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First-in-class thyrotropin-releasing hormone (TRH)-based compound binds to a pharmacologically distinct TRH receptor subtype in human brain and is effective in neurodegenerative models

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ABSTRACT

JAK4D, a first-in-class thyrotropin-releasing hormone (TRH)-based compound, is a prospective therapeutic candidate offering a multifaceted approach to treating neurodegeneration and other CNS conditions. The purpose of these studies was to determine the ability of JAK4D to bind to TRH receptors in human brain and to evaluate its neuropharmacological effects in neurodegenerative animal models. Additionally, JAK4D brain permeation was examined in mouse, and initial toxicology was assessed *in vivo* and *in vitro*. We report that JAK4D bound selectively with nanomolar affinity to native TRH receptors in human hippocampal tissue and showed for the first time that these receptors are pharmacologically distinct from TRH receptors in human pituitary, thus revealing a new TRH receptor subtype which represents a promising neurotherapeutic target in human brain. Systemic administration of JAK4D elicited statistically significant and clinically-relevant neuroprotective effects in three established neurodegenerative animal models: JAK4D reduced cognitive deficits when administered post-insult in a kainate (KA)-induced rat model of neurodegeneration; it protected against free radical release and neuronal damage evoked by intrastriatal microdialysis of KA in rat; and it reduced motor decline, weight loss, and lumbar spinal cord neuronal loss in G93A-SOD1 transgenic Amyotrophic Lateral Sclerosis mice. Ability to cross the blood-brain-barrier and a clean initial toxicology profile were also shown. In light of these findings, JAK4D is an important tool for investigating the hitherto-unidentified central TRH receptor subtype reported herein and an attractive therapeutic candidate for neurodegenerative disorders.

Keywords: Thyrotropin-releasing hormone; Neuroprotection; Neurodegeneration; TRH receptors; JAK4D; Novel central TRH receptor-subtype

1. Introduction

There is pressing unmet clinical and health economic need for neurotherapeutic drugs that prevent or slow disease progression, relieve symptoms and improve quality of life in acute and chronic progressive disorders of the central nervous system (CNS) (Miller, 2012; Pangalos et al., 2007). Given the complexity of multiple pathological processes in neurodegeneration, it is increasingly recognized that pharmacological interventions capable of modifying multiple pathogenic mechanisms may offer therapeutic advantages over strategies targeting a single injury process (Cavalli et al., 2008; Geerts, 2009; Geldenhuys et al., 2011; Stoica et al., 2009). While multifaceted intervention conceivably might be accomplished by a cocktail of targeted drugs, a single multifunctional therapeutic agent would be an attractive alternative. Herein we describe neuropharmacological evaluation of a first-in-class thyrotropin-releasing hormone (TRH)-based compound, hereafter designated JAK4D (Scalabrino et al., 2007) (US Pat. 7713935 B2), which offers such a multifaceted approach.

The central signaling system of the naturally-occurring neuropeptide TRH represents a therapeutic target with multifaceted, neurotherapeutic, homeostatic, and antinociceptive effects independent of TRH's endocrine actions (Boschi et al., 1983; Gary et al., 2003). Potential clinical application of activation of this central system in treating a wide range of CNS disorders has long been recognized (Gary et al., 2003; Griffiths, 1987; Kelly, 1995) and was recently reviewed (Daimon et al., 2013). TRH's remarkable neuroprotective actions include attenuation of multiple constituents of secondary pathways that lead to neuronal cell death (Faden et al., 2005; Jantas et al., 2009) and improvement of cellular bioenergetics, ionic homeostasis, and

cerebral blood flow following neuronal injury (Faden et al., 2005). TRH has also been identified in both the CNS (Gary et al., 2003) and the immune system (Kamath et al., 2009) as a homeostatic regulator, or ‘normalizer’ of dysregulated physiologic systems, a property of increasing relevance in the pharmacotherapy of neurodegenerative disorders.

Harnessing the clinical potential of TRH has hitherto been limited by its rapid degradation (by the highly specific TRH-degrading ectoenzyme (TRH-DE), EC 3.4.19.6), poor bioavailability, and unwanted neuroendocrine side effects (Kelly, 1995). Previously-developed TRH analogs have not sufficiently overcome these impediments: structural features compromising receptor affinity and efficacy, as well as endocrine effects, remained (Scalabrino et al., 2007).

Nevertheless, clinical trials of TRH and TRH analogs have already demonstrated positive effects in many CNS disorders (Gary et al., 2003; Kelly, 1995), with a good safety record (Kubek and Garg, 2002).

Our earlier studies identified JAK4D as the lead compound of a set of first-in-class neuropeptides that uniquely incorporate a dual-function pharmacophore for potent inhibition of TRH-DE (K_i 151 nM) together with selectivity and potent affinity for central TRH receptors in rat brain (K_i 6.8 nM) (Scalabrino et al., 2007). This compound is readily synthesized and chemically stable, displays high plasma stability, and is effective in mimicking and enhancing well-defined central behavioral actions of TRH in rat without evoking endocrine effects (Scalabrino et al., 2007). By overcoming the above mentioned limitations of native TRH and prior analogs, JAK4D presents an innovative means to selectively deliver the centrally-mediated therapeutic benefits of TRH in treating neurodegeneration and other CNS disorders.

Building on our previous findings, the principal aims of this current work were to determine the ability of JAK4D to bind to target receptors in human brain and to evaluate its neuropharmacological effects in neurodegenerative animal models. Herein it is shown that JAK4D binds selectively to a native TRH receptor in human hippocampus. This human brain receptor is pharmacologically distinct from the human pituitary TRH receptor and is reported here for the first time. We also demonstrate that JAK4D displays important statistically significant neuroprotective effects in three animal models of neurodegeneration, crosses the blood-brain-barrier (BBB), and has a favorable toxicological profile.

2. Materials and Methods

2.1 Chemicals

JAK4D was custom synthesized by American Peptide Company (Sunnyvale, California, USA) under a CDA with Trinity College Dublin. All other chemicals were purchased from sources given in the methods or from Sigma Aldrich, Ireland.

2.2 Radioligand Binding Studies

To verify that JAK4D binds to TRH receptors in human CNS tissue, radioligand binding assays were undertaken. Confirmation that a compound binds to its targeted receptor in native human tissue is critical for progression to clinical development, especially since the apparent pharmacology of recombinant receptors may not reflect that of the native human target (George et al., 2002).

[³H][3-Me-His²]TRH (NET 705, 57.4-62.0 Ci/mmol, Perkin Elmer, UK) was used to label TRH receptors in human hippocampal and pituitary tissue from individual non-identifiable donors aged 45-55 years with no history of neurological disease. These tissues were kindly donated to The University of Miami Brain Endowment Bank. Membranes from these tissues were prepared at the University of Miami and purchased from Tissue Solutions Limited, Scotland. Membranes were prepared essentially as described by (Hirst et al., 2000) except homogenization and re-suspension buffers were the same as those we employed previously (Hogan et al., 2008; Kelly et al., 2005, 2002). Membranes were stored at -80 °C pending assay.

Binding assays were conducted essentially as previously described (Hogan et al., 2008; Kelly et al., 2005, 2002). All assays were carried out at 4°C in 0.02 M sodium phosphate buffer (pH 7.5) containing 177 µM bacitracin and 0.1% bovine serum albumin. In saturation binding experiments approximately 225 µg of membrane protein were incubated in triplicate with increasing concentrations of [³H][3-Me-His²]TRH (0.6 - 20 nM) for 5 h in a final volume of 100µl. Non-specific binding (NSB) was determined in the presence of 10 µM TRH. Competition experiments were conducted under similar conditions by incubating membrane suspensions in either duplicate (pituitary) or triplicate (hippocampus) with 10-12 nM [³H][3-Me-His²]TRH and increasing concentrations of competitor (10⁻¹⁰ to 10⁻³ M TRH or JAK4D). Competitors were dissolved in dimethyl sulfoxide (DMSO) at 10⁻² M and diluted with assay buffer such that the final concentration of DMSO in all samples did not exceed 1%, a concentration at which specific binding of [³H][3-Me-His²]TRH is not affected (Kelly et al., 2002). Bound and free ligand were separated by vacuum filtration using a Brandel Cell

Harvester and GF/B filters washed with ice-cold 0.9% NaCl solution. The radioactivity retained on the filters was measured by liquid scintillation counting in a TRI-Carb 2100 TR Packard Liquid Scintillation Analyzer. Protein was measured by the method of Bradford.

2.3 Animals

All animal experiments were carried out under license in accordance with EC directive 86/609/EEC and local regulations for the care and use of animals in research with full commitment to replacement, reduction and refinement, and to minimizing animal suffering. All animal models employed were well-established models providing relevant, clear, measurable readouts. Animals were housed in perspex cages under standard conditions in a temperature controlled (21 ± 1 °C), artificially lighted room (12 hr light cycle) and were allowed access to food and water *ad libitum*.

2.4 Systemic Kainate (KA) Model

Neuroprotective effects of JAK4D were initially assessed in a rat model of kainate (KA)-induced neurodegeneration: systemic administration of the excitotoxic glutamate analog KA was used to produce deficits in learning and memory characteristic of human temporal lobe epilepsy (TLE), and the ability of JAK4D to improve functional recovery from KA-induced learning deficits was evaluated using the Morris water maze (MWM) test (D'Hooge and De Deyn, 2001; Gobbo and O'Mara, 2005).

Male Wistar rats (approximately 250 g) (Comparative Medicine, Trinity College Dublin, Ireland) naïve to drug treatment were used. Three independent experiments were undertaken by three

different investigators blinded to treatment. Rats were randomly assigned to five different groups. On Day 1 of the experiment, all animals received two i.p. injections. Two groups received saline and three groups received KA (AG Scientific, USA) (12mg/kg i.p. in saline). Two hours later the two saline groups received a second injection of either saline or DMSO:saline (40:60), and the three groups receiving KA were administered either saline, DMSO:saline (40:60) or JAK4D (1mg/kg i.p. in DMSO:saline 40:60). KA-treated rats were observed to develop characteristic KA-induced behaviors, including wet-dog shakes, head bobbing, forelimb clonus, foaming at mouth and seizures, for 2-3 h following KA injection. One animal died within the first 24 h following KA treatment. Following day one, all animals were allowed to recover for 3 days before commencement of MWM testing. During this time, animals were given a daily injection as per their second injection on day one, i.e., either saline, DMSO:saline (40:60) or JAK4D (1mg/kg i.p. in DMSO:saline 40:60).

On Day 4, testing was commenced using standard MWM trials as previously described (Gobbo and O'Mara, 2005). Daily injections of treatments as above were continued through the second day of water maze testing. Each animal undertook 5 trials per day, with approximately 20 sec between each trial, for 5 consecutive days. Swim paths, escape latency and swim speed were recorded by an automated tracking system (EthoVision; Noldus, Wageningen, Netherlands). On the day following the last day of escape latency testing, a probe test was carried out to assess reference memory at the end of learning (Vorhees and Williams, 2006). In this test the platform was removed from the pool and rats were placed in the pool and allowed to swim for a 60 sec period. Swim paths were digitally tracked and total time spent in the former zone of the platform was recorded.

Statistical analyses of MWM data were performed by an independent biostatistician. Data from the three independent experiments were pooled and analyzed with ANOVA using SPSS with factors representing Day, Group and Day x Group interactions. Since there was no heterogeneity between control groups in which animals received a first injection of saline followed by a second injection of either saline (n=13) or DMSO:saline (40:60) (n=9), these were collapsed for analysis. Likewise, data from the two groups receiving KA followed by saline (n=10) or DMSO:saline (40:60) (n=13) were also collapsed for analysis. A repeated measures design was used, employing the Greenhouse-Geisser three-step approach to significance testing where relevant. In this case, we report the uncorrected degrees of freedom, the corrected p value and the epsilon value of the correction factor. Post hoc tests were calculated to follow up significant effects.

2.5 Intrastratial Microdialysis KA Model

The formation of highly reactive oxygen species (hROS), which provides a measure of oxidative stress commonly associated with the pathogenesis of many neurodegenerative disorders (Cavalli et al., 2008; Freinbichler et al., 2011; Zhou et al., 2008), can be evoked in a dose-dependent manner by local application of an excitotoxic concentration of KA into rat striatum. hROS can be sensitively quantified as OH-TA, the hydroxylated derivative of sodium terephthalate (TA²⁻) (Freinbichler et al., 2011, 2008). This method was used to assess the ability of JAK4D to protect against *in vivo* free radical formation induced by intrastratial administration of KA (1 mM) during brain microdialysis. Histochemical *ex vivo* tissue analysis was employed to assess related apoptotic striatal tissue damage.

Male Wistar rats (200-220 g) anesthetized with chloral hydrate (400 mg/kg i.p.) were placed in a stereotaxic frame. A microdialysis guide cannula (concentric design, CMA/Microdialysis AB, Stockholm, Sweden) was implanted vertically in the right neostriatum (stereotaxic coordinates for neostriatum, relative to Bregma, were AP 0.7, L 3.2, DV 5.5 mm). Approximately 48 h later, microdialysis experiments were performed on freely-moving rats, always starting at 9:00 am, by inserting the microdialysis probe (4mm exposed surface; CMA/Microdialysis AB, Stockholm, Sweden) into the guide cannula. The perfusion fluid, artificial cerebrospinal fluid (aCSF), comprised: 140 mM NaCl, 3 mM KCl, 1.2 mM CaCl₂, 1.0 mM MgCl₂, 1.2 mM Na₂HPO₄, 0.27 mM NaH₂PO₄ and 7.2 mM glucose (pH 7.4), plus 1 mM sodium terephthalate (TA²⁻) used as a chemical trap for highly reactive oxygen species (hROS) (Freinbichler et al., 2011).

After a 60 min stabilization period, the dialysate samples were collected every 20 min. Initially, two fractions were collected to determine basal levels. At 60 min, the two KA-treated groups received an i.p. injection of either vehicle (15% DMSO in saline) or JAK4D (10 mg/kg in vehicle); the control group, not receiving KA, was administered JAK4D i.p. (10 mg/kg in vehicle). The rats were anesthetized (chloral hydrate 400 mg/kg i.p.) just before KA (1 mM) was applied to the neostriatum for 20 min from 100-120 min through the dialysis probe to the two KA-treated groups. Reverse phase high pressure liquid chromatography analysis of 2-hydroxyterephthalate (OH-TA) in microdialysates was carried out using a Shimadzu instrument (Italia S.r.l., Milan, Italy) with a fluorescence detector ($\lambda_{\text{ex}} = 315 \text{ nm}$; $\lambda_{\text{em}} = 435 \text{ nm}$) (Freinbichler et al., 2011).

48 h after KA administration, animals were killed by decapitation, the brain removed, and the isolated neostriatum fixed in 4% paraformaldehyde in phosphate buffer solution for 72 h. Following paraffin inclusion, slices of the neostriatum (5 micron thick) were cut with a microtome. Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) immunohistochemistry, using the Klenow fragELMT DNA Fragmentation Detection Kit (Inalco, Milan, Italy), was applied to identify apoptotic nuclei. For Caspase-3 analysis, slices, after deparaffinization and rehydration, underwent permeabilization with 0.1% Triton X-100, followed by inactivation of endogenous peroxidase (30 min in 0.3% H₂O₂ in PBS) and serum blocking (5% donkey serum in PBS). They were then incubated overnight with the primary antibody (1:200 Anti-Active Caspase 3 (Promega)) and the next day for 1 h with the secondary antibody (1:500 Biotin Conjugated Donkey Anti-rabbit p AB (Jackson ImmunoResearch)). For both TUNEL and Caspase-3, quantitative image analysis was performed using the public domain software Scion Image (Scion Corporation, Frederick, MD, USA) using a Nikon Eclipse E800 microscope (Nikon Instruments S.p.A., Firenze, Italy) equipped with a JVC SLR camera. For TUNEL and Caspase-3 analyses, three to six and three to four fields were counted respectively, corresponding to approximately 2000–5000 nuclei. For IBA1 immunolabeling to detect microglial activation, sections were first deparaffinized, gradually hydrated, and finally washed twice in 0.01M PBS solution pH 7.4. The tissue was first treated with 0.3% H₂O₂ for 10 min to block endogenous peroxidase activity, then with 5% blocking donkey serum in PBS for 5 min to reduce background or unspecific staining. Sections were incubated overnight at 4°C with the primary antibody (1:500, Anti-IBA1 (WAKO Chemicals GmbH, Germany)) diluted in 0.1M PBS, 5% donkey serum. The following day, sections were washed twice with PBS and then incubated for 10 min with the secondary antibody (1:500, Biotinylated anti-Rabbit IgG Antibody

(Jackson ImmunoResearch Laboratories, USA)) in 0.1M PBS. The antigen-antibody complex was then visualized by the Vectastain ABC peroxidase kit (1:200, Vector Laboratories, USA). Finally, sections were washed and developed using 0.05% DAB (Sigma-Aldrich, Milan, Italy).

2.6 G93A-SOD1 Transgenic Mouse Model

Therapeutic effects of JAK4D were also assessed in the transgenic G93A-SOD1 mouse, an established model of Amyotrophic Lateral Sclerosis (ALS) (Gurney et al., 1994; Ludolph et al., 2010, 2007), employing rotarod performance, body weight, and histological analysis of spinal cord tissue as readouts of potential therapeutic benefit from JAK4D treatment.

A randomized, blinded, gender-, weight- and age-matched study in male G93A-SOD1 mice was performed according to current guidelines for preclinical animal research in ALS. Gender-specific testing was undertaken, given recognized gender differences in disease survival and onset (Kalmar et al., 2008).

For *in vivo* measurements, 26 male G93A-SOD1 transgenic familial ALS mice (high copy number; B6SJLTg (G93A-SOD1) 1Gur/J; The Jackson Laboratory, Bar Harbor, ME, USA) were shipped at 5 weeks of age and allowed one week to acclimate before being assigned to the study. Treatment with JAK4D (2mg/kg i.p. 5 days/week) or vehicle (DMSO: Saline 20:80) was initiated in 2 randomly assigned groups (n=13 per group) 6 weeks after birth and continued until end stage. End stage was defined by death or as the inability of an animal to right itself within 30 s if laid on either side (both sides were tested).

Beginning at 9 weeks of age, motor function was assessed with a rotarod apparatus (Ugo Basile, Italy), using an accelerated rotarod protocol (2-40 rpm over a ramp period of 180 s). During an adaptation period of 3 days, all but one mouse remained on the rotarod for the defined maximum test duration of 500 s. From the outset, one mouse, although physically in good condition and not displaying any neurological signs of ALS until week 17, would not stay on the rotarod for 500 s and was excluded from rotarod analysis. Rotarod testing was assessed twice per week until the animals could no longer carry out the task. Mice carried out the test three times on each day of testing with at least a 10 min rest period between tests. Two investigators performed the experiment, one blinded to treatment.

The body weight of all mice was measured on intake and then weekly from 7 weeks of age until 12 weeks of age when it was measured twice per week. Weighing was always performed between 10 am and 1 pm to avoid diurnal variations. All mice were on a tekland global 18% diet (Harlan U.K.).

Separately, for histological analyses, male G93A-SOD1 transgenic familial ALS mice were treated from 6 to 16 weeks of age with either JAK4D (2 mg/kg i.p. 5 days/week) or vehicle (DMSO: Saline 20:80) (n=5 per group). Five sibling non-carriers (controls) were similarly treated with vehicle. At 16 weeks mice were transcardially perfused with 0.1M PBS followed by 4% buffered paraformaldehyde and spinal cords were rapidly removed following euthanasia. Spinal cord was fixed in formalin for a minimum of 48 h. Following fixation caudal lumbar spinal cord was paraffin embedded and sections (4 micron thick) were cut and stained with haematoxylin and eosin. For evaluation, all ventral horn neurons were counted from within the

ventral horn below a lateral line across the spinal cord from the central canal (Klivenyi et al., 1999). Total counts for both ventral horns were established. Evaluations were undertaken by an accredited research pathologist blinded to the study protocol.

2.7 Brain Permeation

Passage of JAK4D across the blood-brain-barrier (BBB) is favored by its greater lipophilicity compared to TRH (as reflected by respective retention times of 17 min and 9 min on reverse phase high pressure liquid chromatography (Scalabrino et al., 2007)). Moreover, passage is strongly indicated by the behavioral and neuropharmacological effects elicited by JAK4D ((Scalabrino et al., 2007) and herein). To examine brain permeation more directly, JAK4D levels in mouse brain and spinal cord were assessed using LC-MS-MS following a single peripheral injection. An advantage of LC-MS-MS over the use of a radiolabelled compound in such an experiment is that it allows the intact molecule to be unambiguously identified. Capillary depletion, modified for mouse brain, was carried out according to the method of Pardridge adapted from (Triguero et al., 1990) to measure JAK4D levels in brain capillaries and parenchyma following peripheral administration.

2-4 month-old C57/BJ6 (Harlan, UK) male mice were injected either i.v. (via tail-vein) or i.p. with 320 µl of JAK4D (2mg/kg) in 20% DMSO in saline (n = 3 for each group). 10 min post i.v. injection and 5 and 10 min post i.p. injection, mice were anesthetized, the thorax opened, and blood collected from the vena cava using a heparinized syringe. The blood was slowly transferred to an eppendorf containing 7.5 µl heparin and centrifuged at 14,000 rpm (Hettich Mikro 22R) for 5 min at 4 °C. 50 µl of translucent plasma was removed and transferred to a

cryovial and snap frozen in liquid nitrogen. Mice were perfused for 5 min with 0.9% saline. After decapitation the brain was removed, weighed and quickly transferred to a glass homogenizer on ice. The tissue was homogenized with 11 full strokes in a 4-fold volume of cold phosphate-buffered saline (PBS). 1.2 volumes of 40% cold dextran in PBS buffer were added and the tissue was further homogenized using 3 full strokes. 0.2 ml of homogenate was removed, transferred to a cryovial, and snap frozen in liquid nitrogen for LC-MS-MS analysis to give total brain JAK4D levels. The remaining homogenate was divided between two 2 ml microcentrifuge tubes and centrifuged at 4 °C for 10 min at 6000 rpm (Hettich Mikro 22R). The resulting parenchyma layers in each microcentrifuge tube were removed using a Pasteur pipette, combined, and transferred to a cryovial and snap frozen until analysis. The same was carried out for the dextran layers. The remaining pellets containing the brain capillaries were frozen at –20 °C until analysis. Spinal cord was also removed following perfusion, transferred to a cryovial, and snap-frozen in liquid nitrogen. Samples (plasma, spinal cord, homogenate, parenchyma layer, and brain capillaries) were then transported on dry ice to Charles River, Edinburgh, U.K. for analysis by LC-MS-MS.

A research-grade Turbo Ion Spray LC-MS-MS assay was developed and employed by Charles River Preclinical Services U.K. to measure JAK4D in the mouse tissue samples described above. Standard and quality control samples were prepared to give a range of matrix concentrations of 0.5-50,000 ng/ml. In the initial set up, samples of mouse brain and plasma were ‘spiked’ with 10 to 30,000 ng/ml of JAK4D. The amount of JAK4D detected gave values between 96% and 118% of theoretical. Chromatography gave a clean, sharp peak for JAK4D and the calibration curve was linear over the range 5-50,000 ng/ml plasma. The limit of detection was 0.5 ng/ml.

2.8 Toxicology Studies

Adverse events in response to systemic administration of an acute single dose or chronic daily dosing of JAK4D were monitored during the course of *in vivo* studies. Food and water intake and postmortem liver weight were measured in rat to provide information regarding tolerability and toxicity of chronic daily dosing of JAK4D (5 mg/kg i.p. in DMSO:saline 40:60) over a period of 24 days. Cytotoxicity was examined in Caco-2 cells and erythrocytes. Caco-2 monolayers were prepared according to previous methods (Ryan et al., 2009). Cytotoxic effects of JAK4D (3.63×10^{-4} M) were examined in these cells by measuring lactate dehydrogenase (LDH) release (Sigma-Aldrich, UK). Erythrocytes were prepared from blood obtained from male Wistar rats via cardiac puncture using heparinized syringes immediately following euthanasia. These were collected by centrifugation (2000g, 5 min, 4 °C) and washed 3 times with a solution of sodium chloride (150 mM) in 35 mM sodium phosphate buffer, pH 7.0. The final pellet was resuspended in the same buffer to give 4% w/v erythrocyte suspension. 100 μ L samples of suspension (n=6) were plated in 96-well microtitre plates and incubated with 5% DMSO, with or without JAK4D (3.63×10^{-4} M) for 60 min at 37 °C. Following centrifugation at 1000 g for 5 min, aliquots of the supernatant were transferred to a fresh 96-well microtitre plate in which hemoglobin was measured spectrophotometrically at 414 nm. Positive and negative controls in this test were TritonX-100 (which gave 100% hemolysis) and phosphate buffered saline (which gave 0% hemolysis). Percent hemolysis was calculated as described by (Shin et al., 2001).

2.9 Statistical Analysis

Unless otherwise stated, non-linear regression analyses of data were carried out using the computer program Prism (Graph Pad Software Inc.). Statistical analyses were performed using two-tailed Student's t-test when comparing two groups, or analysis of variance (ANOVA), followed by *post-hoc* test as specified in the text, for comparison of multiple groups. To compare independent groups where outcome measures were not normally distributed, non-parametric tests were carried out employing SPSS (Inc. Chicago, Illinois, U.S.A.). The Mann-Whitney test was used for pair-wise comparisons and Kruskal-Wallis where there were three or more groups.

3. Results

3.1 JAK4D binds to human central TRH receptors revealing a novel TRH receptor subtype

In human hippocampal tissue, [^3H][3-Me-His²]TRH specific binding was saturable (Fig. 1A) with a K_D of 8.97 ± 2.90 nM ($n=3$), a value comparable to that reported previously for human amygdala (Manaker et al., 1986; Parker and Capdevila, 1984). Displacement of [^3H][3-Me-His²]TRH binding from human hippocampal and pituitary tissue by TRH and JAK4D is shown in Fig. 1B and 1C. TRH fully displaced [^3H][3-Me-His²]TRH binding from matched human hippocampal and pituitary membranes with similar affinities (hippocampal $\text{pIC}_{50} = 6.09 \pm 0.20$; pituitary $\text{pIC}_{50} = 6.84 \pm 0.20$ ($n=3$)). JAK4D displayed very weak competition in human pituitary tissue, with 10^{-5} M displacing less than 25% of [^3H][3-Me-His²]TRH binding. In contrast, in hippocampal tissue, JAK4D was more potent than TRH in displacing [^3H][3-Me-His²]TRH ($\text{pIC}_{50} = 7.79 \pm 0.15$ ($n=3$)). Corresponding K_i values for TRH and JAK4D in human hippocampus were 3.62×10^{-7} and 7.19×10^{-9} M, respectively. These results confirm the ability

of JAK4D to bind selectively with high affinity to native hippocampal TRH receptors in human tissue and show for the first time that these TRH receptors are pharmacologically distinct from those in human pituitary.

3.2 JAK4D provides significant protection against kainate-induced cognitive deficits in rat

Fig. 2A shows mean escape latency for vehicle-, KA+vehicle-, and KA+JAK4D-treated groups over five days of MWM testing from three independent studies. A significant main effect of Day on escape latency was found ($F_{4, 1228} = 51.805$, $p < 0.0001$). The Day x Group interaction revealed a significant difference in learning for the different groups ($F_{8, 1228} = 4.261$, $p < 0.0001$). The post hoc contrast test for group comparisons showed for days 1-5 a statistically significant difference in mean values between KA+vehicle (n=23) and saline+vehicle (n=22) groups ($p < 0.0001$, Tukey HSD post ANOVA test) and between KA+vehicle and KA+JAK4D (n=17) groups ($p = 0.005$, Tukey HSD post ANOVA test). Comparison of the escape latency slopes over the course of the testing period showed that JAK4D treatment, administered two hours post KA, resulted in a prolonged, two-fold reduction in KA-induced cognitive deficits (Fig. 2A). There were no significant differences in swim speed between groups on each day of testing (two-way ANOVA, Tukey post ANOVA test), indicating that escape latency was not influenced by swim speed.

Data from the probe test, assessing the ability of the animal to recall where the platform had been located in the escape latency test, were combined from the three independent studies by standardizing the time spent in the platform zone by KA-treated animals as a proportion of the mean of the vehicle-treated control group (Fig. 2B). Non-parametric analysis of combined groups revealed a statistically significant difference between KA+vehicle and KA+JAK4D

groups ($p=0.011$; $n=40$; Mann Whitney U test). Thus, JAK4D treatment significantly attenuated both KA-induced recall deficits and KA-induced deficits in acquisition in this model.

3.3. JAK4D protects against free radical release and cell death induced by intrastriatal KA

Systemic administration of JAK4D (10 mg/kg i.p.) 40 min prior to KA application significantly reduced the formation of OH-TA (Fig. 3A), which reflects the production of hROS in rat striatal extracellular space (one-way ANOVA, $F_{2,13} = 9.3$, $p=0.0043$, post hoc Tukey multiple comparison test, $p<0.05$ for KA+vehicle vs KA+ JAK4D and $p<0.01$ for KA+vehicle vs control, $n=4-5$).

Ex vivo analysis of striatal tissue showed that JAK4D pre-treatment also significantly reduced KA-induced apoptosis, as measured by TUNEL immunolabeling (one-way ANOVA, $F_{2,13} = 21.3$, $p=0.0002$, post hoc Tukey multiple comparison test, $p<0.001$ for both, KA+vehicle vs KA+ JAK4D and KA+vehicle vs control, $n=4-5$) and caspase-3 activation (one-way ANOVA, $F_{2,13} = 24.39$, $p<0.0001$, post hoc Tukey multiple comparison test, $p<0.001$ for both KA+vehicle vs KA+ JAK4D and KA+vehicle vs control, $n=4-5$). For statistical analysis the number of TUNEL-positive cells or caspase 3-activated cells were expressed as a percentage of total cell counts (Fig. 3B left and right panel, respectively). Qualitative immunohistochemical examination of striatal sections obtained from KA-treated rats indicated the occurrence of microglial activation, identified by the presence of cells showing increased IBA1 immunoreactivity, characterized by enlarged and/or amoeboid cell bodies and partial or complete loss of thin elongated processes (Fig. 3C). Tissue from rats pre-treated with JAK4D showed less intense immunolabelling. In striatum of control rats, no activated microglial cells were observed.

3.4 JAK4D significantly reduces motor decline, weight loss and cell death in ALS mouse model

Mean rotarod performance was essentially constant until onset of symptoms (Fig. 4A).

Concurring with (Knippenberg et al., 2010) symptoms emerged at 100 days as a sudden 11% reduction in motor performance in both groups. Following onset, motor function and mean body weight rapidly deteriorated in vehicle-treated animals (Fig. 4A and B). Analysis of data to first death (day 118) shows that, after disease onset (day 100), JAK4D treatment consistently and significantly improved rotarod performance and reduced weight loss compared to that of vehicle treatment. Analysis of rotarod data from onset to first death using two-way repeated measures ANOVA (including both treatment group and trial as factors) revealed that the overall interaction between Day and Treatment Group was significant ($p=0.008$). Post-hoc analysis (Newman-Keuls) showed that JAK4D treatment significantly improved rotarod performance compared to vehicle-treated animals on consecutive test days 105, 107, 112 and 114 ($p<0.01$, see Fig. 4A). In particular, rotarod performance in the JAK4D-treated group was enhanced by 21% at first measurement (day 105) following onset and by 109% on last day of measurement (day 114) before first death.

Comparison of changes in mean weight in the period from symptom onset to first death between groups (treatment versus vehicle) was carried out using one-way repeated measures ANOVA. Results showed a significant effect for group versus time interaction (Wilk's Lambda =0.360, $F_{(4,21)} p<0.0001$, see Fig. 4B), indicating a significant difference in mean change of weight over time between the two groups. The multivariate eta squared value was 0.640, signifying a large effect for JAK4D treatment on this outcome measure. Median survival for the control group was

126 days (95% CI 123.4-128.6, SE 1.3), comparable to 127 days reported by (Gill et al., 2009). For the JAK4D-treated group, median survival was extended to 132 days (95% CI 130.0-133.1, SE 0.5). The difference between the two groups did not reach statistical significance (log-rank test $p=0.520$; Fig. 4C), most likely due to reduced power.

One-way ANOVA comparing mean neuron counts in the ventral horns of the caudal lumbar spinal cord sections from vehicle-treated and JAK4D-treated G93A-SOD1 mice and sibling non-carrier controls at sixteen weeks of age showed a statistically significant between-group difference ($p=0.002$). Post-hoc comparisons using Tukey HSD showed that the mean neuron count in the vehicle-treated G93A-SOD1 mice (81.4, SD 22.2) was significantly lower than that observed in both the control group (126.8, SD 6.4, $p=0.002$) and the JAK4D-treated group (109.8, SD 13.6, $p=0.033$). The mean count in the JAK4D-treated group did not differ significantly from the control group ($p=0.232$, see Fig. 4D). These data demonstrate that JAK4D treatment attenuates neuronal cell loss in G93A-SOD1 mice.

3.5 JAK4D crosses the blood brain barrier

Values shown for JAK4D in plasma were 3.7% to 4.7% of the single injected dose (2 mg/kg), equivalent to approximately 2.1-3.1 μM in a 25 g mouse (Table 1). Capillary depletion produced a clear separation of brain capillaries from parenchyma, but the presence of varying amounts of residual dextran (used in the separation procedure) in both layers precluded reliable measurement of wet tissue weights, thus accurate calculation of JAK4D concentration in these two fractions was not possible. Nevertheless, it is clear that the amounts of JAK4D (in ng) in parenchyma + capillaries are sensibly consonant with the values obtained for whole brain (Table 1). Calculated

concentrations based on wet weight of tissue show JAK4D present in the range of 10-22 nM in brain and spinal cord. Therefore, following peripheral administration of a single dose, intact JAK4D crosses the BBB and within minutes reaches CNS concentrations commensurate with its affinity for the targeted receptor.

3.6 JAK4D displays a clean initial toxicology profile

No adverse events were observed in response to either an acute single dose or daily dosing (for up to 3 months) of JAK4D in rat (up to 5 mg/kg i.p.) or mouse (up to 2 mg/kg i.p.), including wild type C57 B1/6J mice and G93A-SOD1 mice. Daily administration of JAK4D (5 mg/kg i.p.) in rat for 24 days had no effect on food or water intake or liver weight (Table 2). Toxicological screening undertaken using standard methods employing Caco-2 monolayer cells and erythrocytes indicate that JAK4D is not cytotoxic (Table 2).

4. Discussion

Neuropeptides such as TRH offer many advantages as neurotherapeutic molecules. Among these are diverse pleiotropic actions, exceptional potency (typically displaying nM affinity compared to μ M affinity associated with classical neurotransmitters and, as agonists, requiring only 5-20% receptor occupancy to be effective), and safety generally (Gonzalez-Rey et al., 2007; Portelli et al., 2012; Vlieghe et al., 2010). Neuropeptides, though, are often susceptible to proteolytic degradation, which is a major impediment to their therapeutic use (Vlieghe et al., 2010). In the case of TRH, susceptibility to degradation and potential for endocrine side effects have critically limited its clinical application (Kelly et al., 2000, 2005; Kelly, 1995; Scalabrino et

al., 2007). By overcoming the inadequacies of TRH and prior TRH analogs, JAK4D now presents the possibility of realizing the benefits offered by activation of the central TRH signaling system in the treatment of CNS disorders (Scalabrino et al., 2007).

Two G Protein-Coupled Receptor (GPCR) subtypes, designated TRHR1 and TRHR2, have been identified to date in mediating TRH actions in non-human mammalian species (Cao et al., 1998; Itadani et al., 1998; Sun et al., 2003). Recent knock-out studies in mouse indicated that TRHR2 receptors account for no more than 5% of total binding in brain and that the CNS effects of TRH and TRH analogs were lost in mice in which TRHR1 was deleted (Thirunarayanan et al., 2013). In addition, a third putative TRH receptor (xTRHR3) was cloned in *Xenopus laevis* (Bidaud et al., 2002). It was subsequently suggested that xTRHR3 is likely a receptor for another peptide, since the affinity of xTRHR3 for 3-Me-His²TRH was $>0.1\mu\text{M}$ and very low affinity was exhibited for TRH and TRH analogs (Lu et al., 2003). In later studies, xTRHR3 was found to be distributed exclusively in the intermediate lobe of *Xenopus laevis* pituitary and to be involved in adaptation of frog skin to background color (Bidaud et al., 2004). TRHR3 sequences have also been identified in teleosts, and putative TRHR3 sequences have been found in some mammals, for example dog, but not in humans, (Saito et al., 2011). In humans only one of these receptors, TRHR1, has been found. Yamada et al (1997) identified a novel gene TRHR transcript in tissue from human pituitary tumors. This was formed from alternative splicing of TRHR1 mRNA and corresponded to a C-terminal truncated protein, which they designated HTRHR2. When expressed in Chinese hamster ovary cells, this protein, however, displayed neither significant binding of [³H][3-Me-His²]TRH nor an intracellular calcium response to TRH administration. It is known that human TRHR1 is approximately 90% homologous with rat TRHR1 at the cDNA

and amino acid level (Sun et al., 2003). It is also recognized that TRHR2 is not present in humans (Gloriam et al., 2007; Sun et al., 2003). We also confirmed independently using the blastp algorithm [<http://blast.ncbi.nlm.nih.gov/>] that no ortholog exists in human for mouse or rat TRHR2. Phylogenetic analyses indicate the molecular evolution of TRHR in vertebrates derives from a common ancestral origin (Saito et al., 2011); with the lack of TRHR2 in primates, chicken and fugu suggesting that it has been lost independently in three lineages (Gloriam et al., 2007).

TRH displays a similar affinity for TRHR1 and TRHR2, as does 3-Me-His²TRH, although more potently than TRH. Therefore, these ligands cannot be used to discriminate pharmacologically between the two receptors (Kelly et al., 2002; O'Dowd et al., 2000; Sun et al., 2003). Our previous studies in rat show that JAK4D displaces [³H][3-Me-His²]TRH binding to native cortical and hippocampal tissue, yet it does not displace this radiolabel in rat pituitary tissue (Hogan et al., 2008; Scalabrino et al., 2007), in which TRHR1 is highly expressed (Sun et al., 2003). If TRHR1 were the only TRH receptor in human tissue, it could be surmised from these rat studies that JAK4D would not bind to TRH receptors in either human pituitary or brain. Indeed, we found JAK4D displays very weak displacement of [³H][3-Me-His²]TRH in human pituitary tissue. The opposite was found in human hippocampal tissue, with JAK4D binding with nanomolar affinity to TRH receptors. These findings are important because, not only do they confirm that JAK4D binds to target receptors in human brain, they reveal for the first time that [³H][3-Me-His²]TRH labels a population of TRH receptors in human brain that are pharmacologically distinct from those in human pituitary. Furthermore, JAK4D is shown to be a

high-affinity, discriminating ligand for these human brain sites. We conclude from these results that JAK4D is binding to a hitherto-unidentified TRH receptor subtype in human brain.

The nature of the hippocampal TRH receptor subtype is presently unclear and awaits structural identification, which would enable structural studies, such as site-directed mutagenesis, to be undertaken, leading to a greater understanding of ligand-receptor interactions, as well as interspecies comparisons to be made. The hippocampal TRH receptor subtype could be a variant of TRHR1 or a different, previously-unknown binding site for TRH. (Daimon et al., 2013) surmised that, similar to gonadotropin-releasing hormone receptor, TRHR1 in humans may exist in more than one conformation and that different agonists may bind selectively to one or other conformer leading to activation of different downstream signals. Alternatively, differences in signal activation may be attributable to differences in the quaternary structure of receptors, e.g., polymeric assemblies (George et al., 2002). This possibility of dissimilar conformers or assemblages for TRH receptors, enabling one gene product to give rise to different pharmacological outcomes, warrants further investigation. JAK4D, given its selectivity for this central TRH receptor, would be a useful experimental tool in such studies.

Discovery of this novel TRH receptor subtype in human brain is a major finding. It opens up new opportunities for exploring the neuropharmacological actions of TRH and, importantly, identifies an attractive neurotherapeutic receptor target. The high affinity binding of JAK4D to TRH receptors in human brain is also a positive indicator for the translation potential of this compound.

Since animal models of CNS disorders cannot wholly reflect the human condition, efficacy in more than one clinically-relevant model, including preferably a non-transgenic and a transgenic model, together with evidence of disease-relevant mechanism of action, increases confidence in the translation potential of a drug candidate (Enna and Williams, 2009; Markou et al., 2009).

Both are demonstrated herein for JAK4D.

In the rat model characterized by KA-induced deficits in hippocampal-dependent learning and memory, JAK4D treatment administered two hours post-insult significantly improved functional recovery. The pathophysiology that underpins such cognitive deficits, which are the hallmark of this model, is unclear. Cognitive deficits in response to KA, as well as to other agents that give rise to memory impairment, have been observed to occur with and without hippocampal cell loss (Chronopoulos et al., 1993; Sun et al., 2009; Zhou et al., 2007). This aligns with the notion that perturbations in cellular and network function may be sufficient to produce neurological impairment in the absence of cell loss, as suggested for neurodegenerative disorders, including Alzheimer's disease (AD) (Huang and Mucke, 2012; Palop et al., 2006). Accordingly, it has been proposed that drugs capable of maintaining or restoring the integrity of impaired networks may provide functional benefit against acquired neurological deficits (Huang and Mucke, 2012; Mucke and Selkoe, 2012; Palop et al., 2006). The multifaceted, homeostatic therapeutic effects associated with activation of the central TRH signaling system, which oppose CNS perturbations, may be relevant to providing cellular and network resilience (Gary et al., 2003). Whatever the underlying pathobiological mechanisms, the therapeutic benefits achieved by JAK4D show that it significantly reduces excitotoxic-induced cognitive deficits, even when administered post insult. Such a therapeutic scenario may be relevant in neurodegenerative

conditions in which hippocampal pathology has been shown to play a role, such as in AD (Palop and Mucke, 2009), and C9orf72-related motor neuron disease and frontotemporal lobe dementia (Al-Sarraj et al., 2011; Cooper-Knock et al., 2012), as well as possibly chronic traumatic encephalopathy (CTE) (McKee et al., 2013).

In a second model, systemic administration of JAK4D is shown to protect against free radical production and neuronal damage brought about by intrastriatal infusion of KA by microdialysis in conscious rats. In this study, central administration of an excitotoxic concentration of KA reliably induced apoptotic cell death in striatal tissue, as evidenced by TUNEL and caspase-3 activation. JAK4D treatment reduced both parameters at a level of statistical significance. JAK4D also appeared to decrease microglial activation, a recognized pathogenic factor in CNS disorders. Additionally, the increase in hROS induced by KA excitotoxicity was significantly decreased, suggesting that reduction of oxidative stress is one means by which JAK4D may exert neuroprotective effects. This result also provides a possible mechanistic rationale in support of the therapeutic potential of JAK4D in neurodegenerative disorders.

Further proof of concept is provided by demonstration of significant beneficial effects in the transgenic G93A-SOD1 mouse model of ALS. This mouse is genetically engineered to express multiple copies of the mutant human SOD1 gene, an etiological factor in a portion of patients with familial ALS (Kenna et al., 2013). In line with critical appraisal of this aggressive model (Scott et al., 2008), it is increasingly recognized that readouts such as rotarod performance and body weight may be more indicative predictors of therapeutic benefit than survival (Knippenberg et al., 2010; Scott et al., 2008). Our results show that JAK4D treatment elicits statistically

significant and clinically-relevant positive effects in these two important functional measures of therapeutic benefit in G93A-SOD1 mice, accompanied by attenuation of neuronal loss in lumbar spinal cord. The significant reductions in motor decline and weight loss observed in JAK4D-treated versus vehicle-treated G93A-SOD1 mice highlight the potential of JAK4D to slow disease progression and thereby improve functionality and hence quality of life of ALS patients.

The presence of the BBB represents a crucial hurdle in accomplishing drug delivery to the brain (Pardridge, 2009, 2005). Using LC-MS-MS, we demonstrated directly the ability of JAK4D to cross the BBB and to reach its target at viable pharmacological levels. In addition to its activity at targeted receptors, it is conceivable that the activity of JAK4D in inhibiting TRH-DE might also play a contributory role in the manifestation of the neuropharmacological effects of this dual-action peptide. However, since the K_i for the receptor is in the nanomolar range and that for the enzyme is two orders of magnitude higher it may be anticipated that the interaction with the receptor would predominate. As we have noted previously, inhibition of TRH-DE by JAK4D might also be anticipated to increase the amount of endogenous TRH available to the pituitary (Scalabrino et al., 2007). As anterior pituitary basal TRH-DE levels are very low, TRH-DE inhibition is expected to have minimal influence on pituitary hormone secretion (Scalabrino et al., 2007). We have previously shown that TSH levels are unaffected in rat by JAK4D treatment (Scalabrino et al., 2007) reflecting both this and the low affinity of JAK4D for the pituitary TRH receptor.

Toxicity is another major impediment to CNS drug development (Kola and Landis, 2004).

Peptide-based compounds, such as JAK4D, typically are highly specific and have low systemic

toxicity (Vlieghe et al., 2010). TRH for instance has been shown to have a good safety profile and to be tolerated well by most patients, from prenatal to elderly (Kubek and Garg, 2002). The ability of JAK4D to counter weight loss in the G93A-SOD1 mouse shows that it does not induce hypermetabolism, concurring with our earlier finding that this compound does not stimulate TSH release in rat (Scalabrino et al., 2007). Initial observations point to a clean safety profile for JAK4D, both *in vitro* and *in vivo*.

In summary, we describe a first-in-class, centrally-acting TRH-based compound, which offers an innovative, multifunctional approach to treating CNS disorders. Demonstrated herein for JAK4D are: (i) selective binding with high affinity to a hitherto-unidentified receptor subtype in human brain, which is pharmacologically distinct from the human pituitary TRH receptor and which represents a promising, druggable neurotherapeutic target; (ii) significant therapeutic benefit in three CNS disease-related animal models with multiple clinically-relevant efficacy endpoints; (iii) pharmacological responses appropriate to the pathological condition; (iv) ability to cross the BBB; and (v) a clean initial safety profile. All are key indicators for building confidence in the potential for successful translation of JAK4D from preclinical studies to human clinical trials. Set against a background of pre-clinical and clinical knowledge of TRH, these findings are a solid foundation of evidence and compelling rationale for continued development of JAK4D as an attractive and much-needed neurotherapeutic drug candidate in the CNS arena.

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Disclosure Statement

J.A.K. is the named inventor on granted patents and patent applications relating to JAK4D and is a director and shareholder of NeuroPath Therapeutics Limited, a University spin-out company which holds a licence from Trinity College Dublin for JAK4D and related intellectual property. C.H.W., J.H., and G.R.S. are shareholders of NeuroPath Therapeutics Limited.

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Table 1

Amounts of JAK4D in whole brain, whole spinal cord, capillaries, parenchyma, and plasma of mice at indicated times following i.p. or i.v. injection (2 mg/kg).

Post-injection time and route	Brain		Spinal Cord		Capillaries	Parenchyma	Plasma
	ng	nM	ng	nM	ng	ng	ng/ml
5 min post-i.p.	6.4±1.2	22±3.3	0.63±0.2	12.7±1.5	1.9±0.8	3.2±0.4	2353±133
10 min post-i.p.	3.6±1.0	12.2±3.6	0.9±0.14	13.4±1.9	2.6±0.3	3.3±0.2	2163±46
10 min post-i.v.	3.8±0.5	12.2±1.5	0.5±0.1	9.7±1.6	0.47±0.1	3.15±0.6	1863±159

Values are mean ± s.e.m. (n =3 in all cases). Concentrations (nM) are based on wet weight of tissue, assuming 1g is equivalent to 1ml. It was not possible to calculate molar concentrations in capillaries or parenchyma (see text).

Table 2

Effects of JAK4D on cell viability and rat physiological parameters.

Treatments	Caco 2 cells	Erythrocytes	Food intake (g)	Water intake (ml)	Liver weight (g)
Vehicle	26.5±5.9	5.7±1.2	29.4±1.7	47.6±4.4	11.37±0.31
Vehicle + JAK4D	29.2±5.8	6.2±1.6	26.7±1.3	43.6±4.3	10.49±0.42

For cell data, vehicle is 5% DMSO in HBSS + 10 mM HEPES buffer and the concentration of JAK4D is 3.63x10⁻⁴ M; values are % damaged relative to a positive control (Triton X-100) (n = 6). Data for rat shows average food and water intake per animal per day, as well postmortem liver weights. Animals were administered daily i.p. injections of either vehicle (DMSO:saline, 40:60, n=7) or JAK4D (5 mg/kg in DMSO:saline,40:60, n=8) over a period of 24 days.

FIGURE LEGENDS

FIGURE 1: Binding to TRH receptors in human tissue. (A) Saturation binding of [^3H][3-Me-His 2]TRH to human hippocampal membranes showing total (TB), non-specific (NSB) and specific binding (SB). Points are mean $\pm$ s.e.m. of n=3 individual donors. The specific binding isotherm was analyzed using non-linear regression. Also shown are competitive radioligand binding curves for the displacement of [^3H][3-Me-His 2]TRH binding in human hippocampal and pituitary membrane suspensions by: (B) TRH and (C) JAK4D. Points are mean $\pm$ s.e.m. of n=3 individual donors.

FIGURE 2: Effects of JAK4D on KA-induced memory loss in rat. (A) Spatial learning in the rat determined by mean escape latency in MWM over 5 days of testing. Data are expressed as mean $\pm$ s.e.m. pooled from three independent experiments (n=17-23). KA treatment significantly impaired memory and learning (p<0.0001 vs vehicle treatment, Tukey HSD post ANOVA test), showing only an 18% decrease in escape latency over the five days compared to a 60% decrease for vehicle-treated animals. JAK4D treatment significantly reduced the KA-induced cognitive deficits (p=0.005 vs KA+vehicle; Tukey HSD post ANOVA test), reflected by a 36% decrease in escape latency over 5 days. (B) Tukey box and whisker plots of probe trial performance from the three independent experiments. The time spent in the platform area by KA-treated groups was standardized as a proportion of the control mean time. The median standardized time in KA-treated rats was 0.076 compared to 0.30 for KA+JAK4D-treated rats; the difference between these two groups was statistically significant (p=0.011, Mann Whitney U test, n=40). Circles indicate outliers.

FIGURE 3: Effects of JAK4D on responses to intrastriatal microdialysis of KA in rat. **(A)** Changes in the extracellular concentration of OH-TA (nM) versus time. Points represent mean $\pm$ s.e.m. (n=4-5). Basal levels were unaffected by JAK4D pre-treatment in the control group. In KA-treated groups 1 mM KA was applied during the 100 min fraction collection period and the effect of KA stimulation was evaluated in terms of the KA-stimulated area under the concentration-time curve minus its basal value over the period 140-260 m. OH-TA levels were significantly increased compared to controls in KA+vehicle-treated animals; this increase was significantly reduced by pre-treatment with JAK4D ($p<0.01$ for KA+vehicle vs control and $p<0.05$ for KA+vehicle vs KA+ JAK4D, Tukey multiple comparisons test, n=4-5). **(B)** *Ex vivo* analysis of striatal tissue showed that JAK4D significantly reduced the observed KA-induced apoptosis as measured by TUNEL and caspase-3 activation ($***p<0.001$, Tukey multiple comparisons test, n=4-5). **(C)** Typical images of IBA1 immunolabelling for the three treatment groups. JAK4D pre-treatment appears to reduce KA-induced microglial activation, as indicated by the presence of a greater number of cells more intensely immunopositive for IBA1 in the KA-stimulated animals compared to the JAK4D pre-treated group. IBA1-immunolabelling was undetectable in the control group.

FIGURE 4: Effects of JAK4D in G93A-SOD1 ALS mice. All comparison analyses were carried out from symptom onset (day 100) to first death (day 118). **(A)** Latency to fall off rotarod measured in triplicate for vehicle- and JAK4D-treated mice (n=13 and 12, respectively). Data represent mean $\pm$ s.e.m. for each group. JAK4D treatment significantly improved rotarod performance following onset ($**p<0.01$ versus vehicle treated group, Newman-Keuls post hoc

test). **(B)** Changes in mean weight for vehicle- and JAK4D-treated groups over time. Points represent mean values (n=13 for each group). JAK4D treatment significantly reduced change in weight loss over time from onset to first death ($p<0.0001$). **(C)** Kaplan-Meier survival plot for vehicle- and JAK4D-treated ALS mice (log-rank test $p=0.520$). **(D)** Histological analysis of caudal lumbar cord. Significant differences were found in neuron cell counts between sibling non-carrier controls and vehicle-treated G93A-SOD1 mice, as well as between vehicle-treated G93A-SOD1 mice and JAK4D-treated G93A-SOD1 mice (** $p<0.01$, * $p<0.05$ Tukey's multiple comparisons test, n=5 per group).

Fig 2. single column fitting

Fig 3. Two column fitting

A **B** **C**

Fig 4. Two column fitting

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Highlights

- First-in-class TRH-based compound JAK4D detects new receptor subtype in human brain
- JAK4D elicits statistically significant effects in neurodegenerative animal models
- JAK4D crosses the blood-brain-barrier and has a clean initial toxicology profile
- JAK4D is a tool to study central pharmacologically-distinct TRH receptor subtype
- JAK4D is an attractive therapeutic candidate for neurodegenerative diseases