activity observed in the FST.

Lithium restores antidepressant response to imipramine in ACTH pre-treated animals

Using the FST we first sought to affirm that ACTH pre-treatment blocked the antidepressant-like immobility reducing effects of imipramine, as reported previously[5–9]. Imipramine has been observed to reliably reduce immobility in healthy control animals[30], including those used in our previous research[9]. Consistent with this, we found that administration of imipramine elicited an antidepressant-like effect in control animals, observed via significantly reduced immobility time in the FST compared to those administered vehicle saline. In contrast, imipramine did not reduce immobility time in ACTH-treated animals. Instead, these animals displayed significantly increased immobility time, coupled with a corresponding decrease in climbing time, compared to ACTH-treated animals administered vehicle saline. When lithium was co-administered with imipramine, a robust antidepressant-like effect was observed in ACTH-treated animals. Specifically, these animals exhibited significantly shorter immobility duration and longer climbing duration than ACTH animals that received imipramine alone, or vehicle saline.

Together, these behavioral data suggest that, consistent with previous findings, ACTH pre-treatment adversely affects the efficacy of imipramine in the FST, promoting antidepressant non-response[9]. Furthermore, these results indicate that lithium co-administration may rescue the typical antidepressant actions of imipramine in ACTH pre-treated animals[6]. Biological correlates for the observed behavioral response may offer some insight into the mechanisms of both ACTH-induced antidepressant treatment resistance, and restoration of therapeutic efficacy via lithium augmentation; this is explored in the next section.

Insulin signalling upregulated by lithium in ACTH pre-treated rats

ILPFC insulin signalling upregulated in ACTH pre-treated rats administered adjunctive lithium

Here we assessed levels of insulin signalling proteins: mTOR, Akt, and GSK3β, as well as *p*Akt, *p*GSK3β, and *p*mTOR in the ILPFC, using Western blot. The ILPFC *p*Akt/Akt ratio was significantly elevated in lithium-treated ACTH animals. ILPFC *p*mTOR/mTOR ratios were significantly lower in ACTH animals administered vehicle

saline, and were significantly elevated again in animals co-administered lithium. *p*mTOR and *p*Akt levels were significantly reduced in the ILPFC of ACTH pretreated animals administered imipramine alone; while animals co-administered lithium had similar levels of activation to the control saline groups.

ILPFC GSK3β levels were significantly lower in ACTH animals co-administered lithium and imipramine compared to those that received imipramine alone. While *p*GSK3β levels and pGSK3β/GSK3β ratio were very slightly elevated in lithium-treated animals, this effect was non-significant. As such, the pattern of increased phosphorylation (and deactivation) of GSK3β consistent with the purported mechanisms of lithium, was less evident. It is interesting that fewer significant differences or patterns emerged for GSK3β protein levels across treatment conditions, despite previous research establishing that lithium inhibits GSK3β activity, both directly and indirectly[15]. It is important to note that lithium may not be acting exclusively via GSK3β given inhibition of inositol monophosphatase (IMPase) is an additional putative mechanism of lithium action that occurs independent of insulin signalling, yet contributes directly to therapeutic response[38]. Further, other brain regions may show differential insulin signalling responses to lithium with respect to FST outcomes. For example, imipramine co-administered with lithium was previously reported to normalize cell proliferation in the hippocampus of ACTH pretreated animals[39,40]. This effect was similarly associated with FST antidepressant efficacy, yet the effect of lithium on hippocampal GSK3 and/or IMPase function, and its contribution to these effects remains to be determined.

Nevertheless, lithium is known to inhibit GSK3β both directly (via competition with Mg^{2+}), and indirectly via Akt[41], promoting mTOR activation[42]. The observed increased levels of mTOR and Akt phosphorylation in the ILPFC of lithium-treated animals are indeed consistent with insulin signaling pathway activation[16], and both Akt and mTOR activation have been implicated in treatment response previously[19,25] making them important candidates for further investigation.

Insulin-evoked PBMCs mTOR activation is upregulated by lithium and correlates with antidepressant response in ACTH pre-treated rats

In our final experiment, we isolated PBMCs from each animal to assess if this accessible peripheral tissue could be used as a proxy marker for predicting early treatment response to lithium, with specific respect to lithium's augmentation of cellular responses to insulin. mTOR activity is affected by various cellular inputs,

ranging from whether certain amino acids and growth factors are present, to the energy and nutrient status of the cell[38]. Taking measurements at baseline (t_0) and after 5 min exposure to insulin (t_5), we investigated the effects of treatment condition on levels of mTOR and *p*mTOR in PBMCs.

The observed patterns of protein levels across treatments were somewhat complex. We found significant differences in ΔmTOR in the PBMCs of ACTH-vehicle animals following 5 min of insulin challenge, in contrast to other treatment groups. Change in *p*mTOR levels was significantly lower in the PBMCs of ACTH animals co-treated with imipramine and lithium relative to ACTH animals receiving only imipramine, suggesting possible normalization. Exploring this relationship further, as shown in Fig. 5c, d, linear regression unveiled significant large positive correlations with immobility time and change in mTOR levels. Moderate to large negative correlations were also observed between immobility duration and *p*mTOR. Interestingly, the observed relationship was limited to those animals that received lithium, and not in those that received imipramine alone.

mTOR plays a critical role in integrating intracellular and extracellular signals to regulate cellular metabolism, growth proliferation, and survival[21], all of which are critical for establishment of effective antidepressant responses and modulated in part by insulin action in the brain. We herein propose that quantification of functional mTOR response to insulin, following acute exposure to lithium treatment, may be one path towards identifying individuals with increased likelihood of achieving a therapeutic response. Given the role that mTOR plays as a cellular sensor responding to energy and stress, modulating synaptogenesis and apoptosis, it is well positioned to serve as an ideal candidate for the evaluation of molecular responses to pharmacotherapies, such as lithium at the cellular level.

Concluding remarks

In summary and consistent with previous studies, ACTH pre-treatment was found to block the immobility reducing effects of imipramine (10 mg/kg) in the FST. The effects of imipramine were rescued by the co-administration of lithium (100 mg/kg) in these animals. mTOR and Akt phosphorylation ratios were increased in the ILPFC of lithium-treated animals. Insulin stimulation (10 mg/mL for 5 min) of isolated PBMCs yielded some interesting differences in protein response. ACTH pretreated animals that received imipramine exhibited increased total *p*mTOR activation following insulin challenge. Augmentation with lithium normalized *p*mTOR

levels, distinguishing responsive lithium-treated animals from those resistant to imipramine. Moreover, immobility duration was highly correlated with insulin-stimulated mTOR and *p*mTOR levels in lithium-treated animals' PBMCs. We propose that PBMC insulin challenge may be a useful probe for predicting antidepressant response to lithium, and potentially other therapies.

As previously discussed, initial research utilizing this model has consistently reported that chronic administration of ACTH renders animals resistant to the therapeutic effects of tricyclic antidepressants (such as imipramine and desipramine) in the FST[5–9]. While some studies have reported 'depressive-like' effects of ACTH treatment in this model (e.g.[8]), the majority, this study included, reported no significant increase in immobility duration following ACTH treatment alone[5–7,9]. In this context, the FST should be viewed as a tool for probing antidepressant-like responses, rather than a model of depressive behaviour per se. While ACTH model does not exhibit high face validity for depression-like behaviour, in contrast to other paradigms, its use of the established predictive validity of the FST makes it useful for assessing antidepressant efficacy. As such, the ACTH model can be utilized to focus specifically on resistance and response to antidepressants.

Table 1.

Descriptive statistics for OFT behaviors

	Treatment														
	Saline						ACTH								
	Saline			Imipramine			Saline			Imipramine			Imipramine+Lithium		
Behavior	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*
Distance	27.31	0.34	11	24.96	1.56	11	28.16	0.92	12	24.66	0.73	11	19.36	1.96	10
Velocity	75.92	0.92	11	69.48	4.36	11	78.28	2.56	12	68.66	2.05	11	53.79	5.45	10
Center duration	40.59	2.09	11	44.30	5.17	11	39.48	3.82	12	43.58	2.18	11	24.35	2.41	10

Table 2.

Descriptive statistics for FST behaviors

	Treatment											
	Saline						ACTH					
	Saline			Imipramine			Saline			Imipramine			Imipramine+Lithium		
Behavior	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*
Immobility	74.97	7.37	10	40.86	4.58	11	66.69	7.37	12	95.39	8.16	11	26.15	7.05	10
Climbing	116.50	7.02	11	152.90	6.96	11	135.80	7.84	12	103.50	5.18	11	183.70	9.46	10
Swimming	53.15	5.46	11	46.20	6.31	11	37.47	6.38	12	41.13	5.08	11	30.11	5.04	10

Table 3.

Mean levels of insulin signaling pathway proteins in lLPFC

	Treatment														
	Saline						ACTH								
	Saline			Imipramine			Saline			Imipramine			Imipramine+Lithium		
Protein	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*
Akt	0.99	0.07	11	1.11	0.03	9	1.25	0.05	11	0.94	0.05	11	0.79	0.08	10
*p*Akt	1.40	0.06	11	1.43	0.06	11	1.35	0.06	12	1.06	0.05	10	1.47	0.05	8
*p*Akt/Akt	1.31	0.10	9	1.27	0.08	9	1.09	0.06	11	1.13	0.07	10	1.92	0.26	10
mTOR	0.89	0.08	10	0.87	0.10	9	1.16	0.11	12	0.88	0.03	9	0.62	0.06	8
*p*mTOR	1.20	0.08	11	1.38	0.05	10	1.02	0.09	12	0.84	0.03	9	1.15	0.08	10
*p*mTOR/mTOR	1.39	0.13	11	1.45	0.17	11	0.85	0.05	11	0.95	0.07	10	1.71	0.27	10
GSK3β	1.50	0.09	11	1.49	0.11	11	1.45	0.10	12	1.56	0.09	11	1.12	0.02	7
*p*GSK3β	1.03	0.09	11	1.04	0.10	11	0.99	0.09	12	0.94	0.09	11	1.12	0.15	10
*p*GSK3β/GSK3β	0.65	0.04	10	0.71	0.07	11	0.59	0.02	9	0.57	0.03	8	0.89	0.16	9

Table 4.

Mean level of mTOR signaling proteins following PSMC insulin challenge.

	Treatment											
	Saline						ACTH					
	Saline			Imipramine			Saline			Imipramine			Imipramine+Lithium		
Protein	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*	*mean*	*S.E.M*	*n*
mTOR	0.0021	0.0008	9	0.0017	0.0018	8	-0.0059	0.0014	10	0.0018	0.0016	9	-0.0029	0.0005	8
pmTOR	0.0035	0.0017	9	-0.0014	0.0029	8	-0.0018	0.0013	10	0.0040	0.0008	9	-0.0027	0.0013	10

Table 5.

Linear regression for immobility duration versus mTOR signaling proteins following PBMC insulin challenge.

		mTOR				pmTOR			
	Treatment	r^2	F	p		r^2	F	p	
Saline									
	Saline	0.0068	$F_{(1,7)}$=0.0500	0.8329		0.0368	$F_{(1,7)}$=0.2673	0.6211	
	Imipramine	0.0432	$F_{(1,6)}$=0.2707	0.6215		0.0597	$F_{(1,6)}$=0.3812	0.5596	
ACTH									
	Saline	0.0178	$F_{(1,8)}$=0.1415	0.7132		0.0945	$F_{(1,8)}$=0.8351	0.3875	
	Imipramine	0.0712	$F_{(1,7)}$=0.5366	0.4877		0.0756	$F_{(1,7)}$=0.5726	0.4739	
	Imipramine+Lithium	0.7923	$F_{(1,8)}$=30.52	0.0006	***	0.4083	$F_{(1,8)}$=5.521	0.0467	*

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EXAMPLE 2

Introduction

As many as a third of all patients diagnosed with depression prove to be resistant to available antidepressant medication following up to a year of four sequential 12-week treatment trials (1–4). Patients with symptoms that fail to improve in response to at least two antidepressant trials of adequate dose and duration are referred to as having treatment-resistant depression (TRD), and often suffer a particularly heavy disease burden that includes years spent dealing with severe, non-remitting symptoms (2). Currently, we understand very little about why some patients respond to antidepressants while others continue to experience symptoms or relapse after a period of time, even when continuing to receive treatment (3, 5). The so-called "first-line" antidepressants prescribed as part of routine standard of care for depression target the monoaminergic system and include selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), norepinephrine reuptake inhibitors (NRIs), and tricyclic antidepressants (TCAs), among others (6, 7).

In contrast, emerging therapies for TRD target mechanisms beyond the monoaminergic system, including the glutamatergic system, which in turn rapidly modulates downstream neurotrophic and metabolic processes (8–11). In particular, the discovery and use of ketamine, an n-methyl-d-aspartate (NMDA) glutamate receptor antagonist, as an effective and fast-acting treatment for TRD is considered one of the most significant paradigm-shifting developments in psychiatry in recent years (12–16). Multiple randomized trials have shown that ketamine at sub-anesthetic doses is an efficient, rapid-acting antidepressant for a subset of patients with unipolar or bipolar TRD, and that it has distinct antisuicidal and antihedonic effects (17–19). However, the mechanisms moderating response to ketamine are unclear. Clinical and preclinical studies have systematically provided essential insights into ketamine's rapid antidepressant mechanisms of action, yet there remains some controversy as to the role of crucial biological factors as potential moderators of this response (5, 15). Insight into these mechanisms may reveal critical information about the underlying pathophysiology of TRD, and help to identify the individuals for whom ketamine treatment may be most effective (8, 13).

Early work from rodent studies indicates that ketamine rapidly engages cellular growth pathways to promote dendritic growth and synapse formation (9, 10, 20). This

rapid promotion of dendritic spine formation may explain how ketamine can rapidly alleviate depressive symptoms within hours of administration (20–22). In these preclinical studies, ketamine was shown to activate mammalian target of rapamycin (mTOR), a signaling molecule that functions as a lynchpin in the regulation of cellular metabolism and cell growth (21, 23, 24). Activating mTOR via phosphorylation initiates a signaling cascade that results in the inhibition of GSK3 (25) and subsequent promotion of neural and synaptic plasticity (26). Other drugs with known efficacy in TRD also activate mTOR, including lithium or metabotropic glutamate receptor 2/3 (mGLUR2/3) antagonists (9, 25–27), whereas first-line antidepressants, such as SSRIs and TCAs, do not (7, 21). However, to date, there have been no studies that link ketamine's metabolic regulatory mechanism to its antidepressant effects, nor do we understand why some individuals with TRD improve in response to ketamine when many other treatment attempts fail.

One of the challenges of studying depression, and TRD in particular, is identifying animal models and related preclinical assays that recapitulate the critical features of human disease. In an attempt to better understand the mechanisms governing antidepressant resistance and response, we have developed a rodent model of TRD in which chronic adrenocorticotropic hormone (ACTH) exposure induces hypothalamic-pituitary-adrenal (HPA) axis disruption and generates animals that are non-responsive to TCA treatment (23, 28–30). Further, we know that HPA axis disruption is linked to TRD in human patients (31–35). Thus, this model is well-suited to studying the mechanisms of action of treatment resistance and response in TRD, including the possibility that ketamine engages a distinct metabolic mechanism within the context of TRD.

Utilizing this rodent model together with complementary peripheral blood mononuclear cell (PBMC) samples obtained during a clinical trial of ketamine in TRD patients in order to probe peripheral insulin and protein signaling activities, we conducted a translational study of ketamine's antidepressant mechanism of action. Using the ACTH pretreatment model of TCA resistance, we have previously shown that roughly half of pretreated (i.e., TCA-resistant) animals display improvement in depression-like behaviors in response to ketamine, consistent with reports for human TRD patients (5, 7, 23). Similar response rates to ketamine are observed in non-ACTH-treated animals. Here, we further investigate the role of mTOR and insulin signaling in

response to ketamine among preclinical samples and human TRD patients in order to explore how ketamine impacts underlying pathophysiology.

Materials and Methods

Animals

Male and female Wistar rats were used in this study, weighing 150-200g at the time of testing. Animals were housed either individually or in pairs as described below. Rooms were temperature-controlled (20-22°C) with a 12h light-dark cycle (lights: on 06:30; off 18:30). Food and water were available *ad libitum*. All procedures were carried out in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Mayo Clinic. Animal use was minimized according to animal welfare guidelines.

Treatments

A modified version of the ACTH treatment protocol described previously was used with minor adjustments to conditions and treatments (23). Male animals were randomly assigned to be administered either ACTH or saline (0.9%) for 15 days. Following the ACTH treatment protocol, a subset of animals (n=48) underwent behavioral testing. An additional cohort of male and female (n=100) Wistar rats were administered ACTH for 15 days, then randomly assigned to one of four treatment conditions: ketamine (10mg/kg), metformin (200mg/kg), ketamine-metformin co-treatment, or saline (0.9%). Animals received ketamine and metformin treatments on days 17 and 18 only.

Behavioral Testing

In the first cohort, animals (N=48) were randomly assigned to 1) remain treatment naïve, 2) social isolation and ACTH administration (100μg/d) for 14 days, or 3) social isolation and control saline (0.9%) administration for 14 days. All animals other than the treatment naïve group were randomly assigned to receive either acute sub-anesthetic ketamine hydrochloride (10 mg/kg) or control vehicle saline (0.9%) i.p. 60-min prior to the behavioral tests. Animals partook in a 15-min initial forced swim exposure on day 14 of the treatment protocol. On day 15, a final 6-min forced swim test (FST) was conducted and animals were humanely euthanized by anesthetic overdose (0.7cc FatalPlus®; Vortech Pharmaceuticals, Dearborn, MI, USA) 30-min after testing. Brains were harvested immediately following euthanasia and frozen on dry ice and then

stored at -80°C until dissection. Buffy coat and serum were isolated from cardiac blood by centrifuging at 3300rpm for 10-min and then stored at -80°C until analysis.

In the second cohort examining metformin synergistic effects on ketamine, baseline blood glucose measurements via tail vein and non-ACTH drug treatments were performed on day 17 before drug treatments and on day 18 following drug treatments and behavioral tests. Immediately following ketamine and metformin treatment, rats were monitored in their home cages for one hour for observation of adverse effects. One hour following drug administration on day 18, rodents partook in the FST. Thirty minutes following the FST, animals were euthanized with an overdose of FatalPlus® (0.7cc; Vortech Pharmaceuticals, Dearborn, MI, USA), and brains were extracted and preserved as above. Cardiac blood for each animal was divided, with half of the blood frozen on dry ice and the other half immediately prepared for PBMC isolation and insulin assay (described below).

Drugs

The drugs used in this study included: adrenocorticotropic hormone-(1-24) (ACTH) (AnaSpec, San Jose, CA, USA), 100μg/day dissolved in distilled water, Ketaset® ketamine hydrochloride (Fort Dodge Animal Health, IA, USA) 10 mg/kg diluted in 0.9% saline, metformin (200mg/kg; Sigma-Aldrich, St. Louis, MO, USA); control vehicle 0.9% saline (Fisher Healthcare, Hanover Park, Illinois, USA), and FatalPlus® (Vortech Pharmaceuticals, Dearborn, MI, USA), (constituents: pentobarbital sodium 390 mg/ml; propylene glycol 0.01 mg/ml; ethyl alcohol 0.29 mg/ml; benzyl alcohol (preservative) 0.20 mg/ml) 0.70 cc. All drugs were delivered via intraperitoneal (i.p.) injection.

Human Subjects

Twelve adults (18-64 years of age) with treatment-resistant unipolar or bipolar major depression were administered intravenous (IV) ketamine (0.5 mg/kg, over 100 minutes) in two phases—an acute phase and a continuation phase. During the acute phase, subjects were given IV ketamine thrice weekly for up to 2 weeks. Treatment-resistance was defined as having failed to respond to at least two adequate therapeutic trials of antidepressive treatments during the current depressive episode, including conventional antidepressants, mood stabilizers, and electroconvulsive therapy. Participants had a 9-item Patient Health Questionnaire (PHQ-9) suicide item (item 9) score of $\geq$1 at the screening visit. Psychotic symptoms, chronic depression (duration of the current depressive episode >2 years), current alcohol or non-nicotine substance use

disorder (unless remitted for ≥12 months), positive urine drug screen, history of intellectual disorder or developmental delay, medical instability, pregnancy, and involuntary psychiatric hospitalization were exclusionary. Blood pressure was measured every 15 minutes, while heart rate, ECG, and pulse oximetry were continuously monitored during ketamine infusions and through 60 minutes post-infusion. Depressive symptoms were assessed at baseline before infusion, at the end of infusions (100 minutes), and 24 hours post-infusion using the Montgomery Åsberg Depression Rating Scale (MÅDRS). Remission was defined as achieving a MÅDRS total score of ≤9, measured 24 hours post-infusion. Remission could occur after any of the six acute-phase infusions, at which point remitted subjects were given once-weekly IV ketamine (same dose and infusion rate) for four weeks during a continuation phase of treatment. Subjects who did not achieve remission were not offered weekly continuation infusions of ketamine. All study participants provided written informed consent. The clinical effectiveness results of this trial have been published (36).

Western blotting

Brains were dissected on a ThermalTray™ LP (BioCision, Mill Valley CA, USA) maintained at -20°C using dry ice. The infralimbic (IL) prefrontal cortex (PFC) and prelimbic (PL) PFC were identified using a rodent brain atlas (37) and stored at -80°C until use. Tissue samples were lysed in radioimmunoprecipitation assay (RiPA) lysis buffer for western blotting. Protein concentration was determined by Bradford protein assay. Equal amounts of protein lysate of the dissected ILPFC, PLPFC, or WBCs were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to polyvinylidene fluoride (PVDF) membrane (Immobilon-P). Membranes were blocked for two hours with tris buffered saline solution with the detergent Tween® 20 (TBST) containing 5% milk (or 5% bovine serum albumin (BSA) for phosphorylated antibodies) before being incubated at 4°C with primary antibodies overnight. Antibodies used included: total Akt (pan), phospho-Akt (Ser473), GSK3α/β, phospho-GSK3α/β (Ser21/9), total mTOR, phospho-mTOR (Ser2448) (Cell Signaling Technology, Beverley, MA, USA); total TrkB, phospho-TrkB (Tyr496), BDNF (Santa Cruz Biotechnology, Santa Cruz, CA, USA); total β-actin (Sigma-Aldrich, St Louis, MO, USA). Blots were washed the following day with TBST and incubated with anti-rabbit HRP-linked secondary antibody (Cell Signaling Technology) for 1 hour. Blots were again washed with TBST and exposed to enhanced chemiluminescence (ECL) substrate (Amersham Biosciences/GE Healthcare,

Piscataway, NJ, USA). Band detection and densitometric analysis were conducted using a Bio-Rad ChemiDoc™ imaging system. Readings were normalized to β-Actin.

ELISA

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to determine insulin (ALPCO, 80-INSRT-E01, E10) concentrations present in the plasma samples. All procedures were carried out per manufacturer instructions.

PBMC isolation

Blood samples were collected immediately before and two hours following first ketamine infusion. Whole blood was mixed with an equivalent volume of Phosphate Buffered Saline (PBS) and carefully layered over Histopaque 1077 (Sigma-Aldrich), then centrifuged at 400xg for 30 minutes at room temperature. The interface was removed (containing PBMCs) and washed with PBS. Cells were slow frozen at -80°C until insulin stimulation assays and western blot analysis were performed.

Ex vivo insulin challenge assays:

PBMC cells were isolated and stimulated with insulin, as previously described in Example 1. In brief, Dulbecco's phosphate-buffered saline (DPBS) (1–4 mL) (Gibco Life Technologies, Rockville, MD, USA) was added to whole blood samples according to sample volume (2–8 mL) and homogenized. The blood/DPBS mixture was added to a polypropylene tube (BD Falcon™: BD Biosciences, Bedford, MA, USA) with 2mL of Histopaque® medium (Sigma-Aldrich) and centrifuged at 400×*g* to facilitate removal of the PBMC layer.

The PBMCs were incubated overnight in T25 tissue culture flasks (BD Biosciences) filled with 5mL Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco Life Technologies) containing 10% fetal bovine serum (FBS), l-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and penicillin/streptomycin solution. Cells were harvested the following day and centrifuged at 1200rpm for 5-min at 4 °C. Media was aspirated to isolate a pellet at the base of the tube. Cells were resuspended in 5% dimethyl sulfoxide (DMSO) (ATTC, Manassas, VA, USA) media and dispensed in 1mL aliquots into cryogenic vials (Corning Inc., Corning, NY, USA) at −80 °C.

PBMC Insulin challenge

PBMCs were thawed, centrifuged, and re-suspended in media without FBS. Cells were then divided and incubated in plates devoid of growth factors to deplete residual insulin, where they were stimulated with insulin (10mg/mL) for 5-min (37 °C;

5% CO_2). Cells were then centrifuged and lysed using 50mL RIPA lysis buffer. Cellular debris was removed via centrifugation (14,000 rpm; 4 °C; 10 min) and the lysate was dispensed into a new tube. ELISA kits were used to examine mTOR (Total) and *p*mTOR (Ser^{2448}) (Cell Signaling).

Statistical Analyses

One or two-way multivariate ANOVAs were conducted for behavioral tests. For statistical robustness, the more conservative Pillai's Trace was interpreted for all multivariate tests to account for any variance-covariance violations observed in Box's *M* test. Levene's test was used to evaluate the assumptions of homogeneity of variance. These assumptions were met unless otherwise specified. Statistical analyses were calculated using the *IBM SPSS Statistics 21.0* software package and *GraphPad Prism 8.2*.

Results

Behavioral responses to ketamine are evident among a subset of ACTH-pretreated animals

Using the FST as an assay of ketamine efficacy as quantified by a reduction in immobility behavior, we tested the rate of response to ketamine among ACTH-pretreated and saline-pretreated (control) animals. We found that in both cohorts, roughly 50% responded to ketamine treatment with an increase in active coping behaviors. These findings were thus consistent with clinical data (below).

Open Field

Two-way multivariate ANOVA was conducted for the effects of treatment, drug, and the interaction effect of treatment × drug on locomotor behaviors in the OFT. The assumption of variance-covariance was violated (Box's M=56.357, $F(18,4071)=2.637$, $p<0.001$). As a result, the more conservative Pillai's Trace was interpreted for statistical robustness.

A statistically significant main effect of drug administration was observed, indicating univariate tests for the effect of ketamine were appropriate, $F(3,34)=3.982$, $p=0.016$; Pillai's Trace=0.260, $\eta p2=0.260$. The observed main effect of treatment on behaviors in the OFT was not statistically significant, $F(3,34)=0.990$, $p=0.409$; Pillai's Trace=0.080, $\eta p2=0.080$. No significant interaction effect of treatment × drug was observed, $F(3,34)=0.618$, $p=0.608$ Pillai's Trace=0.052, $\eta p2=0.052$.

Univariate analyses yielded no significant difference in total distance traveled (saline-saline: M=28.8867, SD=4.59496, n=9, SEM=1.5317; saline-ketamine: M=25.6978, SD=4.61216, n=9, SEM=1.5374; ACTH-saline: M=26.8667, SD=2.86316, n=12, SEM=0.8265; ACTH-ketamine: M=26.1270, SD=3.72969, n=10, SEM=1.1794), F(1,36)=2.471, p=0.125, ηp2=0.064.

A significant difference was observed in the average velocity of animals administered ketamine (saline-ketamine: M=71.8333, SD=13.05469, n=9, SEM=4.3516; ACTH-ketamine: M=71.4000, SD=10.76445, n=10, SEM=3.4040) versus controls (saline-saline: M=83.4222, SD=12.36404, n=9, SEM=4.1213; ACTH-saline: M=76.6167, SD=7.88057, n=12, SEM=2.1797), F(1,36)=5.814, p=0.021, ηp2=0.139.

No significant overall difference was observed for duration of time spent in the center of the open field arena (saline-saline: M=25.9544, SD=21.1814, n=9, SEM=7.0605; saline-ketamine: M=26.8522, SD=12.43154, n=9, SEM=4.1435; ACTH-saline: M=17.776, SD=16.6316, n=12, SEM=4.8011; ACTH-ketamine: M=30.7540, SD=12.02759, n=10, SEM=3.8035), F(1,36)=1.412, p=0.242, ηp2=0.038. Data perusal, however, suggested post hoc tests comparing center duration of the two ACTH-pretreated groups were appropriate. Welch's t-test confirmed a significant difference between the two ACTH-pretreated groups, where animals in the ACTH-saline group spent less time in the central region of the OFT arena compared to ACTH-ketamine animals, t(22)=2.118, p=0.0459, two-tailed, R2=0.1724.

Forced Swim Test

A one-way ANOVA was conducted to explore the effects of ketamine on FST coping behaviors 60-min following a single exposure to ketamine (see figure 6a-d). A Brown-Forsythe test demonstrated no significant differences in standard deviations across groups for each respective behavioral assay. A statistically significant main effect of ketamine was observed on total immobility, $F(6,41)=8.997$, $p<0.0001$; latency to immobility $F(6,40)=10.04$, $p<0.0001$; and swimming $F(6,41)=8.230$, $p<0.0001$. No significant difference was observed for climbing behavior across treatment groups.

Immobility

Tukey's multiple comparisons test demonstrated that social isolation with ACTH treatment (n=8) elicited significantly greater immobility in the FST relative to the non-stress condition (n=8) (mean difference ± SEM: 46.38±13.45; $p<0.05$). No significant difference was observed for saline-treated, socially isolated animals (n=8)

relative to stress naïve animals (n=8) (mean difference ± SEM: 33.25±13.45). Ketamine administration significantly reduced immobility time in a subset of saline pre-treated animals (n=6) comparative to those administered control vehicle saline (n=8) (mean difference ± SEM: 59.71±14.52; $p<0.01$) and those non-responsive to ketamine (n=6) (mean difference ± SEM: -85.17±15.53; $p<0.0001$). No significant difference was observed between saline-treated animals non-responsive to ketamine (n=6) and animals administered vehicle saline (n=8) (mean difference ± SEM: 25.46±14.53). Similarly, ketamine administration significantly reduced immobility time in a subset of ACTH pre-treated animals (n=6) comparative to animals administered control vehicle saline (n=8) (mean difference ± SEM: 47.83±14.53; $p<0.05$) and ACTH pre-treated non-responsive to ketamine (n=6) (mean difference ± SEM: -49.33±15.53; $p<0.05$). No difference was observed between non-responsive ACTH-treated animals (n=6) and animals administered control vehicle saline (n=8) (mean difference ± SEM: 1.50±14.53). In addition to this, saline pre-treated animals responsive to ketamine (n=6) spent significantly less time immobile relative to ACTH-treated controls (n=8) (mean difference ± SEM: 72.83±14.53; $p<0.001$) and ketamine non-responsive ACTH-treated animals (n=6) (mean difference ± SEM: 74.33±15.53; $p<0.001$). Likewise, ACTH-treated ketamine responsive animals (n=6) were significantly more mobile than ketamine non-responsive saline-treated animals (n=6) (mean difference ± SEM: 60.17±15.53; $p<0.01$). Both saline-treated and ACTH-treated animals non-responsive to ketamine (n=6) demonstrated significantly greater immobility relative to stress naïve animals (n=8) (mean difference ± SEM: 58.71±14.53, $p<0.01$; and 47.88±14.53; $p<0.05$)

Latency to Immobility

Tukey's multiple comparisons test demonstrated group differences in total immobility were mirrored in latency to immobility, a complementary measure of antidepressant response. Stress naïve animals took significantly longer to become immobile relative to saline-treated controls (mean difference ± SEM: 65.75±12.74; $p<0.01$); saline-treated ketamine non-responsive animals (mean difference ± SEM: 67.67±13.77; $p<0.01$); ACTH-treated controls (mean difference ± SEM: 67.75±12.75; $p<0.001$); and ACTH-treated ketamine non-responsive animals (mean difference ± SEM: 84.33±13.77; $p<0.0001$). Saline-treated animals responsive to ketamine likewise demonstrated greater latency to immobility relative to saline-treated controls (mean difference ± SEM: 75.95±13.77; $p<0.01$); saline-treated non-responders (mean

difference ± SEM: 77.87±14.72; p<0.01); ACTH-treated controls (mean difference ± SEM: 77.95±13.77; p<0.001); and ACTH-treated non-responders (mean difference ± SEM: 94.53±14.72; p<0.0001). ACTH-treated ketamine responders demonstrated significantly longer duration to immobility relative to ACTH-treated non-responders (mean difference ± SEM: 54.67±14.72; p<0.05) and non-significant increase relative to ACTH-treated controls (mean difference ± SEM: 38.08±13.77; p<0.08).

Swimming

Tukey's multiple comparisons test demonstrated group differences in total time spent swimming were significantly greater among stress naïve animals relative to saline-treated ketamine non-responsive animals (mean difference ± SEM: 58.58±13.77; p<0.01); ACTH-treated controls (mean difference ± SEM: 47.88±12.75; p<0.01); and ACTH-treated ketamine non-responsive animals (mean difference ± SEM: 45.58±13.77; p<0.05). Saline-treated ketamine-responsive animals demonstrated significantly more swimming behavior relative to saline-treated controls (mean difference ± SEM: 48.54±13.77; p<0.05); saline-treated non-responders (mean difference ± SEM: 72.50±14.72; p<0.001); ACTH-treated controls (mean difference ± SEM: 61.79±13.77; p<0.01); and ACTH-treated non-responders (mean difference ± SEM: 59.50±14.72; p<0.01). ACTH-treated ketamine responders also demonstrated significantly more time swimming relative to ACTH-treated non-responders (mean difference ± SEM: 45.67±14.72; p<0.05); ACTH-treated controls (mean difference ± SEM: 47.96±13.77; p<0.05); and saline-treated non-responders (mean difference ± SEM: 58.67±14.72; p<0.01).

Ketamine moderates protein expression in the prefrontal cortex and in PBMCs

One-way ANOVA and subsequent Brown-Forsythe tests were conducted to examine the effects of ketamine on protein expression in the ILPFC, PLPFC, and in PBMC buffy coats following exposure to ketamine (see figure 8a-c). In the ILPFC, statistically significant main effects of treatment were found when examining mTOR, $F(5,42)=6.023$, $p=0.0034$; phosphorylated (p)mTOR/mTOR, $F(5,51)=2.401$, $p=0.0411$; Akt, $F(5,46)=3.222$, $p=0.0029$; GSK3α, $F(5,36)=1.314$, $p=0.0302$; pGSK3α, $F(5,38)=15.310$, $p=0.0136$; pGSK3α/GSK3α, $F(5,37)=4.0952$, $p=0.0323$; GSK3ß, $F(5,38)=2.8321$, $p=0.0012$; pGSK3ß (non-significant trend), $F(5,42)=1.245$, $p=0.0813$; and pGSK3ß/GSK3ß, $F(5,38)=3.5811$, $p=0.0288$. In the PLPFC, statistically significant

main effects of treatment were found for total mTOR, F(5,56)=2.255, p=0.0174; pmTOR/mTOR (non-significant trend), F(5,35)=1.640, p=0.0872; GSK3α, F(5,37)=1.643, p=0.0158; pGSK3ß, F(5,41)=3.007, p=0.0101; and pGSK3ß/GSK3ß, F(5,37)=1.870, p=0.0129. In the PBMC buffy coats, statistically significant effects of treatment were found on expression of mTOR, F(5,42)=2.622, p=0.0012; pmTOR, F(5,36)=4.600, p=0.0001; pmTOR/mTOR, F(5, 5)=1.519, p=0.0092; GSK3α, F(5,38)=3.941, p=0.0416; pGSK3α, F(5,38)=15.310, p=0.0136; pGSK3α/GSK3α, F(5,50)=8.653, p=0.0021.

Infralimbic prefrontal cortex region

Tukey's multiple comparisons revealed that ACTH-ketamine non-responders (n=6) had significantly lower expression of total mTOR compared to ACTH-ketamine responders (n=6) (mean difference ± SEM: 2.67±0.83; p=0.0159) and ACTH-controls (n=9) (mean difference ± SEM: 2.24±0.76; p=0.032). ACTH-ketamine non-responders trended toward greater expression of pmTOR over ACTH-ketamine responders (mean difference ± SEM: 1.544±0.602; p=0.0801). ACTH-ketamine non-responders had significantly greater pmTOR/mTOR compared to ACTH-ketamine responders (mean difference ± SEM: 1.71±0.58; p=0.0300). ACTH-ketamine responders had significantly greater GSK3α expression compared to ACTH-vehicle saline (mean difference ± SEM: 0.756±0.25; p=0.0291). ACTH-ketamine non-responders had significantly greater expression of pGSK3α compared to ACTH-controls (mean difference ± SEM: 1.120±0.37; p=0.0264) and ACTH-ketamine responders (mean difference ± SEM: 1.329±0.40; p=0.0108). ACTH-ketamine non-responders had significantly greater pGSK3α/GSK3α compared to ACTH-ketamine responders (mean difference ± SEM: 1.649±0.48; p=0.0083) and ACTH-controls (mean difference ± SEM: 1.251±0.45; p=0.0493). ACTH-ketamine responders had significantly higher GSK3ß expression compared to ACTH-controls (mean difference ± SEM: 0.828±0.22; p=0.0030). ACTH-ketamine non-responders had significantly higher expression of pGSK3ß/GSK3ß compared to ACTH-ketamine responders (mean difference ± SEM: 0.588±0.20; p=0.0273).

Prelimbic prefrontal cortex region

Tukey's multiple comparisons revealed that ACTH-ketamine non-responders had significantly less GSK3α expression compared to ACTH-ketamine responders (mean difference ± SEM: 0.269±0.09; p=0.0475), as well as lower pGSK3α/GSK3α (mean difference ± SEM: 1.561±0.56; p=0.0467). ACTH-ketamine non-responders had

significantly higher pGSK3ß than ACTH-ketamine responders (mean difference ± SEM: 1.578±0.52; p=0.0255) and ACTH-controls (non-significant trend; mean difference ± SEM: 1.226±0.46; p=0.0641). ACTH-ketamine non-responders had significantly less pGSK3ß/GSK3ß compared to ACTH-ketamine responders (mean difference ± SEM: 1.315±0.39; p=0.0113) and ACTH-controls (mean difference ± SEM: 1.154±0.35; p=0.0144).

Peripheral blood mononuclear cell buffy coat

Tukey's multiple comparisons revealed that ACTH-ketamine non-responders had significantly less mTOR compared to ACTH-ketamine responders (mean difference ± SEM: 0.685±0.69; p=0.0046). ACTH-ketamine non-responders had significantly greater pmTOR/mTOR compared to ACTH-ketamine responders (mean difference ± SEM: 1.534±0.43; p=0.0063) and ACTH-controls (mean difference ± SEM: 1.142±0.39; p=0.0333). ACTH-ketamine non-responders had significantly less GSK3α compared to ACTH-ketamine responders (mean difference ± SEM: 0.629±0.20; p=0.0231). ACTH-ketamine non-responders had significantly greater pGSK3α than ACTH-ketamine responders (mean difference ± SEM: 1.329±0.39; p=0.0108) and ACTH-controls (mean difference ± SEM: 1.121±0.37; p=0.0264). ACTH-ketamine non-responders had significantly greater pGSK3α/GSK3α compared to ACTH-ketamine responders (mean difference ± SEM: 1.879±0.50; p=0.0071), ACTH-controls (mean difference ± SEM: 1.72±0.44; p=0.0043), and saline-ketamine-treated animals (mean difference ± SEM: 1.805±0.44; p=0.0024).

Augmentation of insulin signaling with metformin improves ketamine response rates in ACTH-treated animals and insulin-stimulated mTOR activation.

It follows that normally functioning insulin signaling would be required for ketamine response. To test this idea, we combined co-administered ketamine and metformin, an insulin sensitizer. We hypothesized that the addition of metformin would enhance underlying insulin signaling, thereby facilitating a stronger antidepressant-like effect by ketamine compared to ketamine as a monotherapy.

Forced swim test

An ANOVA was conducted to examine the behavioral expression of antidepressant-like effects of ketamine-metformin co-administration, as well as ketamine and metformin as monotherapies, following chronic ACTH administration in the FST (see figure 8A-C). Statistically significant main effects of treatment were

observed in total immobility, F(3,92)=6.091, p=0.0008; swimming, F(3,90)=4.557, p=0.0051; and climbing behaviors, F(3,79)=7.925, p=0.0005.

Immobility

Following application of Tukey's multiple comparisons, we found that animals receiving ketamine-metformin co-treatment (n=22) had significantly reduced immobility in the FST compared to saline-controls (n=26) (mean difference ± SEM: 56.49±13.93; p=0.0003) and ketamine-treated animals (n=28) (mean difference ± SEM: 33.90±13.70; p=0.0449), but not metformin-treated animals (n=20). Further, metformin-treated animals had significantly less immobility than saline-controls (mean difference ± SEM: 40.66±14.30; p=0.0165).

Swimming

Tukey's multiple comparisons revealed that animals receiving ketamine-metformin co-treatment (n=22) spent significantly more time swimming in the FST than saline-control animals (n=26) (mean difference ± SEM: 39.77±13.07; p=0.0092), and trended toward greater swimming time than ketamine-treated animals (n=28) (mean difference ± SEM: 31.05±12.86; p=0.0523 (non-significant)). Animals administered metformin as a monotherapy (n=18) swam significantly more than saline-controls as well (mean difference ± SEM: 37.26±13.84; p=0.0251).

Climbing

Tukey's multiple comparisons revealed that animals receiving the ketamine-metformin co-therapy spent significantly more time climbing the walls of the FST apparatus than saline-controls (n=20) (mean difference ± SEM: 9.900±2.983; p=0.0055. Animals receiving ketamine as a monotherapy (n=25) had more time spent climbing than saline-controls (mean difference ± SEM: 11.13±2.83; p=0.0007).

Open Field Test

An ANOVA was conducted to examine modifications of mobility in the OFT following treatment administration preceded by chronic ACTH administration (see figure 8D, E) A statistically significant main effect of treatment were observed on total distance traveled F(3,85)=3.031, p<0.0001, but not time spent in the center of arena (p=ns).

Total Distance

Significant effects were found when comparing treatments following application of Tukey's multiple comparisons. Animals receiving the ketamine-metformin co-treatment (n=19) had significantly less total distance traveled than saline-

controls (n=24) (mean difference ± SEM: 11497±1615; p<0.0001), ketamine-treated animals (n=28) (mean difference ± SEM: 7725±1563; p<0.0001), and metformin-treated animals (n=18) (mean difference ± SEM: 5365±1730; p=0.0130). Animals receiving metformin as a monotherapy had significantly less total distance traveled compared to saline-controls (mean difference ± SEM: 6132±1640; p=0.0017). Animals receiving ketamine as a monotherapy trended toward less total distance traveled compared to saline-controls (mean difference ± SEM: 3772±1463; p=0.0568 (ns)).

Glucose and Insulin

ANOVAs were used to examine the effect of treatment on glucose and insulin signaling within peripheral whole blood and plasma, respectively (see figure 8F, G). When examining the total change in glucose levels from before treatment to immediately following behavioral testing, a main effect of treatment was revealed $F(3,81)=1.057$, p=0.0371. Tukey's multiple comparisons revealed that animals treated with the ketamine-metformin co-treatment (n=19) had a significantly higher increase in glucose levels compared to ketamine-treated animals (n=26) (mean difference ± SEM: 14.33±4.926; p=0.0017. No significant effect of treatment was found on total insulin levels.

Insulin-evoked mTOR change in PBMC

The effects of treatment on change of mTOR expression following 5 minutes of insulin stimulation on PBMCs was examined, revealing a significant main effect $F(3,86)=2.357$, p=0.0368) (see figure 8H). Tukey's multiple comparisons were used to examine specific comparisons between groups, revealing that animals receiving the ketamine-metformin co-treatment (n=24) expressed a significantly greater positive change in mTOR following insulin stimulation than ketamine-treated animals (n=24) (mean difference ± SEM: 0.0906±0.0373; p=0.0341). Additionally, ketamine-treated animals had significantly less change in mTOR following insulin stimulation than saline-controls (n=21) (mean difference ± SEM: 0.1027±0.0386; p=0.0185).

Insulin-simulated mTOR activation in PBMCs differentiates clinical response to ketamine.

No significant differences were observed in insulin-stimulated mTOR or pmTOR levels at baseline or following insulin exposure for subjects' PBMC samples collected immediately before ketamine administration. In contrast, for samples obtained immediately following first ketamine infusion, a significant and robust increase in

pmTOR levels was observed in response to insulin stimulation for those patients that achieved clinical remission with ketamine ($p<0.05$; $n=12$) (see figure 9).

Discussion

In this translational study, we demonstrate that variations in insulin signaling are associated with antidepressant response to ketamine in individuals with TRD. We leveraged this mechanism to develop an *ex vivo* PBMC assay to quantify individual cellular responses to insulin following ketamine administration, demonstrating differential insulin-stimulated mTOR responses post-ketamine in responsive and non-responsive individuals. The potential clinical utility of restoring impaired cellular responses to insulin was established through overall improvement of antidepressant response rates upon co-administration of ketamine with metformin. Relative to ketamine monotherapy, the ketamine-metformin co-treatment was effective in elevating glucose availability and mTOR sensitivity to insulin stimulation, concurrent with promotion of a robust antidepressant-like effect. Taken together, these findings highlight an essential connection between antidepressant treatment response to ketamine and insulin action within brain and peripheral tissues. Ketamine also elevated protein signals in insulin-stimulated PBMCs from patients with treatment-resistant unipolar or bipolar depression. This information may be utilized for future translational research and drug discovery efforts.

Ketamine's antidepressant action in ACTH-treated animals is associated with brain insulin response.

In rodents, we showed that approximately half of ACTH-treated animals did not respond to ketamine, and that phosphorylation of insulin signaling proteins, Akt, mTOR, and GSK3, were significantly elevated across both PFC and PBMC tissue in ketamine-treated animals. Akt, mTOR, and GSK3 are critical constituents of the insulin signaling pathway with functional activation well-established to be dependent on variations in insulin sensitivity (26, 38). Further, these molecules play a critical role in the upregulation of structural and functional synaptic plasticity, proposed to form the biological basis of ketamine's rapid antidepressant effects. The observed differences in molecular activation associated with behavioral treatment response data suggest that ketamine's antidepressant actions may be moderated by insulin action. We posit that a critical mechanism of action of ketamine involves the facilitation of cellular metabolic pathways, specifically, insulin-sensitive mTOR signaling. In line with this, the

facilitation of cellular responses to insulin with metformin improved treatment outcomes to ketamine.

Augmentation insulin action with metformin improves ketamine response rates in ACTH-treated animals.

Metformin, a treatment demonstrated to decrease hepatic glucose production and improve peripheral insulin sensitivity (39, 40), increased antidepressant-like responses to ketamine in ACTH-treated animals. Further, ACTH-pretreated animals receiving metformin alone were more active in the FST than ACTH-controls, highlighting the antidepressant-like effects of enhanced insulin signaling in these animals. In combination with ketamine, metformin improved overall response rates while also producing a stronger antidepressant-like response relative to both ACTH-controls and ketamine-treated animals (total immobility time and swimming time). We also found that animals receiving the ketamine-metformin co-treatment had higher pre/post-change in glucose levels following behavioral testing than ketamine-treated animals, suggesting a possible modulation of glucose uptake for bioenergetic output related to insulin production. As an extension of this, we developed an *ex vivo* insulin assay to quantify PBMC responses to insulin following ketamine and metformin exposure, finding that animals receiving the ketamine-metformin co-treatment had significantly higher mTOR expression in response to insulin stimulation than animals receiving ketamine as a monotherapy. The elevation of mTOR expression found here may suggest that although total insulin levels did not differ by treatment group, the ketamine-metformin co-treatment may instead facilitate cellular sensitivity to insulin — thereby enhancing mTOR production for purposes of neural and synaptic plasticity. If successfully extended to the clinical population for validation, this assay may be useful for early screening of antidepressant response to ketamine and metformin due to the accessibility and relative non-invasiveness of PBMC collection.

Ex vivo PBMC insulin stimulation assay reflects ketamine response profile in human subjects with TRD

In human subjects with TRD, mTOR activation following insulin stimulation was shown to be significantly elevated in all but one patient who went on to receive full and sustained remission. This was observed only in samples collected post-ketamine treatment. Overall, these data suggest that early antidepressant response to ketamine may be moderated by its direct facilitation of insulin release and insulin signaling in the brain, and reliably assayed in peripheral PBMCs. It is important to note that pre-

ketamine PBMC insulin response was not significantly different at baseline for individuals responsive to ketamine relative to those failing to receive therapeutic benefit. This similarity of baseline insulin response indicates that insulin stimulation assays will not serve as a valid predictive screen for treatment response to ketamine. However, the observation that this is shifted post-ketamine infusion suggests that insulin action may serve as an important moderator of treatment outcomes to ketamine in patients with TRD. As such, novel treatment approaches can be developed to directly target this mechanism of action to facilitate treatment outcomes, as demonstrated in the rodent model with adjunctive metformin administration.

Significance of mTOR response in brain and blood tissue

In the present study, we have demonstrated a direct association between ketamine's immobility reducing effects in the FST and activation of mTOR signaling in the prefrontal cortex and blood (buffy coat) tissue obtained 30 mins post-FST exposure. Importantly, this effect was shown to be region dependent, with significant differences observed in the ILPFC but not PLPFC region. The ILPFC represents the rodent homolog of the subgenual cingulate (SGC: Brodmann's area 25), a region well-established to be hypermetabolic in depression and to maintain this hyperactivity when treatment response to antidepressants fails (30). Activity in this region also contributes to the regulation of effortful escape behaviors in the FST (31). Metabolic stress due to regional hyperactivity may contribute to the impairment of antidepressant efficacy and serve as a moderator of antidepressant response. That is, in a subset of individuals, neural adaptations to antidepressant actions may be impaired by rate-limiting metabolic deficits in regions of the brain under high energy demand, such as the SGC.

The ILPFC and PLPFC have previously been described to have contrasting functional roles in the stress response (41), fear expression (42), and behavioral flexibility (43). The ILPFC and the PLPFC also differ in their cytoarchitecture (44), interconnectivity with other brain regions (45), modulation of mesolimbic dopamine pathways (46) and in their response to glucocorticoids (47). The data we find here complements theories of a functional dichotomy, as indicated by contrasting directional correlations between protein phosphorylating and behavior for the two regions. Furthermore, it indicates that the critical role of mTOR signaling within ketamine's mechanism of antidepressant action (21) is region dependent. That is, activation of these metabolic and growth signaling systems in the ILPFC, together with their downregulation in the PLPFC, may facilitate increased active coping in ACTH-treated

animals during the FST paradigm. We further demonstrated that differential activation of this pathway in peripheral WBCs in antidepressant response versus non-response to ketamine, with significantly elevated levels of pmTOR and pGSK3 in the former. While this may result from direct NMDA receptor blockade, a hypothesized mechanism of ketamine action (48), it suggests that alternate or complementary functional effects may also contribute. As already described, mTOR and GSK3 are highly responsive to substrate availability and coordinate cellular growth, including synaptic plasticity, in response to energy status. We observed that ACTH-treated animals demonstrated a relative reduction in baseline glucose levels, followed by a ketamine-induced rise post-FST. These results suggest that sufficient nutrient availability is a necessary component of the antidepressant response to ketamine in ACTH-treated animals and complements a theory proposed by Henry Lester and colleagues (2015), who suggest that ketamine's mechanism of action may rely less on extracellular receptors than previously suspected (49). These authors propose instead that ketamine may directly stimulate mTOR regulation of cellular metabolism and growth given its structural similarity to endogenous amino acids (49).

Indeed, there is substantial support for ketamine's stimulation of mTOR signaling and its antidepressant and anxiolytic properties, particularly in the rodent medial PFC and hippocampus (See Browne and Lucki (50) for a comprehensive review). However, the reason we observed such strong, significant correlations between ketamine's antidepressant actions in ACTH-treated animals and ILPFC levels of mTOR, Akt, and GSK3 remain to be determined. As noted above, this contrasts with saline-treated animals, suggesting that stimulation of mTOR signaling is essential for the antidepressant-like effect of ketamine among ACTH-treated animals. This may suggest peripheral alterations in HPA-axis and metabolic function may play a role in ketamine's mechanism of action. Recently, Ostroff and Kathari (2015) have reported that HPA-axis dysregulation (non-suppression with dexamethasone suppression test) in a patient was normalized during a ketamine infusion trial, parallel with induction of antidepressant response (51). Moreover, they observed a return to non-suppression together with depressive symptoms following the trial (51). Complementing this, ketamine has also been shown to normalize circulating ACTH and glucocorticoid levels in a preclinical chronic mild stress model (52). This, together with results of the current study, highlight the importance of understanding the role that ketamine plays in regulating HPA-axis and metabolic dysfunction in facilitating antidepressant responses.

Altered metabolic responses under stress and associated mTOR and GSK3 signaling may represent a significant and understudied mechanism of antidepressant treatment resistance and response given the critical role these signals play in enabling synaptic plasticity. In line with this, scopolamine, the muscarinic cholinergic antagonist with rapid antidepressant effects paralleling those of ketamine, similarly requires the express activation of mTOR complex 1, preferential activation of AMPA receptors, and synaptogenesis for its antidepressant actions (27) (see Drevets, Zarate, and Furey (53) for review). Lithium augmentation, which inhibits GSK3 and indirectly promotes the activation of mTOR (54), in conjunction with low dose ketamine, was recently shown to facilitate and extend ketamine's antidepressant actions (22). Martinowich and colleagues (55) suggest that adaptations such as these lend to Hyman and Nestler's (56) theory of "Initiation and Adaption." The theory posits that psychotropic drugs (in this case, antidepressants) modulate brain function via their molecular targets within critical circuits dysregulated in depression (such as the mesocorticolimbic network). A new adaptive state is set through the reinstatement of homeostasis downstream of cellular signaling cascades and biochemical changes (55), (56). The rapid, antidepressant-like effects of ketamine may circumvent some of the requisite upstream effects generated by typical antidepressants via mTOR-related signaling to affect dendritic protein translation machinery and neuronal plasticity (55). Such changes have been previously implicated not only in ketamine's mechanism of action (e.g. (57); reviewed in (50)), but also to be sufficient for generating the antidepressant-like effects of imipramine (10 mg/kg for 1 week) and fluoxetine (10 mg/kg for 2 weeks) (58). Interestingly, both imipramine and fluoxetine were not previously found to affect mTOR signaling, pointing towards at least one divergent mechanism in the efficacy of ketamine.

Conclusions

Our study presents evidence for three things: (1) A subset of TRD individuals do not respond to ketamine, and this failure results, in part, as a result of impaired cellular responses to insulin and associated insulin signaling dysfunction; (2) Ex vivo PBMC responses to insulin post-ketamine infusion are associated with treatment response to ketamine in TRD patients; and (3) Facilitation of insulin-dependent processes that augment insulin-dependent cell signaling pathways can improve treatment response rates. Taken together, these results highlight underlying pathophysiological differences that may contribute to treatment response outcomes and the potential for the development of new treatments aimed at more uniformly

facilitating overlapping insulin-dependent mechanisms of ketamine and metformin. Such pathophysiology-directed approaches offer a unique opportunity to develop individualized treatments for TRD patient care.

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EXAMPLE 3

Introduction

Ketamine has emerged as a promising intervention for adults with TRD (Newport et al., 2015). The rapid onset and large effect sizes observed in trials of ketamine for TRD (Coyle and Laws 2015; Wilkinson et al. 2017; Bobo et al. 2016) underscore its potential value as a research probe for illuminating biological mechanisms underlying clinical improvement in this difficult to treat patient population. A core feature of depression in general, and TRD in particular, is the tendency to get "stuck" in negative moods (Holtzheimer and Mayberg 2011). Cognitively, patients with severe depression perseverate, or ruminate, on negative thoughts, experience a rigidly negative perspective on themselves and the world, and perceive a sense of narrowed options (Lennings 1994; Cleary 2012). This lack of flexibility may be reflected in cellular markers of limited molecular responsivity to environmental stimuli (adaptability), and in the flexibility of brain signals. Examination of central and peripheral brain flexibility may be a promising approach for understanding neural correlates of clinical response to rapid-acting treatments such as ketamine.

Treatment-related changes in neural flexibility can be measured through quantification of entropy. Entropy is a concept originally born in physics and more recently adapted to information theory, is a univariate measure of signal variability, unpredictability. Although entropy of the brain can be estimated from time-course or matrix of time-courses of a range of methods, the focus here is on rs-fMRI. Large scale studies of rs-fMRI entropy in healthy human subjects have revealed hierarchical brain entropy networks consistent with conventional functional and anatomical brain parcellations (Wang et al. 2014). Multi-scale rs-fMRI entropy studies in healthy subjects have further shown that regional entropy is correlated with network functional connectivity in a frequency specific manner (Wang et al. 2018). Importantly, low frequency rs-fMRI signals represent distributed processing across brain networks: increased low frequency entropy is associated with increased low frequency network connectivity. In contrast, high frequency rs-fMRI signals represent regional signal processing: increased high frequency entropy is associated with decreased high frequency network connectivity.

The cognitive inflexibility associated with depression may be characterized by rigidity in neural signals, whereas increased complexity (less rigidity) may reflect clinical improvement. Entropy of brain signals has been used to estimate the flexibility (Yin et al., 2016), as evidenced by their findings that changes in entropy are evidendent across the lifespan. Increased rs-fMRI entropy has been associated with increased intelligence in healthy individuals, suggesting that intellectual capacity may critically depend on the brain's flexibility and ability to access highly variable and complex neural states (Saxe et al. 2018). Perhaps the most compelling evidence that rs-fMRI entropy is a potential biomarker of functional connectivity flexibility is based on two samples of healthy subjects in which entropy was related to divergent thinking - fluency, flexibility and originality (Shi et al. 2019). Entropy of rs-fMRI data has also been used to characterize signal rigidity in mental health conditions (Bassett et al., 2012). Although there is preliminary evidence that ketamine treatment may lead to increased entropy regardless of conscious state: one EEG study in humans reported significantly increased entropy following the administration of ketamine versus saline in anesthetized adult patients (Hans et al., 2005) and another reported increased spontaneous MEG signal complexity following administration of ketamine in awake adult patients (Schartner et al., 2017), to date, few studies have explicitly investigated the utility of rs-fMRI entropy in the study of depression or its treatment.

Additionally, a lack of adaptability at the cellular level may contribute to poor treatment outcomes in depression, due to failed upregulation of neurotrophic responses to treatment (Price et al. 2018). While such molecular changes in brain cells are difficult to capture in human studies, recent work suggests that using insulin probes to study molecular changes in peripheral blood mononuclear cells (PBMCs) may shed light on treatment-associated changes in pro-trophic events that promote neural flexibility. Ketamine studies using animals have shown that activation of neurotrophic signaling cascades (i.e. brain derived neurotrophic factor and its receptor TrkB), together with the promotion of mammalian target of rapamycin (mTOR) and glycogen synthase-3-beta (GSK3β) phosphorylation, mediates the rapid antidepressant effects including behavioral change and dendritic spine growth (Li et al. 2010; Liu et al. 2013). These neurotrophic responses are critical for synapse formation, synaptic plasticity and neural network remodeling, which are in turn thought to underlie improved mood regulation (Price et al. 2018; Bessa et al. 2009), and cognitive flexibility (Xu et al. 2019) in depression following effective treatment. At the molecular level, insulin serves as a

critical moderator of such neurotrophic growth responses within the brain. Using a rodent model of TRD, we have established that these central pro-trophic insulin signaling responses can be derived in PBMCs upon ex vivo stimulation (Walker et al. 2019).

The current Example examined neuroimaging markers of brain flexibility and associated peripheral pro-trophic molecular indicators of cellular adaptability to identify potential neurobiological correlates of clinical response to ketamine in adolescents with TRD. Markers were obtained during our recently conducted open-label pilot study testing intravenous ketamine as an intervention for adolescents with TRD (Cullen et al., 2018). Clinical results (high tolerability of the ketamine intervention and a significant reduction in depression symptoms for 5 of the participants) have already been published (Cullen et al., 2018). Here we examined rs-fMRI entropy and insulin-stimulated mTOR and GSK3β activation before and after treatment to identify neurobiological correlates of clinical improvement in the context of ketamine treatment in this sample. We predicted that clinical improvement following ketamine treatment would be accompanied by evidence for increased brain flexibility as measured by increased rs-fMRI entropy in specific depression-related regions of interest (ROIs) and by upregulation of insulin-stimulated mTOR and GSK3β phosphorylation in PBMCs. We also postulated that these brain and blood markers would correlate with each other, providing converging evidence for brain flexibility as a correlate of clinical improvement.

Methods

Overview

A detailed description of this sample has been published previously (Cullen et al., 2018). Briefly, this study was approved by the Institutional Review Board of the University of Minnesota (UMN). Participants were recruited via community postings and clinic referrals. Inclusion criteria were age 12–18 years, current diagnosis of Major Depressive Disorder, Children's Depression Rating Scale-Revised (CDRS-R) (Poznanski et al. 1985) raw score >40, and treatment resistance defined as a failure to exhibit a satisfactory response to at least two antidepressant medications. Rigor of antidepressant trials was assessed using the Antidepressant Treatment History Form (Sackeim 2001); past trials were considered sufficient if they scored at least a ''3'' (on a scale of 1 to 4; as an example, a rating of ''3'' for fluoxetine is 4 weeks or more and

dosage 20–39 mg/day, a rating of ''4'' is 4 weeks or more and dosage ≥40 mg/day), or if the trial was truncated due to intolerance (as opposed to an early decision regarding inefficacy). Current psychotropic medications had to be dose stable for 2 months. If participants opted to discontinue any psychotropic medications before the study, we required a washout period of 2 weeks for mood stabilizers and antipsychotic medications, 4 weeks for antidepressants, and 1 week for stimulants. Exclusion criteria were the presence of a current substance use disorder, a primary psychotic disorder, bipolar disorder, autism spectrum disorder, a history of intellectual disability, a neurological disorder, or a significant medical illness.

Baseline clinical assessment and baseline MRI were conducted one day before the first ketamine infusion; post-treatment assessments were completed one day after the last infusion. Blood was drawn directly before the first and two hours after the last infusion.

Clinical Assessments and Ketamine Infusions.

After completing the informed consent and assent (where applicable) process, participants were evaluated using the Kiddie Schedule for Affective Disorders and Schizophrenia, Present and Lifetime Version (K-SADS-PL) (Kaufman et al., 1997). Adolescents and parents were interviewed separately by trained clinicians. Clinicians assessed depression using the CDRS-R (based on both adolescent and parent report) (Poznanski et al., 1984). A consensus meeting following the interviews integrated all available clinical information for diagnostic and inclusion finalization. At post-treatment, CDRS-R was repeated. The primary clinical outcome measure was percent change in depression symptoms as measured by the CDRS-R. Additionally, participants were classified into groups of treatment responders and non-responders based on if they had or did not have at least a 50% reduction in depression symptoms. For all results shown, a CDRS-R score change of 100% would be equivalent to complete remission of depression symptoms.

Each participant underwent six sessions of ketamine (0.5mg/kg) infusions over the course of two weeks following the baseline MRI session. (As noted previously (Cullen et al., 2018), dosing was based on ideal body weight for the first 5 participants and then based on actual body weight for the remaining participants.) We elected to include a series of 6 infusions, rather than a single infusion, based on evidence emerging

around the time of our study that repeated infusions led to higher responses (Shiroma et al. 2014).

Neuroimaging Data Acquisition

Scanning took place before the first ketamine infusion and one day after the 6th ketamine infusion. All neuroimaging data for the study were acquired using a 3T Siemens Prisma scanner at the Center for Magnetic Resonance Research at UMN. We utilized a multiband echo planar imaging (MB-EPI) sequence to improve the spatial and temporal resolution of the acquired fMRI data over conventional methods (Feinberg et al., 2010). Individual rs-fMRI data (eyes open, fixation cross, Multiband Factor [MB]=8, Time Repeat [TR]=710 ms, Echo Time [TE]=30 ms, 2mm isotropic voxel size, 680 volumes [~8 minutes]), along with a B0 field map and high resolution T1 weighted MPRAGE anatomical scan (Magnetization-Prepared Rapid Gradient-Echo, TR=2530 ms, TE=3.65, Inversion Time [TI]=1100 ms, 7 degree flip angle, 1mm isotropic voxel size, 4 minutes), were collected before and after the ketamine intervention for all 13 study participants.

rs-fMRI Preprocessing

rs-fMRI data were preprocessed using FEAT (FMRI Expert Analysis Tool) Version 6.00, part of FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl). The following preprocessing pipeline was applied: motion correction using MCFLIRT (Jenkinson et al., 2002), B0 field-map unwarping and distortion correction using FUGUE, non-brain removal using BET2 (Smith, 2002), spatial smoothing using a Gaussian kernel of FWHM 3mm, grand-mean intensity normalization of the entire 4D dataset by a single multiplicative factor and high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with sigma=50.0s). ICA-based exploratory data analysis was carried out using MELODIC (Beckmann and Smith, 2004) prior to automated identification and removal of artifactual components (Kelly et al., 2010) using FSL FIX. Registration to standard MNI space was carried out using FLIRT (Jenkinson et al., 2002; Jenkinson and Smith, 2001). Image quality of each resting state scan was evaluated using the method of Power et al. (Power et al., 2012). Volumes with framewise displacement (FD) values of more than 0.5 mm and/or temporal derivative of time courses (DVARS) which exceeded 8 after motion correction only were flagged as having excessive motion, along with the previous volume and next 2 volumes. If a

scan had more than 30% of volumes with "excessive motion," that scan was excluded from analysis.

rs-fMRI Entropy Analysis

Time-courses from 132 ROIs (cortical and subcortical regions from the FSL Harvard-Oxford atlas and cerebellar regions from the AAL atlas) were extracted using the Functional Connectivity Toolbox (CONN) in SPM (Whitfield-Gabrieli and Nieto-Castanon, 2012). Entropy analysis was carried out using custom MATLAB scripts, including functions from the Wavelet Toolbox. rs-fMRI time-courses for all 132 ROIs were first bandpass filtered (0.08-0.12 Hz) using a 6th order Butterworth filter. We chose to assess this high frequency range instead of the conventional 0.01-0.1 Hz range based on several studies that have shown higher mean frequency resting-state BOLD activity in limbic regions in depression (Ries et al., 2018; Wu et al., 2008). We focused on the Shannon entropy since it is a well-established concept in information theory and straightforward to compute (Bassett et al., 2012; Shannon, 1997). For this calculation, the Shannon entropy E of a signal s is defined by:

$$E(s) = -\sum_i s_i^2 \log(s_i^2)$$

with the convention $0\log(0) = 0$ (Coifman and Wickerhauser, 1992). Entropy values for each timepoint were calculated for each ROI's band-pass filtered time-series data using the *wentropy* function. We transformed the resulting values by log(-E(s)) to provide a more intuitive metric in which greater entropy is associated with greater complexity. We examined local entropy changes in a subset of 14 ROIs that have been previously implicated in TRD, including the subcallosal cingulate cortex (Mayberg et al., 2005), regions within the default mode network (posterior cingulate, precuneus, and anterior cingulate) which have been implicated in depression and rumination (Greicius et al., 2007; Hamilton et al., 2015; Zhu et al., 2017) and limbic regions known to be implicated in emotion regulation and reward processing (bilateral hippocampus, amygdala, nucleus accumbens [NAc], insula and thalamus) (Phillips et al., 2003a, 2003b).

Individual changes in local entropy were also plotted against CDRS-R score changes and mTOR measures for correlation analysis. Additionally, Student's t-tests (alpha=0.05) were used to compare mean local entropy changes in each of the 14 ROIs

between the responder (n=5) and non-responder (n=6) groups. We examined uncorrected results and also applied a Bonferroni correction ($p < .05/14=0.0036$) to the ROI results to adjust for multiple tests.

PBMC *Assays*

Whole blood was collected immediately prior to the first ketamine infusion and following the last ketamine infusion. Samples were then shipped overnight for PBMC isolation and testing. Upon receipt of the samples, PBMCs were isolated via ficoll gradient and slow frozen as previously described (Walker et al., 2019). Following completion of sample collection from all subjects, cells from each subject were counted and 1 X 10^7 cells were placed into one of four 35mm dishes with 5mls of media. 10μM of insulin was added to two of these, allowing the others to serve as baseline. The cells were incubated for 5 minutes and then immediately centrifuged, and washed once with 10mls of PBS. The cells were then lysed with RIPA lysis buffer to prepare protein whole cell lysates. Relative change in total and phosphorylated levels of mTOR, *p*mTOR, GSK3β, *p*GSK3β were determined post-insulin exposure using ELISA (Cell Signaling Technology, Danvers, MA), in accordance with manufacturer's instructions. We measured the extent to which stimulating the cells with insulin increased the ratio of phosphorylated to non-phosphorylated mTOR (*p*mTOR/mTOR) and GSK3β (*p*GSK3β/GSK3β), before and after ketamine treatment. We considered insulin-mediated upregulation of *p*mTOR/mTOR and *p*GSK3β/GSK3β protein levels, relative to no insulin baseline levels, as variables for correlation analyses with clinical improvement and entropy measures, respectively.

Results

Participants

As previously reported, 13 adolescents completed the 2-week ketamine protocol (mean age 16.9 years, range 14.5-18.8 years, 8 biologically male). Five were considered responders (at least 50% decrease on CDRS-R scores). All 13 adolescents also completed the neuroimaging protocol before and after treatment, but data from two of these participants were excluded due to excessive motion (greater than 30% of the volumes in the pre and/or post scan exceeded our motion threshold described above.) There were 11 participants with usable scans for the entropy analysis and 10 of these

participants had viable blood for the PBMC insulin assays.

Entropy Analysis

After Bonferroni correction for multiple comparisons, of the 14 ROIs examined, only entropy changes in the right NAc significantly correlated with individual changes in CDRS-R scores (r=0.86, p=0.00066) (see Figure 10). All responders showed an increase in right NAc log(-entropy) after treatment (n=5, M=0.35, SE=0.13, change range: 0.046 to 0.77) while all non-responders showed a decrease in right NAc log(-entropy) after treatment (n=6, M=-0.42, SE=0.10, change range: -0.17 to -0.77). Entropy changes in the right NAc were significantly different between responder and non-responder groups (t=4.73, p=0.0011), with a Cohen's effect size value (d=2.84), suggesting a very high practical significance of this difference (see Figure 11).

Insulin-stimulated change in mTOR and GSK3β

Figure 12 summarizes the results of the mTOR analyses. Post-ketamine treatment, responders showed significantly higher (t=2.41, p=0.042) insulin-induced upregulation of *p*mTOR/mTOR (n=5, M=26.12%, SE=4.37%) compared to non-responders (n=5, M=1.05%, SE=9.43%). In contrast, pre-ketamine treatment, no significant group difference in insulin-induced upregulation of *p*mTOR/mTOR was observed. Percent change in CDRS-R score over the course of the study was significantly correlated (r=0.80, p=0.0053) with post-ketamine insulin-induced upregulation of *p*mTOR/mTOR). Further, post-ketamine insulin-induced upregulation of *p*mTOR/mTOR was correlated with post-pre ketamine changes in right NAc entropy (r=0.72, p=0.019).

Similar findings were observed for the GSK3β analyses (Figure 13). Post-ketamine treatment, responders showed significantly higher (t=3.73, p=0.0058) insulin-induced upregulation of *p*GSK3β/GSK3β (n=5, M=16.20%, SE=3.14%) compared to non-responders (n=5, M=-3.99%, SE=4.40%). No significant group difference in insulin-induced upregulation of *p*GSK3β/GSK3β was observed pre-ketamine exposure. Percent change in CDRS-R score over the course of the study was also significantly correlated (r=0.87, p=0.0012) with post-ketamine insulin-induced upregulation of *p*GSK3β/GSK3β. Finally, post-ketamine insulin-induced upregulation of *p*GSK3β/GSK3β was correlated with post-pre ketamine changes in right NAc entropy (r=0.72, p=0.019).

Discussion

This is the first study to report on neurobiological correlates of treatment response to ketamine in adolescents with TRD. In these adolescents, clinical improvements after the ketamine infusions were accompanied by pre-post increases in NAc rs-fMRI entropy and greater post-treatment insulin signaling (*p*mTOR/mTOR and *p*GSK3β/GSK3β). NAc entropy changes were also associated with post-ketamine upregulation of insulin-stimulated *p*mTOR/mTOR and *p*GSK3β/GSK3β levels in PBMCs. This suggests that cellular neurotrophic responses to insulin post-ketamine may have a mechanistic link with the associated effects on NAc entropy. Importantly, we did not observe generalized ketamine-induced changes in rs-fMRI entropy and insulin-stimulated *p*mTOR/mTOR and *p*GSK3β/GSK3β expression; these findings were specific to responders. Collectively these data provide preliminary evidence suggesting that enhancing neural flexibility may be critical for clinical improvement in the context of ketamine treatment in adolescents.

Entropy has emerged as a promising measure for quantifying different brain states based on the variability of neural activity (Carhart-Harris, 2018; Carhart-Harris et al., 2014) and as a metric of neuroplasticity (Foz et al., 2002; Tecchio et al., 2006), across a number of methods. It has been proposed that entropy can reflect the flexibility of a state (Yin et al., 2016; Shi et al. 2019), and that while depression is associated with lower entropy, certain psychoactive drugs can increase brain entropy (Carhart-Harris et al., 2014). A recent MEG study found that ketamine, LSD and psilocybin all increased resting state entropy measures at doses conferring psychoactive effects, suggesting that these dissociative drugs can generally increase the complexity of neural activity (Schartner et al., 2017). These previous studies of entropy focused on real-time changes in brain activity. In contrast, our work investigated sustained changes in entropy, given that the post-treatment assessment was one day after the final ketamine infusion of the six infusions delivered over the course of two weeks. We found that adolescent responders to ketamine showed increased signal complexity in the NAc following the intervention. This could signify enhanced neural flexibility, allowing the adolescent to break out of rigid pathological signaling pathways. Whereas depression research has previously identified neural networks that become entrenched in pathological feedback loops that contribute to ongoing problems with mood and behavior (Hamilton et al., 2015), our results suggest that in adolescents with TRD, a

clinical improvement in the context of ketamine treatment may result from enhanced neural flexibility in key limbic regions, allowing for a therapeutic shift in the behavior of these entrenched neural networks.

In our study, entropy change associated with clinical improvement was particularly noted in the NAc, a subcortical brain region which serves as a nexus point for pathways mediating emotion, cognition and motor function (Nauczyciel et al., 2013). Reduced activation in this region has been associated with severe depression and has been thought to underlie dysfunction in the reward system (Pizzagalli et al., 2009). Furthermore, dendritic atrophy in medium spiny neurons of the NAc is known to mediate stress-induced depression behaviors in animal models (Francis et al., 2017). Thus, recent efforts to treat severe depression in adults using deep brain stimulation have targeted the NAc as well as the ventral striatum (Bewernick et al., 2010, 2012). Numerous studies have also associated the antidepressive effects of ketamine with modulation of NAc activity (Abdallah et al., 2017). Studies in rats have found that ketamine enhances high frequency oscillations (130-180 Hz) and reduces gamma band activity (30-90 Hz) within the NAc (Hunt et al., 2006); these changes could potentially relate to the increased local rs-fMRI entropy that we observed in our group of adolescent responders. Collectively, our findings suggest that ketamine's effects on NAc entropy could be a critical step for facilitating neural flexibility and treatment response in adolescents with TRD.

Finally, based on prior research suggesting that signal transduction along the mTOR pathway mediates ketamine's antidepressant effects (Welberg, 2010), we examined ketamine-associated changes in insulin-stimulated *p*mTOR/mTOR and *p*GSK3β/GSK3β expression. We found evidence for increased *p*mTOR/mTOR and *p*GSK3β/GSK3β as markers of clinical response in this small sample. Furthermore, greater post-treatment *p*mTOR/mTOR and *p*GSK3β/GSK3β levels were associated with increased NAc entropy. Taken together, our results suggest that upregulation of insulin-stimulated mTOR/GSK3β signaling may be an important mechanistic marker of neural flexibility that occurs in concert with increased NAc entropy in the process of adolescents with TRD responding to ketamine.

Conclusion

There is an urgent need for understanding mechanisms of TRD and of novel treatments to address it in adolescents. Our preliminary findings suggest that measures

of central and peripheral neural flexibility (including increased rs-fMRI entropy, particularly in the NAc which was associated with increased mTOR and GSK3β signaling) represent neural correlates of clinical response in adolescents with TRD. These preliminary findings suggest that rs-fMRI entropy and insulin-mediated mTOR/GSK3β signaling could represent candidate neurobiological targets in future research investigating optimization strategies designed to enhance neural flexibility and plasticity in adolescents with TRD. It is possible that these physiological changes are due to ketamine treatment but this conclusion would need to be confirmed with subsequent studies.

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EXAMPLE 4

In this Example, an in-cell western assay was trialled for determination of protein expression from treated PBMCs (see Figures 14 and 15). The In-Cell Western (ICW) Assay is a quantitative immunofluorescence assay performed in microplates (optimized for 96-or 384-well format) that combines the specificity of Western blotting with the replicability and throughput of ELISA.

Protocol

1. Frozen PBMCs are thawed from liquid nitrogen into RPMI media
2. PBMCs are seeded 20,000 to 40,000 cells per well in a 96-well plate
3. PBMCs are treated with different stimuli (e.g. Dexamethasone or LPS), and treated with different drugs for a period of time.
4. Cells are immediately fixed and permeabilised in ice-cold methanol for 20 mins at 4°C
5. Cells are blocked with a suitable blocking solution for 1 hour.
6. After blocking, cells are incubated with up to 2 primary antibodies of interest from two different hosts. For example mouse anti-mTOR and CellTag (a normalisation stain that normalised for seeding density).
7. Secondary incubation is performed with infra-red conjugated (IR-Dye) secondary 800 and 680 antibodies which detects in the 680 nm and 800 nm wavelength and emits red and green fluorescence respectively.
8. The 96-well plate is scanned and imaged into Odyssey scanner.
9. The relative expression fluorescence units are determined and tabulated.

Results of the ICW are provided in Figure 15 confirming that it essentially replicates those results previously seen with western blotting.

EXAMPLE 5

In the present Example, it was tested whether PBMCs isolated from depressive disorder patients that have been treated *in vitro* with ketamine (*i.e.*, an *ex vivo* assay) demonstrate similar results with respect to insulin signalling, as shown for PBMCs isolated from depressive disorder patients after treatment in the earlier Examples. Additionally, the present Example investigated whether additional readouts of insulin signalling and cellular metabolism, such as mitochondrial activity and glucose uptake, were altered in PBMCs with ketamine treatment.

Methods and Results

PBMC Cell Culture:

Cells were isolated from the whole blood of patients with a depressive disorder via ficoll gradient as previously described (Walker et al., 2019; Example 1). Cells were divided across respective assays and sequentially exposed to ketamine (1 μM) and insulin (10 μM).

PBMC glucose uptake:

A 96-well plate was seeded with 1 x 10^4 cells/well. Cells were cultured overnight in growth media. The following day, cells were exposed to fluorescently labelled 2-NBD Glucose (200 μg/ml) or vehicle in 100 μl glucose-free culture medium 5 minutes prior to insulin stimulation. The plate was then centrifuged for five minutes at 400 x g at room temperature and supernatant aspirated (twice). Fluorescence levels were determined using a plate reader (excitation/emission = 485/535 nm).

A significant increase in glucose uptake into the cell, quantified by change in fluorescence intensity (arbitrary units), was observed following exposure to the ketamine/insulin *ex vivo* assay ($p = 0.0418$, number of pairs = 8) (Figure 16).

PBMC Mitochondrial Function:

A 96-well plate was seeded with 1 x 10^4 cells/well. Cells were cultured overnight in growth media. Cells were exposed to ketamine and insulin. Five and 15 minutes post-insulin exposure, cells were lysed using detergent together (50 μL). D-Luciferin (10 μL) was added to react with ATP. Fluorescence levels were determined using a plate reader (excitation/emission = 330/535 nm).

Similar to the glucose uptake response, ketamine/insulin exposure in the *ex vivo* cellular assay significantly increased ATP production in PBMCs ($p = 0.0017$, number of pairs = 8) (Figure 17).

PBMC Insulin Signalling

Methods for quantifying mTOR, Akt and GSK3 in this assay were as described in Tye et al., and in the aforementioned Examples. Insulin-mediated mTOR signalling differentially correlates with antidepressant effects of ketamine in treatment resistant depression. However, this assay itself included *ex vivo* addition of ketamine and lithium to PBMCs from individuals not previously exposed to ketamine *in vivo*.

Following ketamine/insulin stimulation *in vitro*, a significant increase in expression of each of the aforementioned insulin signalling proteins was observed (Figure 18). This result is similar to that observed for PBMCs isolated from patients after ketamine treatment in Examples 2 and 3.

CLAIMS

1. A method of predicting the responsiveness of a neurological disease, disorder or condition in a subject to an agent, said method including the step of determining a level of insulin sensitivity of one or a plurality of blood-derived cells of the subject that have been treated with the agent, wherein the level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased or decreased responsiveness of the neurological disease, disorder or condition to the agent.

2. The method of Claim 1, wherein a relatively increased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent; and/or a relatively decreased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively decreased responsiveness of the neurological disease, disorder or condition to the agent.

3. The method of Claim 1 or Claim 2, further including the step of treating the neurological disease, disorder or condition in the subject.

4. A method of treating a neurological disease, disorder or condition in a subject, the method including the step of determining a level of insulin sensitivity of one or a plurality of blood-derived cells of the subject that have been treated with an agent and based on the determination made, initiating, continuing, modifying or discontinuing treatment with the agent.

5. The method of Claim 3 or Claim 4, further including the step of administering to the subject a therapeutically effective amount of an insulin sensitizing agent.

6. The method of any one of Claims 3 to 5, further including the step of administering to the subject a therapeutically effective amount of the agent when the level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent.

7. The method of Claim 3 or Claim 4, further including the step of administering to the subject therapeutically effective amounts of: (a) the agent; and (b) an insulin sensitizing agent when the level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively decreased responsiveness of the neurological disease, disorder or condition to the agent.

8. The method of any one of the preceding claims, further including the initial steps of:

(a) treating the one or plurality of blood-derived cells with an effective amount of the agent; and/or

(b) treating the one or plurality of blood-derived cells with an effective amount of an insulin agonist.

9. The method of any one of the preceding claims, wherein determining the level of insulin sensitivity comprises determining one or more of a level of insulin signalling, a level of glucose uptake and a level of mitochondrial activity of the one or plurality of blood-derived cells of the subject.

10. The method of Claim 9, wherein determining the level of insulin signalling comprises determining an activity and/or expression level of one or more proteins selected from the group consisting of mTOR, Akt, AMPK, CaMKII, GSK3α, GSK3β and phosphorylated forms thereof.

11. The method of Claim 9 or Claim 10, wherein determining the level of mitochondrial activity comprises determining levels of adenosine triphosphate (ATP) and/or glycerol phosphorylation in the one or plurality of blood-derived cells.

12. The method of Claim 11, further including the initial step of isolating the one or plurality of blood-derived cells from the subject.

13. A kit for predicting the responsiveness of a neurological disease, disorder or condition in a subject to an agent, the kit comprising at least one reagent capable of determining a level of insulin sensitivity of one or a plurality of blood-derived cells of the subject that have been treated with the agent, wherein the level of insulin sensitivity

of the one or plurality of blood-derived cells indicates or correlates with relatively increased or decreased responsiveness of the neurological disease, disorder or condition to the agent.

14. The kit of Claim 13, wherein a relatively increased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively increased responsiveness of the neurological disease, disorder or condition to the agent; and/or a relatively decreased level of insulin sensitivity of the one or plurality of blood-derived cells indicates or correlates with relatively decreased responsiveness of the neurological disease, disorder or condition to the agent.

15. The kit of Claim 13 or Claim 14, further comprising reference data for correlating the level of insulin sensitivity of the one or plurality of blood-derived cells with responsiveness of the neurological disease, disorder or condition to the agent.

16. The kit of Claim 15, wherein the reference data is on a computer-readable medium.

17. The kit of any one of Claims 13 to 16, for use in the method of any one of Claims 1 to 12.

18. The method of any one of Claims 1 to 12 and the kit of any one of Claims 13 to 17, wherein the agent is or comprises a glutamatergic modulator.

19. The method or kit of Claim 18, wherein the glutamatergic modulator is or comprises an N-methyl-D-aspartate (NMDA) receptor antagonist, an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, a metabotropic glutamate receptor antagonist, a kainate receptor antagonist, a glycine transportor-1 antagonist, a dopamine modulator, an immune modulator, an anti-inflammatory agent and/or a lithium compound or salt.

20. A method of screening, designing, engineering or otherwise producing an agent for treating a neurological disease, disorder or condition in a subject, said method including the step of determining whether a candidate molecule is capable of at least

partly increasing or enhancing a level of insulin sensitivity in one or a plurality of blood-derived cells treated with effective amounts of the candidate molecule.

21. The method of Claim 20, wherein determining whether the candidate molecule is capable of at least partly increasing or enhancing the level of insulin sensitivity in the one or plurality of PBMCs comprises determining one or more of a level of insulin signalling, a level of glucose uptake and a level of mitochondrial activity of the one or plurality of PBMCs of the subject.

22. The method or kit of any one of the preceding claims, wherein the one or plurality of blood-derived cells are or comprise peripheral blood mononuclear cells (PBMCs).

23. The method or kit of any one of the preceding claims, wherein the subject is a human.

24. The method or kit of any one of the preceding claims, wherein the neurological disease, disorder or condition is or comprises: a mood disorder, including depressive disorders and bipolar disorders; post-traumatic stress disorder; dementia; Alzheimer's disease; an anxiety disorder, including panic disorders, phobias, obsessive-compulsive disorders, stress disorders and generalized anxiety disorders; suicidality; and any combination thereof.

25. An agent screened, designed, engineered or otherwise produced according to the method of any one of Claims 20 to 24.

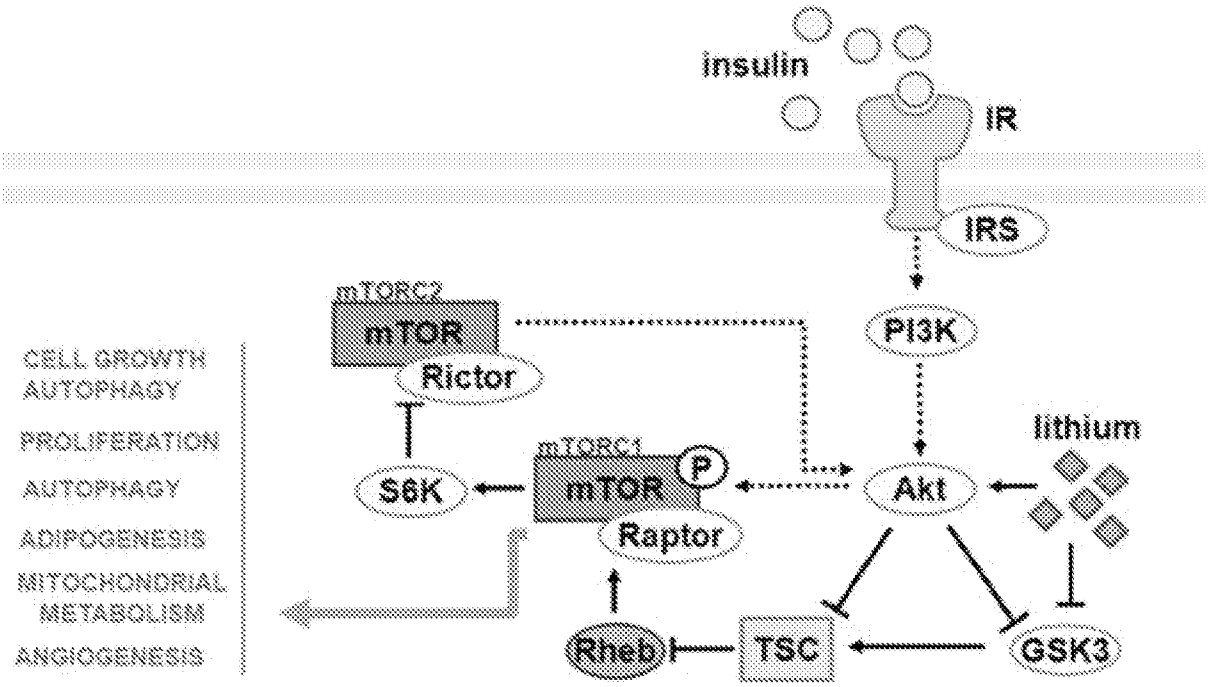

insulin
IR
IRS
PI3K
mTORC2
mTOR
Rictor
mTORC1
mTOR
P
Raptor
S6K
Akt
lithium
Rheb
TSC
GSK3
CELL GROWTH
AUTOPHAGY
PROLIFERATION
AUTOPHAGY
ADIPOGENESIS
MITOCHONDRIAL
METABOLISM
ANGIOGENESIS

FIG. 1

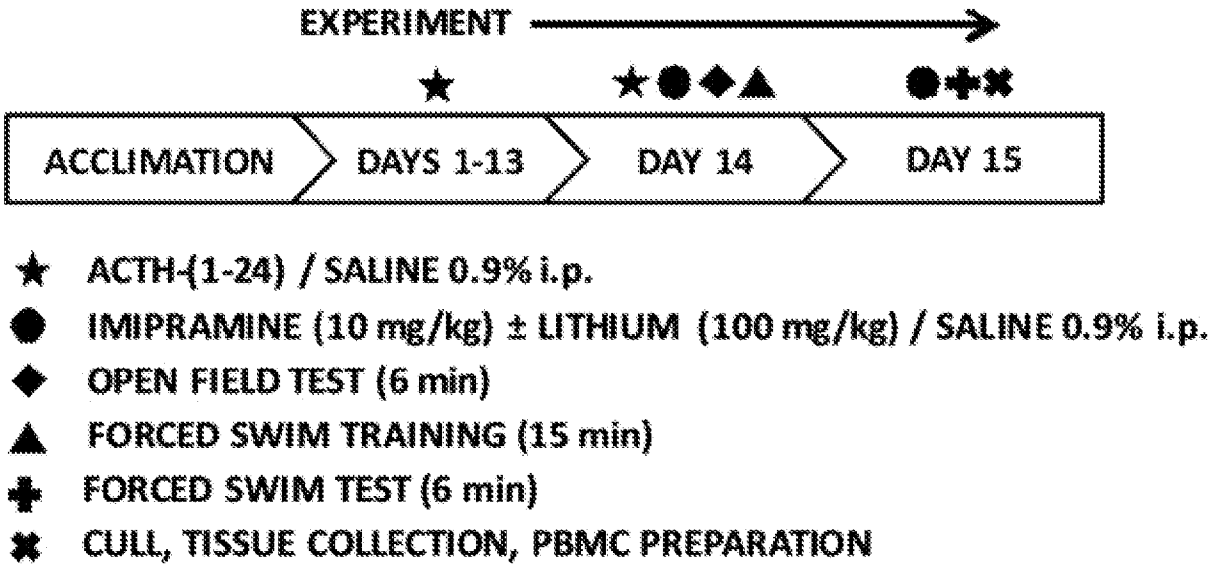

EXPERIMENT
ACCLIMATION
DAYS 1-13
DAY 14
DAY 15
★ ACTH-(1-24) / SALINE 0.9% i.p.
● IMIPRAMINE (10 mg/kg) ± LITHIUM (100 mg/kg) / SALINE 0.9% i.p.
◆ OPEN FIELD TEST (6 min)
▲ FORCED SWIM TRAINING (15 min)
✚ FORCED SWIM TEST (6 min)
✖ CULL, TISSUE COLLECTION, PBMC PREPARATION

FIG. 2

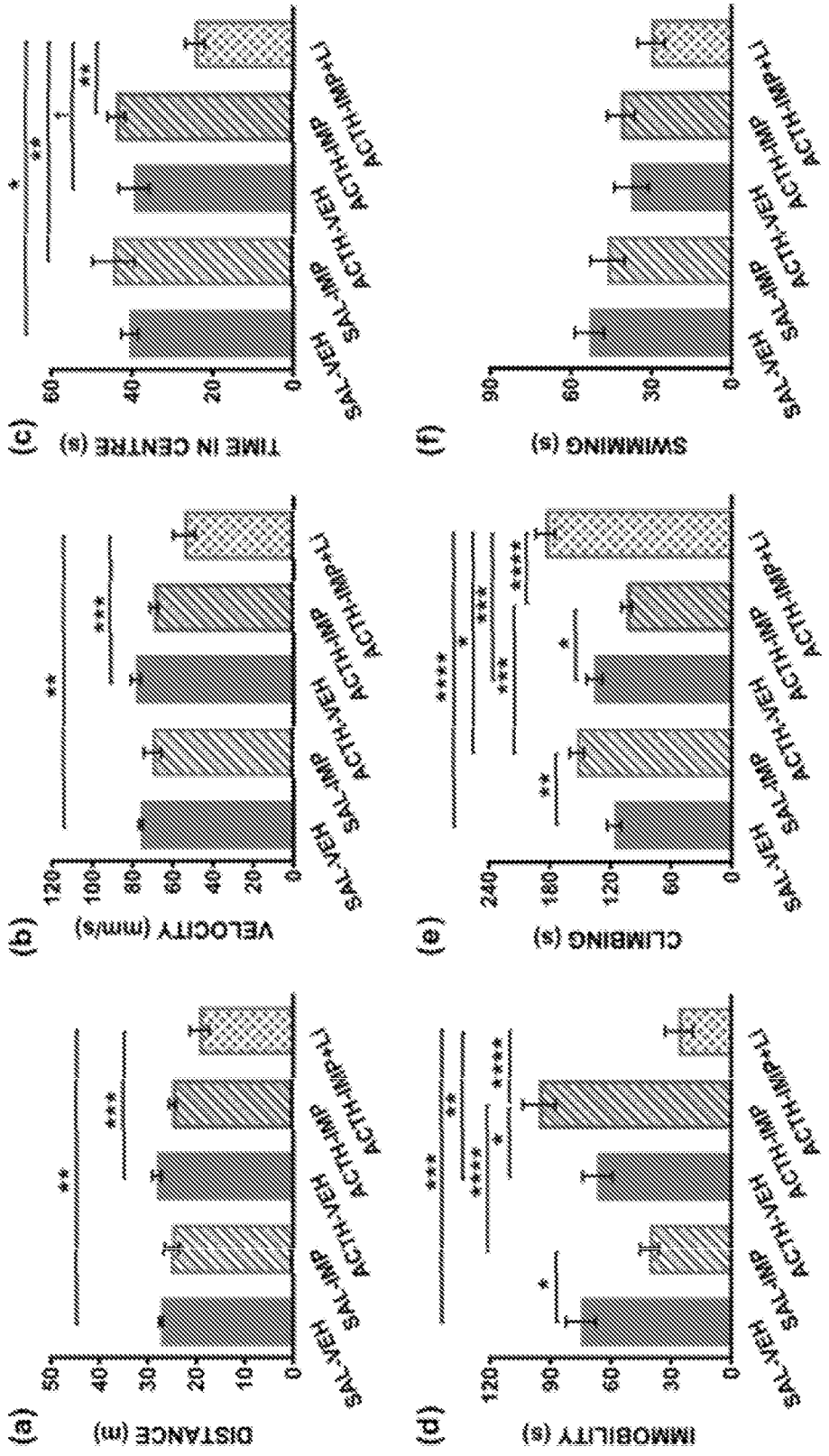

(a)
DISTANCE (m)
(b)
VELOCITY (mm/s)
(c)
TIME IN CENTRE (s)
(d)
IMMOBILITY (s)
(e)
CLIMBING (s)
(f)
SWIMMING (s)
SAL-VEH
SAL-AMP
ACTH-VEH
ACTH-AMP
ACTH-AMP+LI

FIG. 3

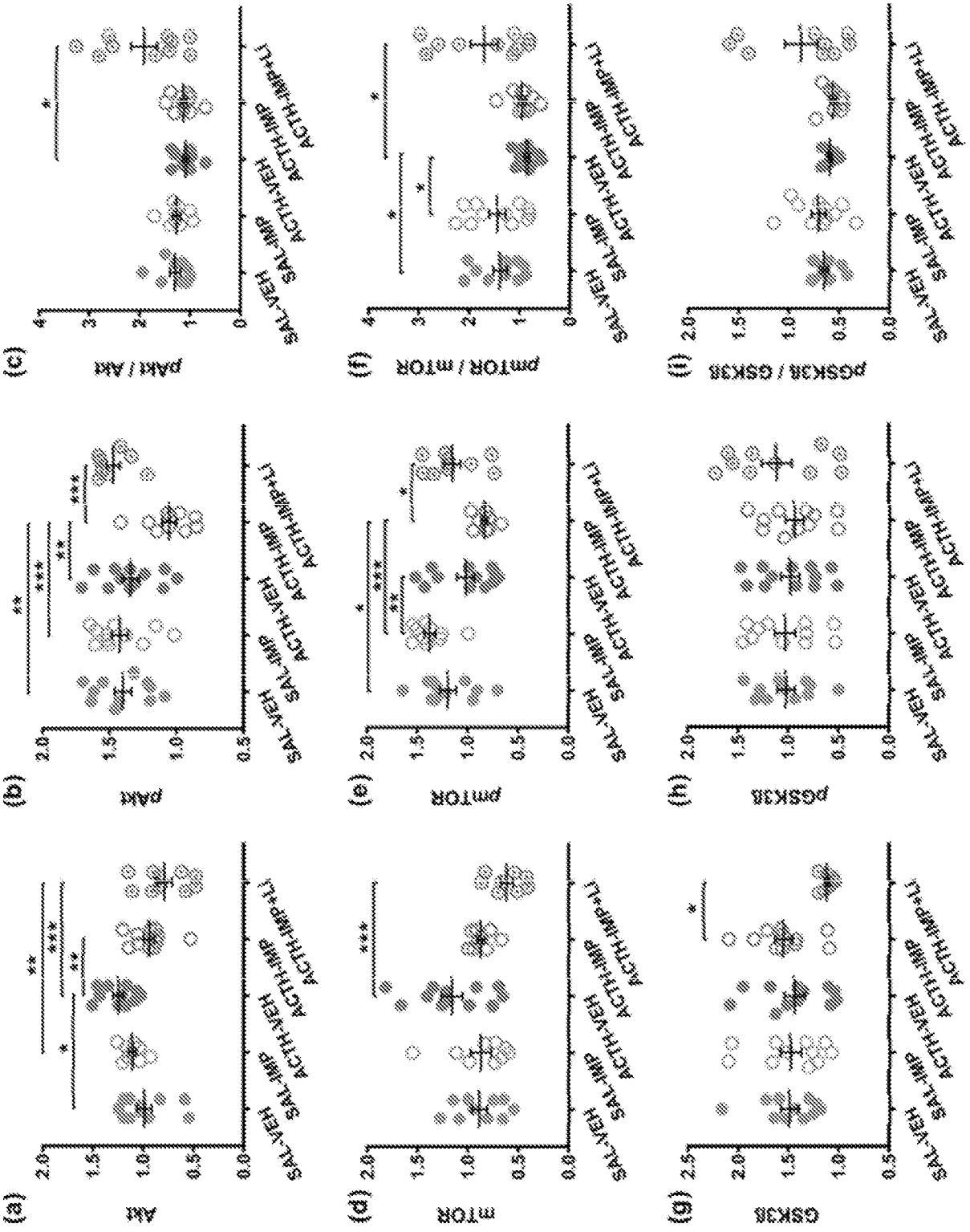

(a)
Akt
(b)
pAkt
(c)
pAkt / Akt
(d)
mTOR
(e)
pmTOR
(f)
pmTOR / mTOR
(g)
GSK3β
(h)
pGSK3β
(i)
pGSK3β / GSK3β
SAL-VEH
SAL-IMP
ACTH-VEH
ACTH-IMP
SAL-VEH
SAL-IMP
ACTH-VEH
ACTH-IMP

FIG. 4

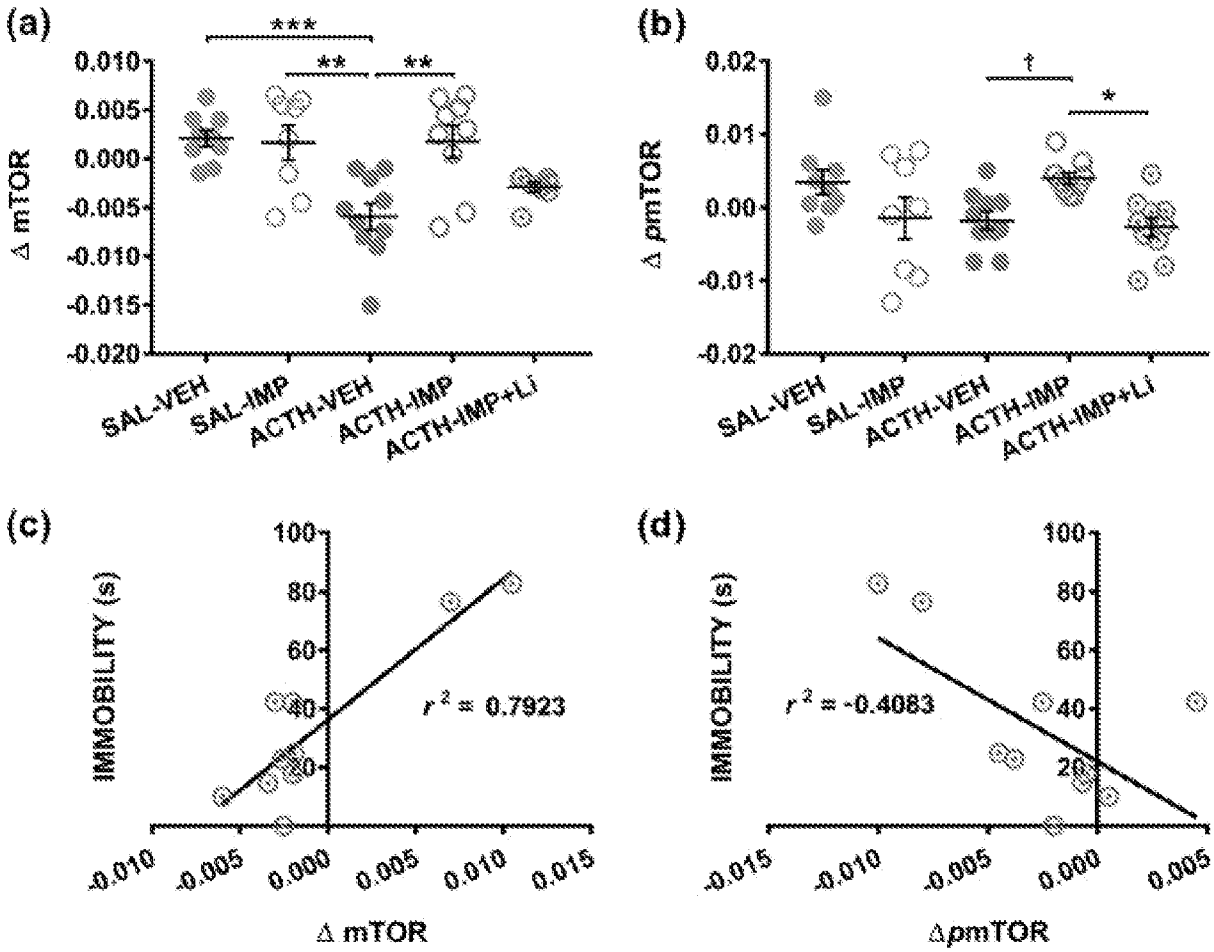

(a)
***
**
**
0.010
0.005
0.000
-0.005
-0.010
-0.015
-0.020
Δ mTOR
SAL-VEH
SAL-IMP
ACTH-VEH
ACTH-IMP
ACTH-IMP+Li
(b)
†
*
0.02
0.01
0.00
-0.01
-0.02
Δ pmTOR
SAL-VEH
SAL-IMP
ACTH-VEH
ACTH-IMP
ACTH-IMP+Li
(c)
100
80
60
40
20
IMMOBILITY (s)
r² = 0.7923
-0.010
-0.005
0.000
0.005
0.010
0.015
Δ mTOR
(d)
100
80
60
40
20
IMMOBILITY (s)
r² = -0.4083
-0.015
-0.010
-0.005
0.000
0.005
ΔpmTOR

FIG. 5

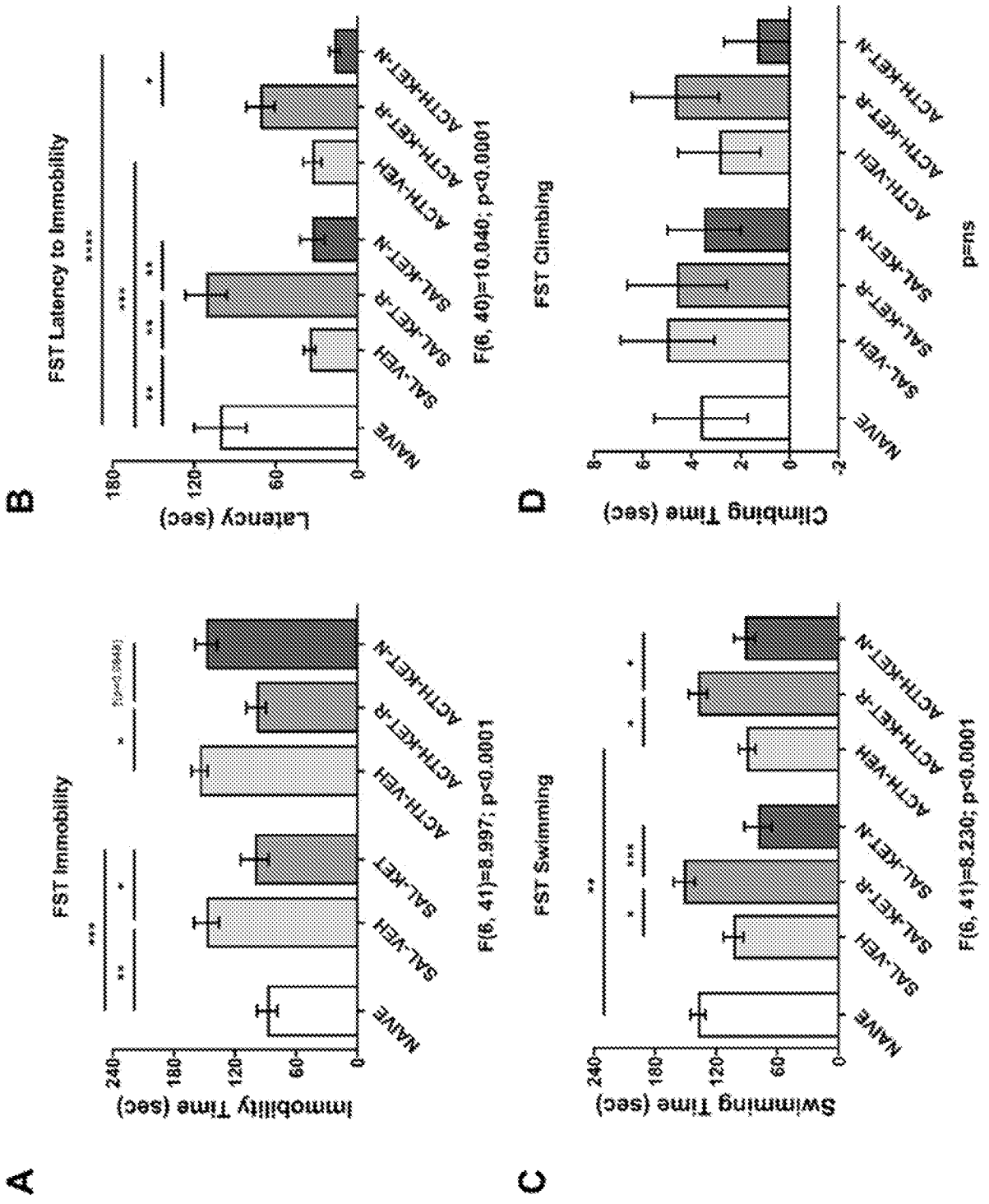

A
FST Immobility
Immobility Time (sec)
F(6, 41)=8.997; p<0.0001
B
FST Latency to Immobility
Latency (sec)
F(6, 40)=10.040; p<0.0001
C
FST Swimming
Swimming Time (sec)
F(6, 41)=8.230; p<0.0001
D
FST Climbing
Climbing Time (sec)
p=ns
NAIVE
SAL-VEH
SAL-KET-R
SAL-KET-N
ACTH-VEH
ACTH-KET-R
ACTH-KET-N

FIG. 6

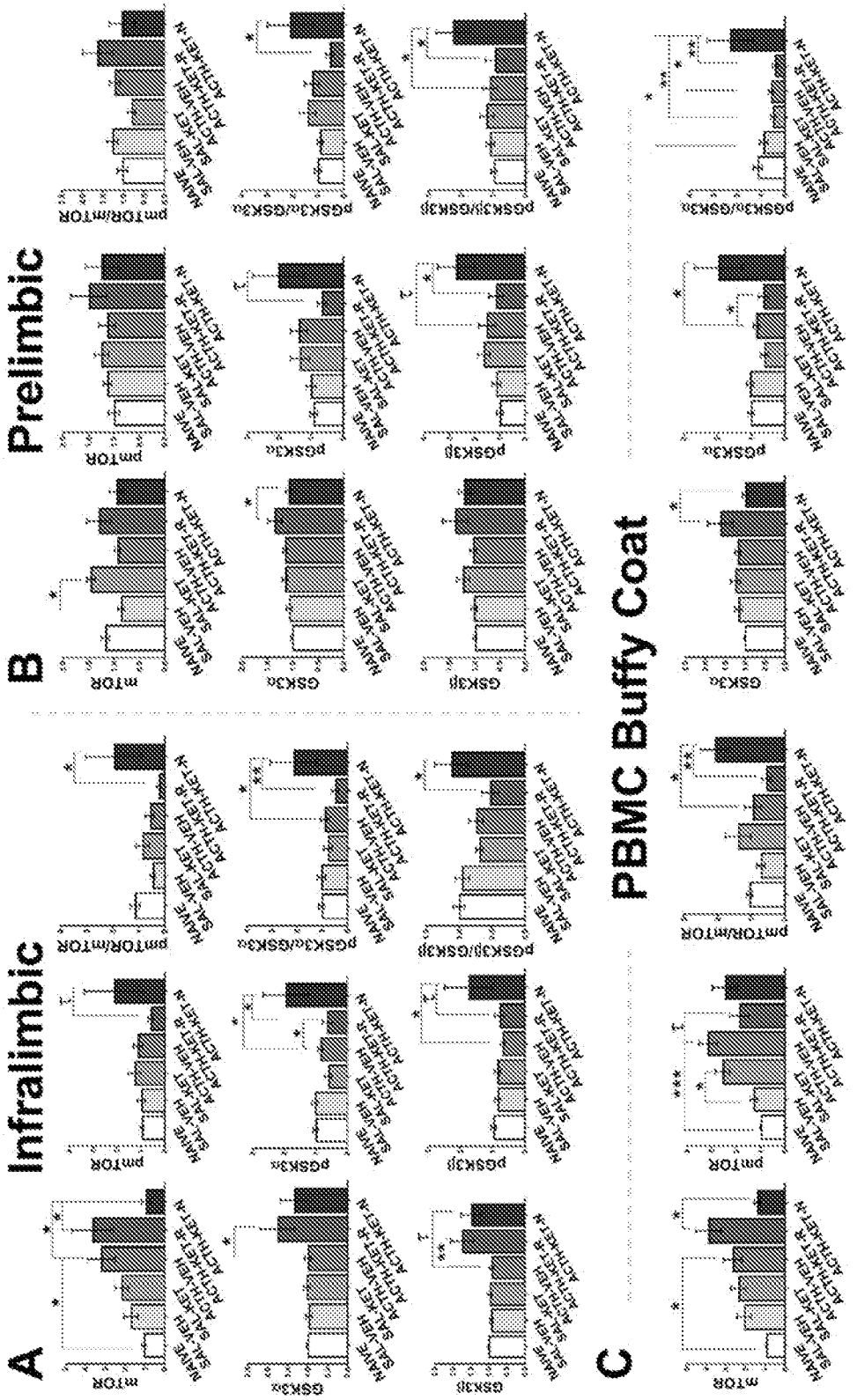

A
Infralimbic
B
Prelimbic
C
PBMC Buffy Coat
mTOR
pmTOR
pmTOR/mTOR
GSK3α
pGSK3α
pGSK3α/GSK3α
GSK3β
pGSK3β
pGSK3β/GSK3β
NAIVE SAL-VEH ACTH-VEH SAL-KET ACTH-KET-S ACTH-KET-R

FIG. 7

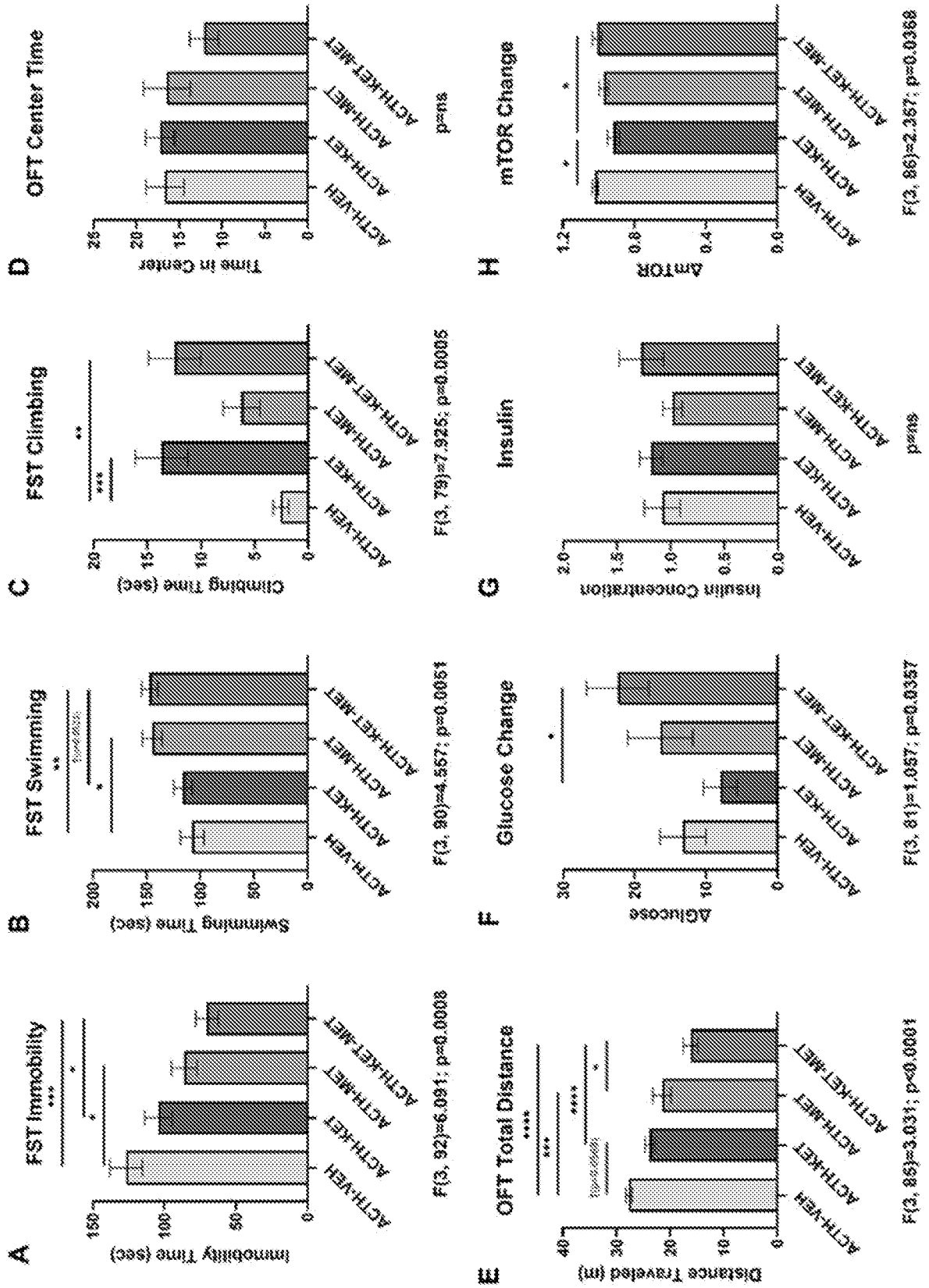

A
FST Immobility
Immobility Time (sec)
F(3, 92)=6.691; p=0.0008
B
FST Swimming
Swimming Time (sec)
F(3, 90)=4.557; p=0.0051
C
FST Climbing
Climbing Time (sec)
F(3, 79)=7.925; p=0.0005
D
OFT Center Time
Time in Center
p=ns
E
OFT Total Distance
Distance Traveled (m)
F(3, 86)=3.031; p<0.0001
F
Glucose Change
ΔGlucose
F(3, 81)=1.057; p=0.0357
G
Insulin
Insulin Concentration
p=ns
H
mTOR Change
ΔmTOR
F(3, 86)=2.367; p=0.0388
ACTH-VEH
ACTH-KET
ACTH-MET
ACTH-KET-MET

FIG. 8

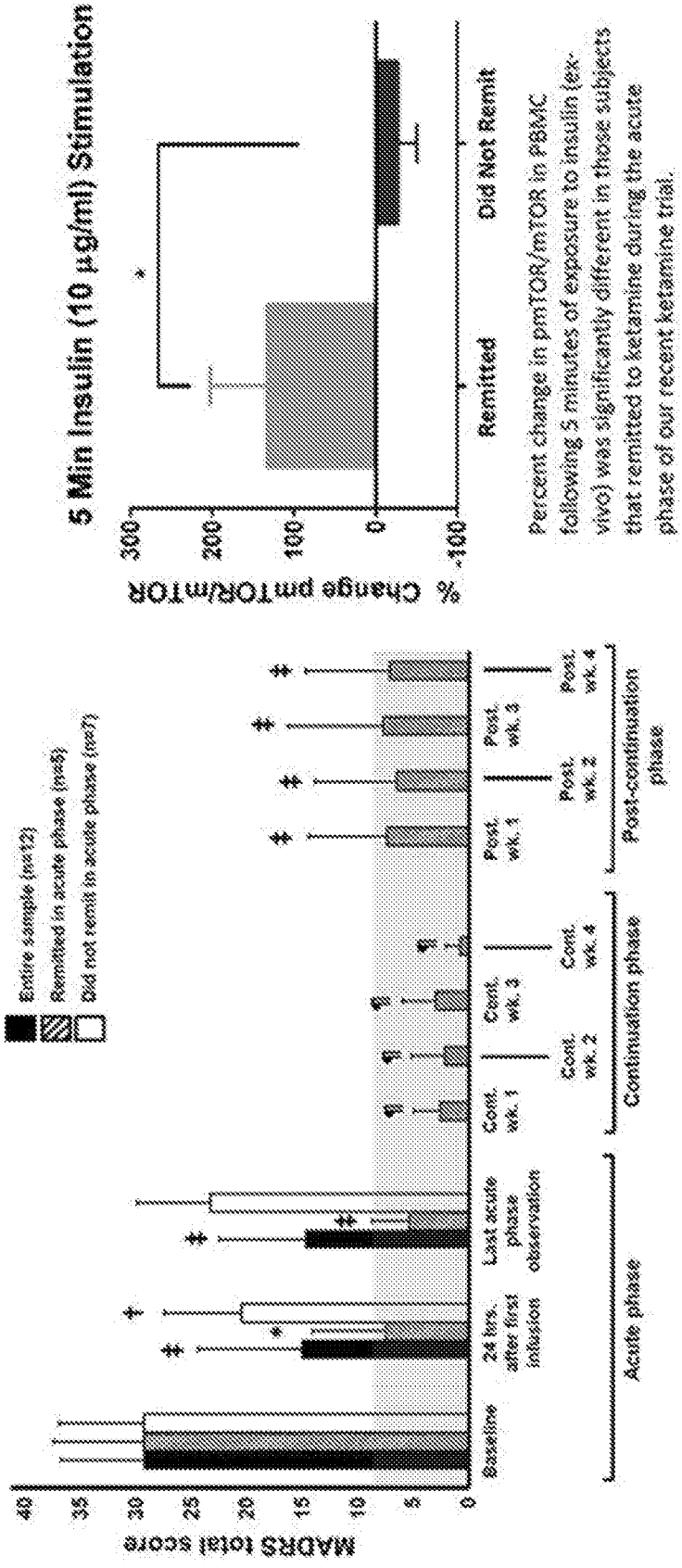

5 Min Insulin (10 µg/ml) Stimulation
% Change pmTOR/mTOR
300
200
100
0
-100
*
Remitted
Did Not Remit
Percent change in pmTOR/mTOR in PBMC following 5 minutes of exposure to insulin (ex-vivo) was significantly different in those subjects that remitted to ketamine during the acute phase of our recent ketamine trial.
Entire sample (n=12)
Remitted in acute phase (n=5)
Did not remit in acute phase (n=7)
MADRS total score
40
35
30
25
20
15
10
5
0
Baseline
24 hrs. after first infusion
Last acute phase observation
Cont. wk. 1
Cont. wk. 2
Cont. wk. 3
Cont. wk. 4
Post. wk. 1
Post. wk. 2
Post. wk. 3
Post. wk. 4
Acute phase
Continuation phase
Post-continuation phase

FIG. 9

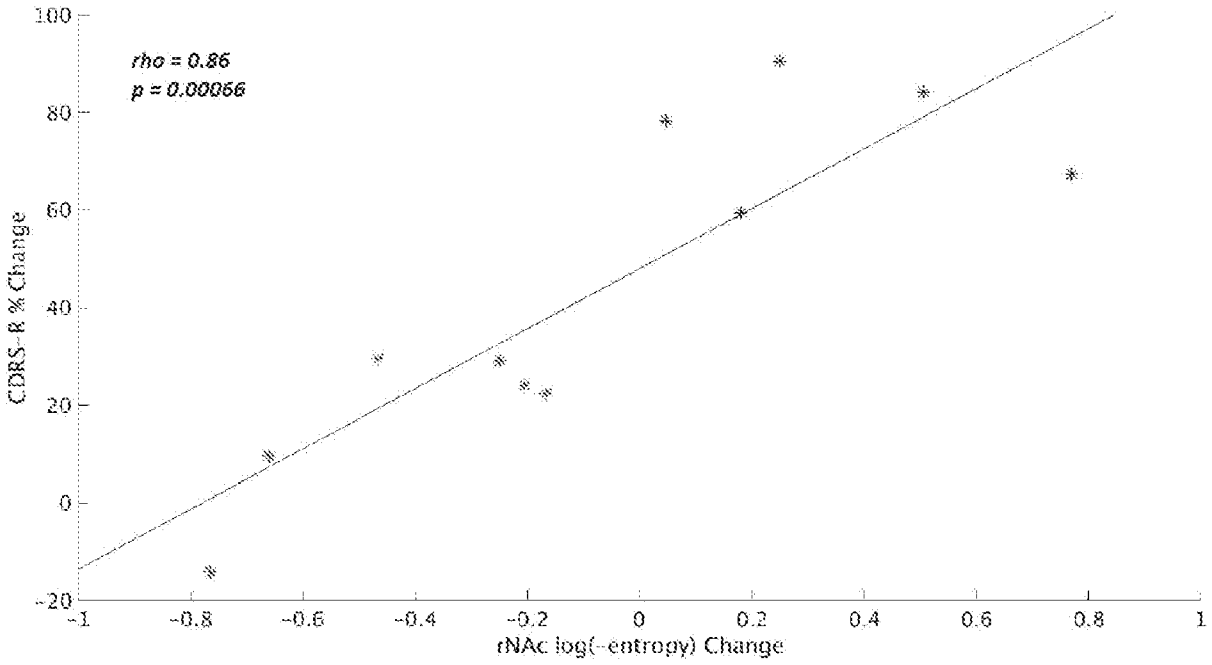

rho = 0.86
p = 0.00066
CDRS-R % Change
rNAc log(−entropy) Change
100
80
60
40
20
0
−20
−1
−0.8
−0.6
−0.4
−0.2
0
0.2
0.4
0.6
0.8
1

FIG. 10

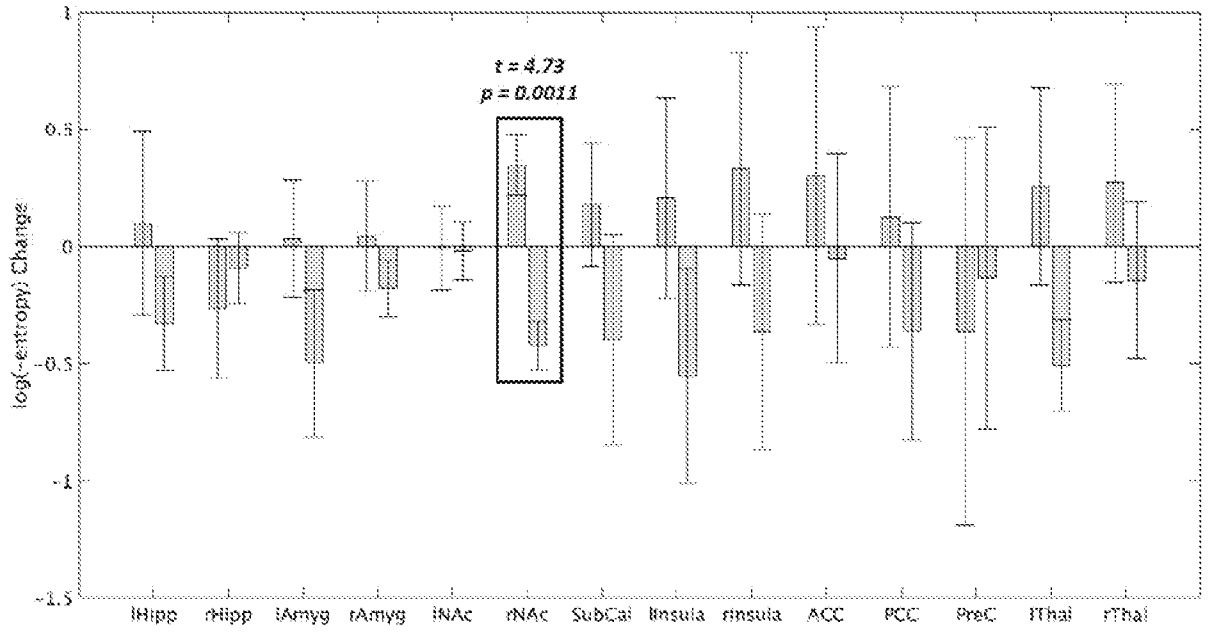

t = 4.73
p = 0.0011
log(−entropy) Change
1
0.5
0
−0.5
−1
−1.5
lHipp
rHipp
lAmyg
rAmyg
lNAc
rNAc
SubCal
lInsula
rInsula
ACC
PCC
PreC
lThal
rThal

FIG. 11

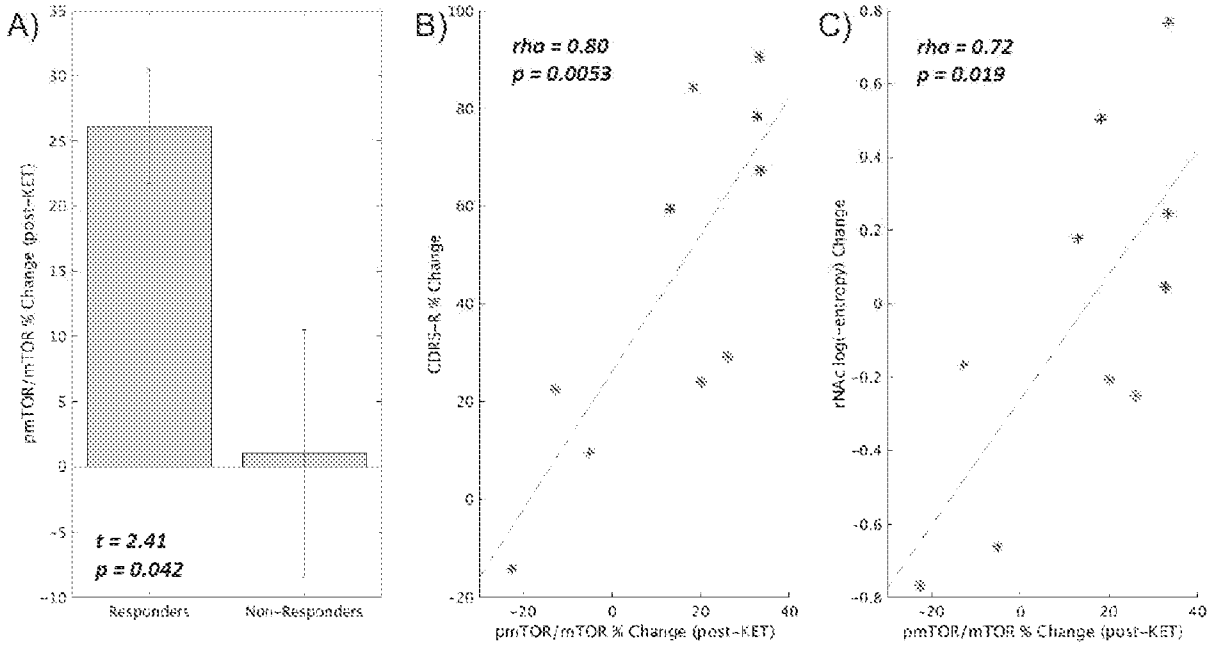

A)
pmTOR/mTOR % Change (post-KET)
t = 2.41
p = 0.042
Responders
Non-Responders
B)
rho = 0.80
p = 0.0053
CDRS-R % Change
pmTOR/mTOR % Change (post-KET)
C)
rho = 0.72
p = 0.019
rNAC (log(-entropy)) Change
pmTOR/mTOR % Change (post-KET)

FIG. 12

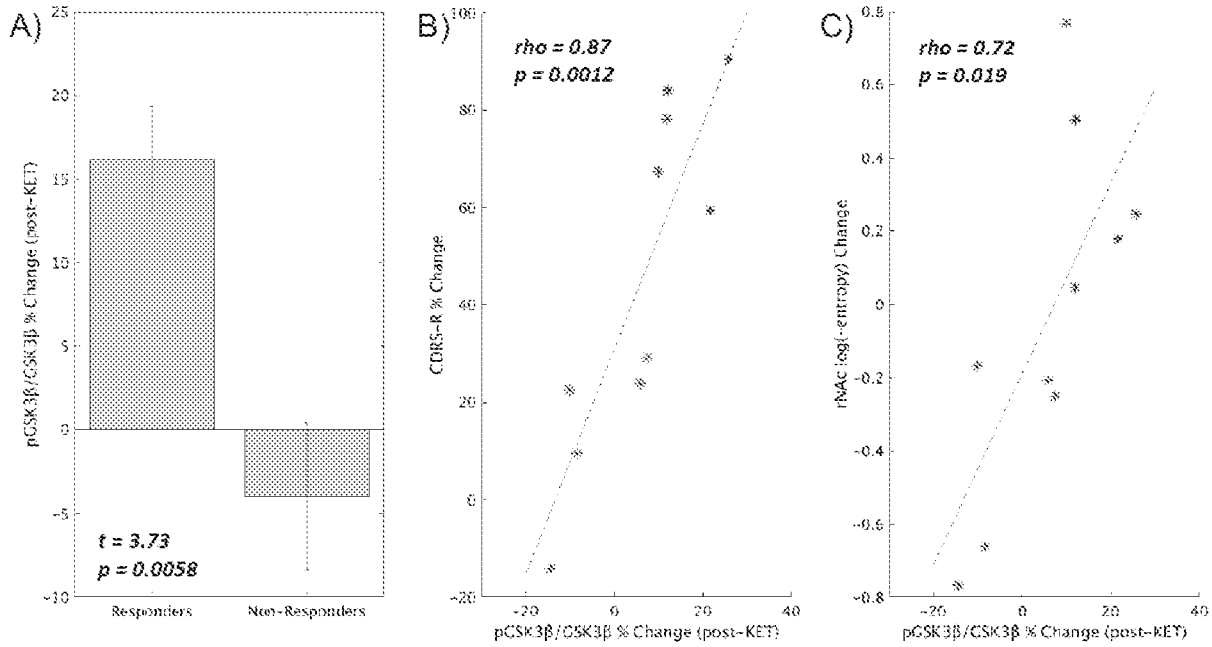

A)
pGSK3β/GSK3β % Change (post-KET)
t = 3.73
p = 0.0058
Responders
Non-Responders
B)
rho = 0.87
p = 0.0012
CDRS-R % Change
pGSK3β/GSK3β % Change (post-KET)
C)
rho = 0.72
p = 0.019
rNAC (log(-entropy)) Change
pGSK3β/GSK3β % Change (post-KET)

FIG. 13

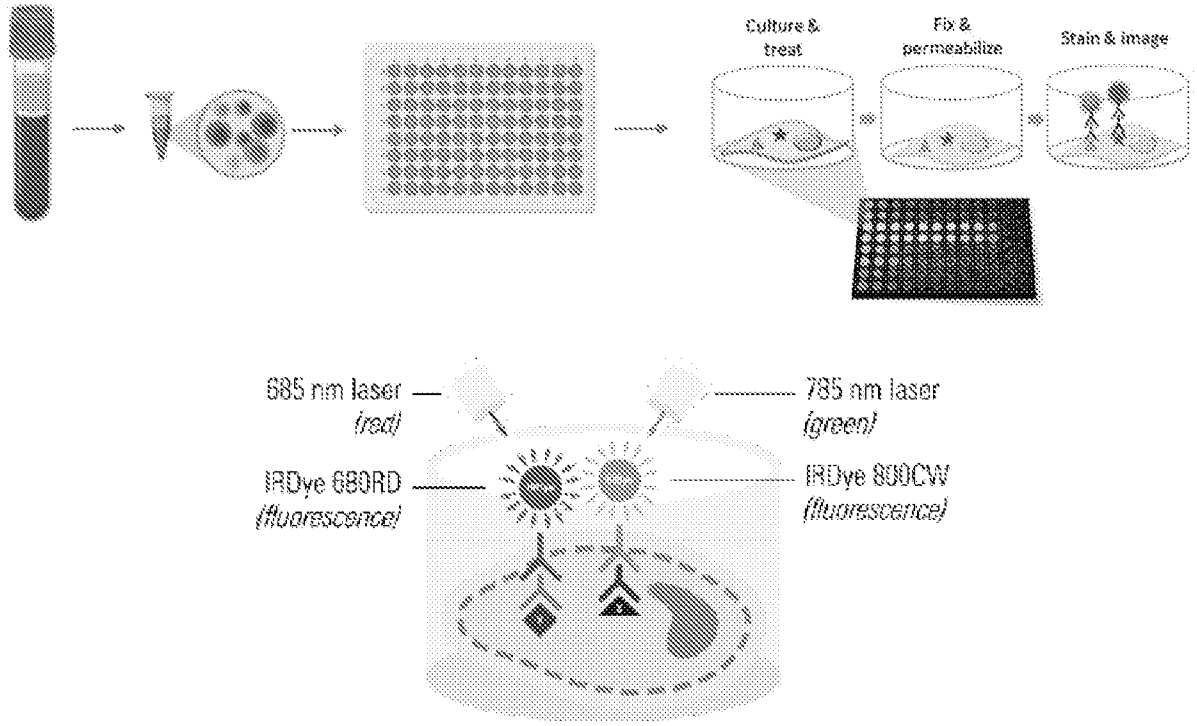

Culture & treat
Fix & permeabilize
Stain & image
685 nm laser (red)
785 nm laser (green)
IRDye 680RD (fluorescence)
IRDye 800CW (fluorescence)

FIG. 14

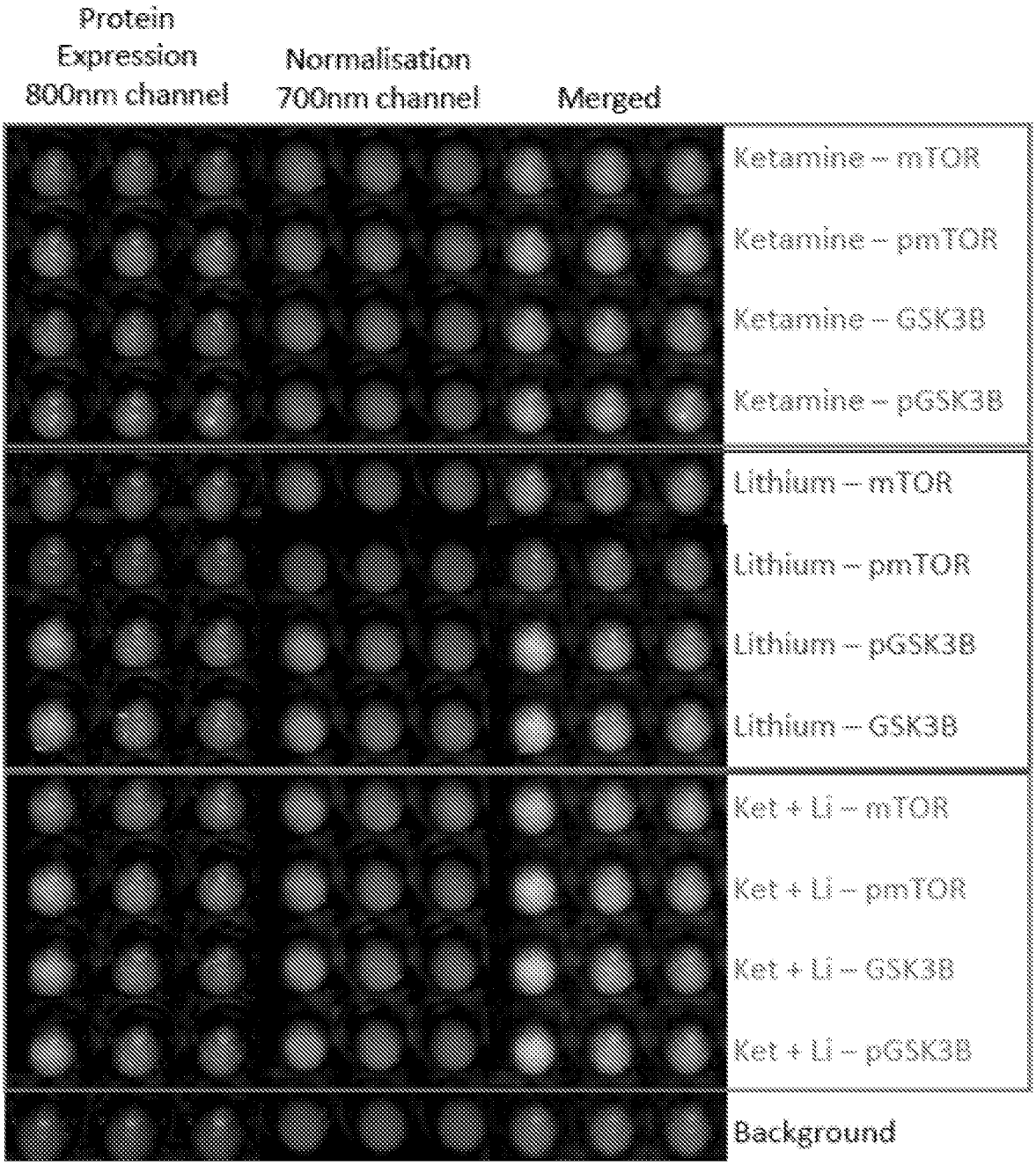

Protein Expression 800nm channel
Normalisation 700nm channel
Merged
Ketamine – mTOR
Ketamine – pmTOR
Ketamine – GSK3B
Ketamine – pGSK3B
Lithium – mTOR
Lithium – pmTOR
Lithium – pGSK3B
Lithium – GSK3B
Ket + Li – mTOR
Ket + Li – pmTOR
Ket + Li – GSK3B
Ket + Li – pGSK3B
Background

FIG. 15

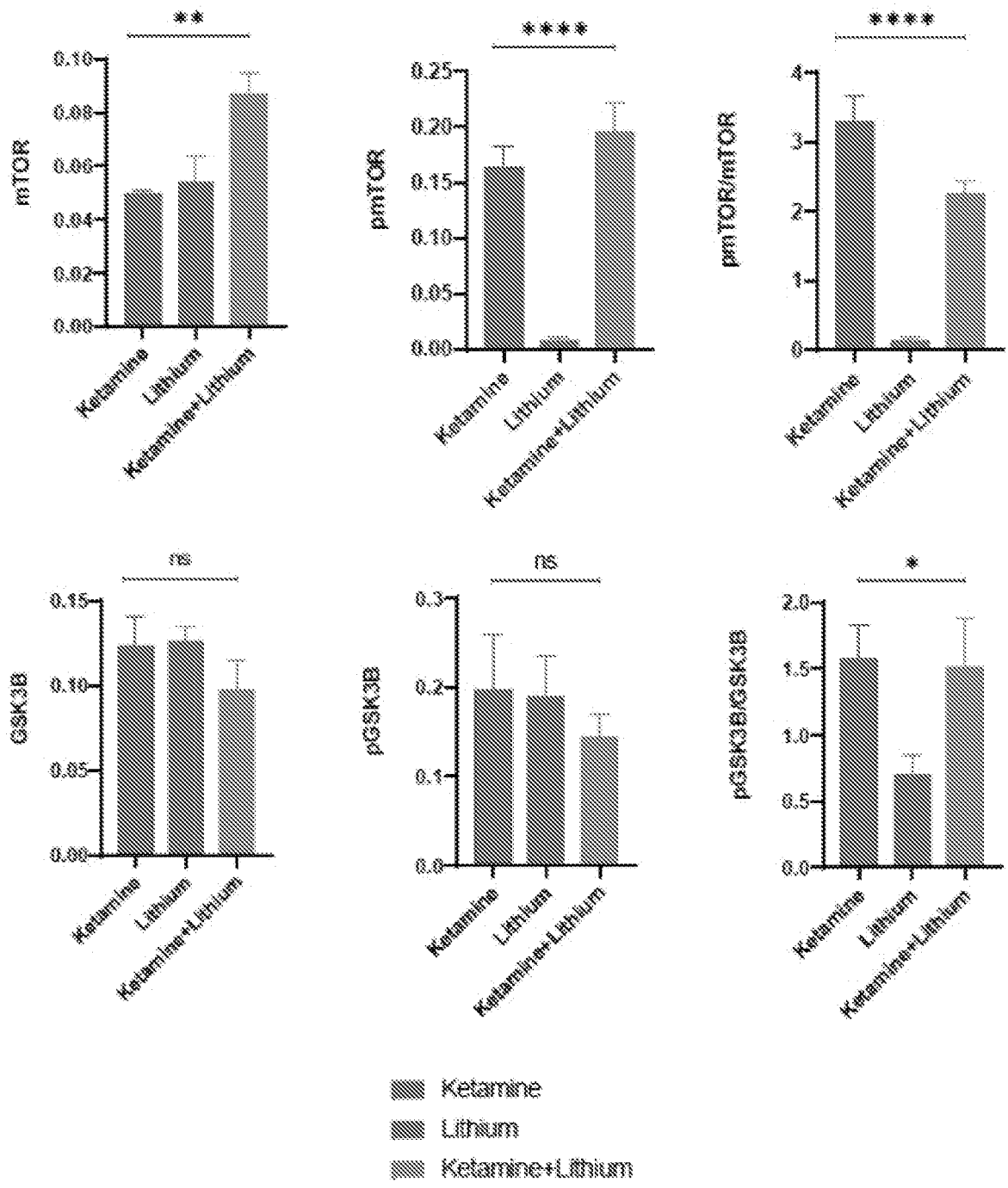

**
mTOR
0.10
0.08
0.06
0.04
0.02
0.00
Ketamine
Lithium
Ketamine+Lithium
****
pmTOR
0.25
0.20
0.15
0.10
0.05
0.00
Ketamine
Lithium
Ketamine+Lithium
****
pmTOR/mTOR
4
3
2
1
0
Ketamine
Lithium
Ketamine+Lithium
ns
GSK3B
0.15
0.10
0.05
0.00
Ketamine
Lithium
Ketamine+Lithium
ns
pGSK3B
0.3
0.2
0.1
0.0
Ketamine
Lithium
Ketamine+Lithium
*
pGSK3B/GSK3B
2.0
1.5
1.0
0.5
0.0
Ketamine
Lithium
Ketamine+Lithium
Ketamine
Lithium
Ketamine+Lithium

FIG. 15 cont'd

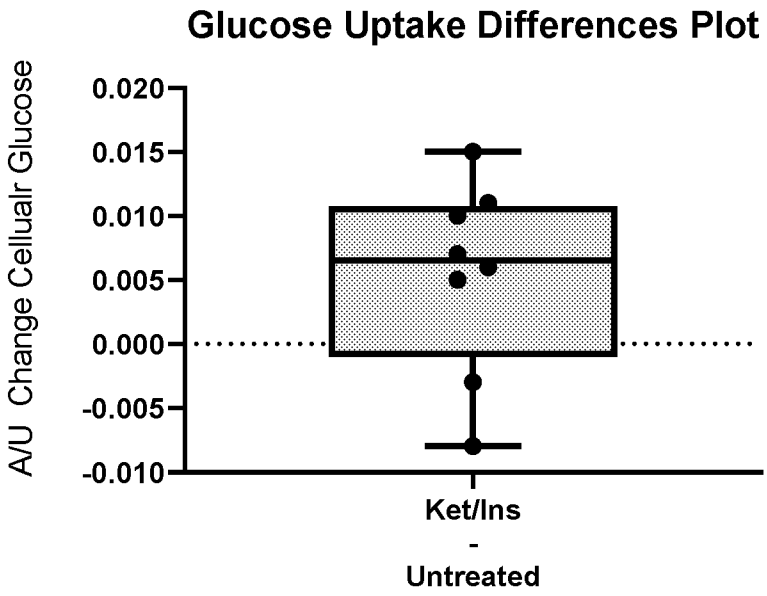

Glucose Uptake Differences Plot
A/U Change Cellualr Glucose
0.020
0.015
0.010
0.005
0.000
-0.005
-0.010
Ket/Ins
-
Untreated

FIG. 16

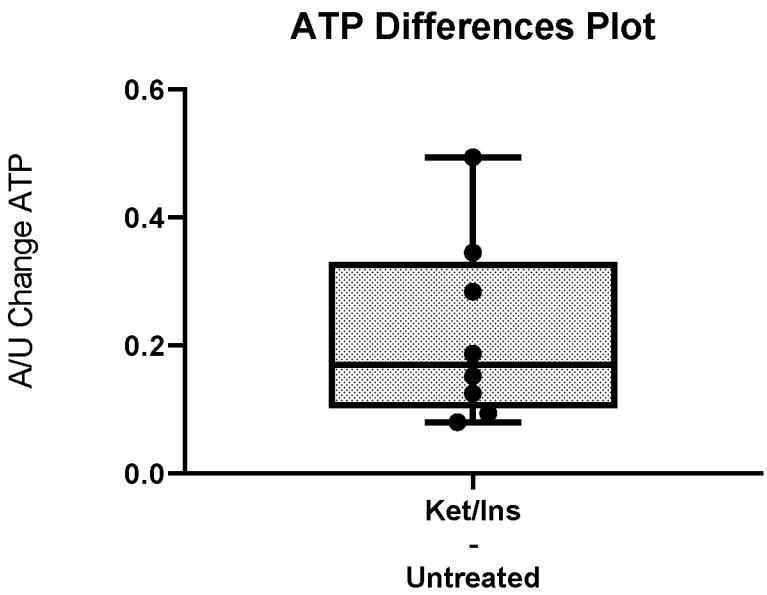

ATP Differences Plot
A/U Change ATP
0.6
0.4
0.2
0.0
Ket/Ins
-
Untreated

FIG. 17

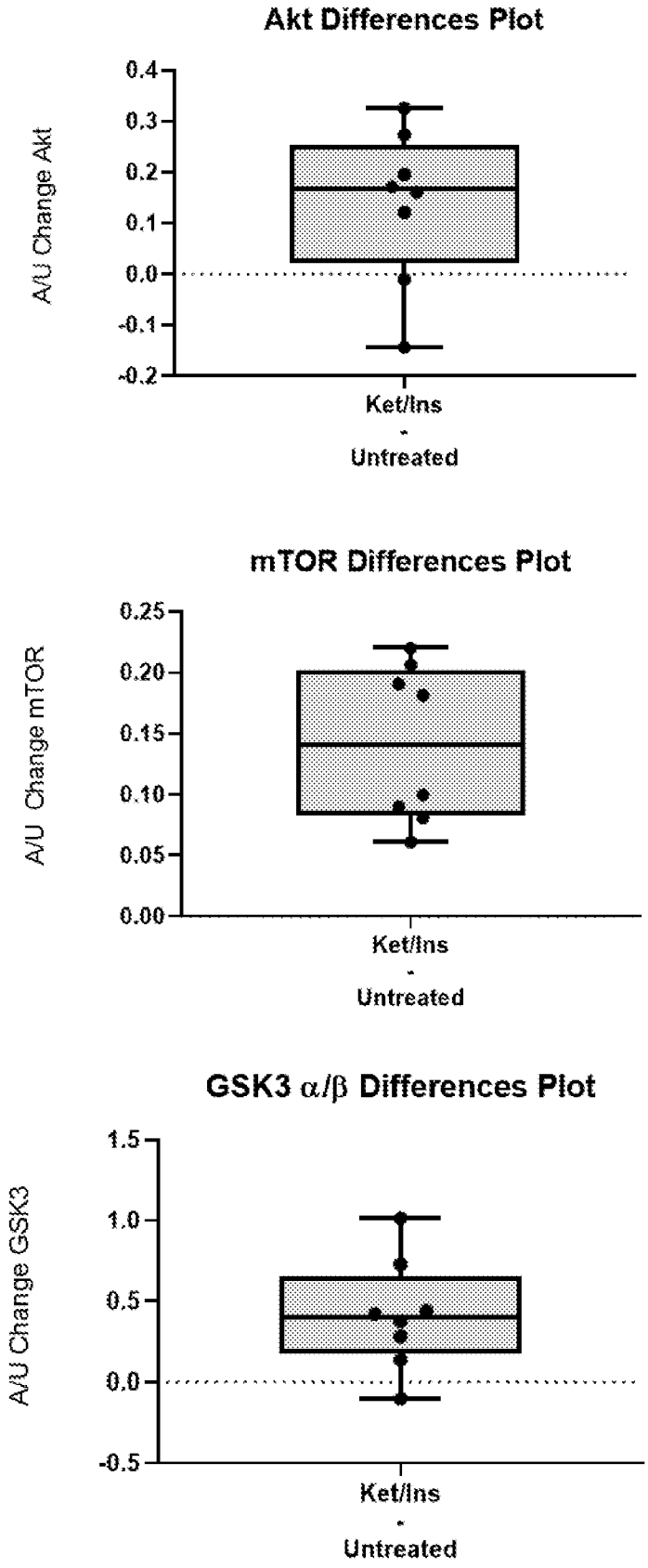

Akt Differences Plot
A/U Change Akt
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
Ket/Ins
-
Untreated
mTOR Differences Plot
A/U Change mTOR
0.25
0.20
0.15
0.10
0.05
0.00
Ket/Ins
-
Untreated
GSK3 α/β Differences Plot
A/U Change GSK3
1.5
1.0
0.5
0.0
-0.5
Ket/Ins
-
Untreated

FIG. 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2020/050240

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/53 (2006.01) ***A61K 33/14 (2006.01)*** ***A61K 31/135 (2006.01)*** ***A61K 31/55 (2006.01)*** ***A61K 31/155 (2006.01)*** ***A61P 25/24 (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)

Search conducted in databases WPIAP, EPODOC, MEDLINE, full text English language databases, CAPLUS, BIOSIS and EMBASE using the following keywords: Peripheral blood mononuclear cell, PBMC, T cell, B cell, plasma cell, NK cell lymphocyte, monocyte, depression, anxiety, bipolar, mood, post-traumatic stress, suicide, obsessive compulsive, stress disorder, phobia, panic, neurological, neurodegenerative, Alzheimer's, dementia, Parkinson's, schizophrenia, insulin, glucose uptake, mTOR, FRAP, RAFT1, Akt, CaMKII, GSK3α, GSK3β, mitochondria, ATP, glycerol phosphorylation and related terms.

Search of the applicant and inventor names using databases AusPat, Patentscope, PubMed and IP Australia Internal databases.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 May 2020	20 May 2020
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Shawn Lyons AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832081

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WALKER A.J. et al., "Insulin-stimulated mTOR activation in peripheral blood mononuclear cells associated with early treatment response to lithium augmentation in rodent model of antidepressant-resistance", Translational Psychiatry, March 15 2019, Vol. 9, No. 1, 113 See the abstract; the section entitled "Insulin-evoked PBMCs mTOR activation is upregulated by lithium and correlates with antidepressant response in ACTH pre-treated rats" on page 9; Figure 5; the section entitled "PBMC insulin challenge" on page 6; Figure 1	1-25
X	TYE S. et al., "Differential MTOR activation and insulin signaling in peripheral blood distinguishes lithium responsive and non-responsive bipolar patients", Bipolar Disorders, March 2018, Volume 20, Issue S1, Special Issue: 20th Annual Conference of the International Society for Bipolar Disorders, Abstract RC-06 See the entire abstract	1-25
X	PRICE J.B. et al., "Prospective Biomarker for Early Treatment Response to Lithium in Antidepressant-resistant Animals", Biological Psychiatry, May 2016, Vol. 79, No. 9, Supplement 1, page 199S, Abstract 577 See the entire abstract	1-25
A	ZEMDEGS J . et al., "Metformin Promotes Anxiolytic and Antidepressant-Like Responses in Insulin-Resistant Mice by Decreasing Circulating Branched-Chain Amino Acids", The Journal of Neuroscience, 24 July 2019, Vol. 39, No. 30, pages 5935-5948 See the abstract; Figure 6	5 and 7
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This Annex lists known 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/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.