A small molecule p75<sup>NTR</sup> ligand normalizes signalling and reduces Huntington’s disease phenotypes in R6/2 and BACHD mice

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Abstract

Decreases in the ratio of neurotrophic versus neurodegenerative signalling play a critical role in Huntington’s disease (HD) pathogenesis and recent evidence suggests that the p75 neurotrophin receptor (NTR) contributes significantly to disease progression. p75<sup>NTR</sup> signalling intermediates substantially overlap with those promoting neuronal survival and synapse integrity and with those affected by the mutant huntingtin (muHtt) protein. MuHtt increases p75<sup>NTR</sup>-associated deleterious signalling and decreases survival signalling suggesting that p75<sup>NTR</sup> could be a valuable therapeutic target. This hypothesis was investigated by examining the effects of an orally bioavailable, small molecule p75<sup>NTR</sup> ligand, LM11A-31, on HD-related neuropathology in HD mouse models (R6/2, BACHD). LM11A-31 restored striatal AKT and other pro-survival signalling while inhibiting c-Jun kinase (JNK) and other degenerative signalling. Normalizing p75<sup>NTR</sup> signalling with LM11A-31 was accompanied by reduced Htt aggregates and striatal cholinergic interneuron degeneration as well as extended survival in R6/2 mice. The p75<sup>NTR</sup> ligand also decreased inflammation, increased striatal and hippocampal dendritic spine density, and improved motor performance and cognition in R6/2 and BACHD mice. These results support small molecule modulation of p75<sup>NTR</sup> as an effective HD therapeutic strategy. LM11A-31 has successfully completed Phase I safety and pharmacokinetic clinical trials and is therefore a viable candidate for clinical studies in HD.

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Introduction

Huntington’s disease (HD) is a neurodegenerative disorder characterized by motor, cognitive and psychiatric disturbances arising from preferential damage to medium spiny neurons (MSNs) in the striatum as well as cortical and hippocampal neurodegeneration (1,2). The disease is caused by a mutation that extends the trinucleotide CAG repeat region in exon 1 of the HTT gene generating a mutant huntingtin (muHtt) protein with a polyglutamine expansion (3). MuHtt reduces neurotrophic support, largely due to deficient brain-derived neurotrophic factor (BDNF) signalling via its tropomyosin receptor kinase B (TrkB) receptor, and this deficit is thought to have a prominent causal role in developing fundamental HD pathologies (4,5).

Neurotrophins (NTs) bind to specific Trk receptors and with lower affinity to the 75 kDa pan-NT receptor (p75NTR) (6,7). p75NTR lacks intrinsic catalytic activity but has the ability to regulate diverse cellular processes including active cell death via recruitment of intracellular adaptor proteins and modulation of other receptors. NT-p75NTR binding can potentiate Trk function and thereby promote trophic signalling, depending on the cellular context and Trk abundance, and can activate degenerative pathways with a co-receptor sortilin, which is up-regulated in HD, and may also independently promote survival or degenerative signalling (8–11). Lack of NTs leads to a ‘dependence receptor’ mode of p75NTR signalling that activates degenerative pathways (7). States in which an NT and/or Trk deficiency exist along with increased p75NTR expression may be particularly deleterious (12–14). This state occurs in HD patients and multiple HD mouse models, wherein p75NTR levels are increased in striatum and hippocampus and TrkB levels and/or downstream signalling are decreased (12,15–18). This imbalance contributes to altered p75NTR signalling which impairs TrkB signalling and negatively impacts structural and functional synaptic plasticity in HD mouse models (12,15,17,19). p75NTR signalling intermediates overlap many of those that are responsible for neuronal survival and synapse integrity as well as those affected by muHtt and/or other HD-related mechanisms (e.g. excitotoxicity, inflammation; Fig. 1) (7,12,19,20). Pharmacological and/or genetic inhibition of some of these signalling intermediates restored BDNF-dependent long-term potentiation (LTP) in indirect pathway spiny projection neurons in striatum of the BACHD mouse model of HD (17). Normalizing hippocampal p75NTR expression via genetic means or treatment with fingolimod, a sphingosine-1 phosphate receptor modulator, reduced degenerative signalling and restored both structural and functional aspects of synaptic plasticity and memory in HD mouse models (15,19). These studies strongly suggest p75NTR as a viable HD therapeutic target especially if its pro-survival signalling can be activated while its deleterious signalling is inhibited.

The hypothesis that a p75NTR ligand may inhibit degeneration and promote neuronal function in HD through interactions with the many signalling pathways affected by muHtt (as in Fig. 1) was tested directly in this study using a small molecule p75NTR ligand, LM11A-31. LM11A-31 binds to p75NTR and not Trk receptors, down-regulates p75NTR-related degenerative signalling, up-regulates trophic signalling (21–23) and prevents neurodegeneration in vitro studies (23–28) and animal models of multiple neurological disease states (29–31), including post-traumatic brain injury and Alzheimer’s disease (30,32–34). Here we report that oral administration of LM11A-31 to two HD mouse models normalizes p75NTR signalling, reduces key HD neuropathologies, including Htt aggregation and dendritic spine loss, and improves cognition and motor performance, providing evidence that targeting p75NTR may be an effective strategy for the treatment of HD.

Results

LM11A-31 decreases p75NTR-associated degenerative signalling and increases survival signalling

p75NTR up-regulation and/or TrkB down-regulation in the striatum and hippocampus disrupts neurotrophic support, contributing to reduced survival signalling and augmented degenerative signalling in HD patients and multiple mouse models (11,12,15,19). However, this imbalance had not been previously determined in the R6/2 mouse model of HD. Therefore, levels of full-length p75NTR and TrkB proteins were examined in the striatum of 11-12 week old R6/2 and wild-type (WT) mice treated for 7 weeks with vehicle or LM11A-31. p75NTR levels were increased by 29 ± 8% (mean ± s.e.m) (Fig. 2A) in vehicle-treated R6/2 mice compared to WTs, while TrkB levels were reduced by 20 ± 4.6% (Fig. 2B), as shown previously (18). Consequently, the ratio of p75NTR to TrkB was substantially increased in R6/2 striatum. LM11A-31 did not affect levels of either receptor in either genotype.

NTs and other ligands bind to p75NTR promoting intracellular domain interactions with cell adaptor proteins that regulate downstream signalling and endocytosis. p75NTR differs from most other growth factor receptors in that it is not phosphorylated upon activation and therefore cannot be monitored in this manner to assess target engagement. Ligand binding can induce regulated intramembrane proteolysis which generates a C-terminal fragment (CTF), which is further cleaved to produce an intracellular domain-fragment (ICD) (35–37). It was therefore of interest to determine whether LM11A-31 would promote, inhibit or otherwise modulate p75NTR proteolysis in HD mice, which would also provide an indication of compound function in the brain region of interest. LM11A-31 was previously shown to increase CTF and ICD levels indicating in vivo target engagement in an Alzheimer’s disease mouse model (33). Thus, to assess in vivo target engagement in HD mice and to begin to examine the effects of LM11A-31 on HD-related signalling, the ligand’s impact on p75NTR cleavage was assessed. Immunoblotting with an antibody detecting the p75NTR cytoplasmic domain revealed that R6/2-vehicle mice exhibited decreased striatal CTF but not ICD levels relative to WTs (Fig. 2C). Furthermore, LM11A-31 normalized CTF in R6/2 mice and increased ICD levels in both WT and R6/2 mice, demonstrating that the compound promoted p75NTR processing, which indicates that the ligand engaged its intended target (36,38).

MuHtt disrupts multiple signalling pathways intersecting with those regulated by p75NTR (Fig. 1), including c-Jun kinase (JNK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Rho kinase, as well as AKT (6,7,39). Thus, the nature and extent of LM11A-31 effects on key intermediates in these pathways was determined. AKT pathway activation, which is neuroprotective, is reduced in striatum of HD patients and rodent models in advanced disease stages (20,40). Decreased phospho(p)-AKT in the R6/2 striatum was significantly increased by 49 ± 15% (mean ± s.e.m) with LM11A-31 treatment (Fig. 2D). JNK signalling is associated with cell death and is up-regulated by muHtt in striatum of HD rodent models, including R6/2 mice (20,41–43). LM11A-31 prevented JNK activation in R6/2 striatum (Fig. 2E). An inhibitor of kappa beta kinase (IKK)/NF-κB signalling mediates neuroinflammation and,
promotes cell survival when regulated (44). However, excessive activation of NFκB signalling is observed in HD patients and mouse models and contributes to deleterious neuroinflammation (44–46). NFκB is sequestered by an inhibitor of nuclear factor κB (IκBα) which prevents it from translocating to the nucleus to activate inflammation-promoting gene expression programs (47–49). IκBα levels are reduced in the R6/1 hippocampus (15) and, here, are shown to decrease by 29 ± 6% (mean ± s.e.m.) in R6/2 striatum (Fig. 2F), which could cause elevated NFκB signalling; LM11A-31 mitigated this deficit. The Rho kinase pathway is modulated by p75 NTR signalling (50,51) and increased activity/signalling of the small GTPase RhoA has been implicated in HD pathogenesis (15,17,19,52–54). p75 NTR directly activates RhoA, which activates Rho-associated kinase (ROCK), triggering phosphatase-and-tensin-homolog-deleted-on-chromosome 10 (PTEN) to inhibit phosphoinositide 3-kinase (PI3K) activation of AKT (Fig. 1) (6,51,55,56). ROCK1 levels are increased in brains from HD patients and R6/2 mice (54) and PTEN is increased in inhibitory spiny projection neurons in striatum of BACHD mice (17). In this study, both ROCK1 and PTEN were up-regulated in the R6/2 striatum and these increases were attenuated by LM11A-31 treatment (Fig. 2G and H). Another downstream target of RhoA is p21-activated kinase 1 (PAK1), which, when activated, is neuroprotective and involved in regulating the actin cytoskeleton and neuroplasticity, including LTP consolidation, and increased post-synaptic density (PSD) levels (57–59). R6/2 mice given vehicle had reduced pPAK1 and PSD-95 levels which were normalized by LM11A-31 (Fig. 2I and J).

Striatal levels of p75NTR and the effects of LM11A-31 on its key signalling pathways were also investigated in 9 month-old BACHD mice. Similar to the R6/2 striatum, p75NTR levels are up-regulated in BACHD mice and are unaffected by LM11A-31 treatment (Fig. 2K). It was previously shown that TrkB levels were unaltered in BACHD mice at this age (18). While pAKT levels were similar in striatum of WT and BACHD mice, they were increased in BACHD mice treated with LM11A-31 (Fig. 2L). Both pJNK and PTEN were increased in BACHD-vehicle mice versus WTs, and LM11A-31 significantly reduced pJNK but not PTEN levels (Fig. 2M and N).

LM11A-31 mitigates intranuclear Htt aggregates and striatal DARPP-32 deficits

MuHtt translocates from the cytoplasm to the nucleus of neurons in numerous brain areas of HD patients and mouse models and forms aggregates (60,61). LM11A-31 significantly reduced the number and total area occupied by neuronal intranuclear Htt aggregates in the striatum and cortex but neither of these measures was affected in the hippocampus of 11 week-old R6/2 mice (Fig. 3A–D). In addition, aggregate size was significantly decreased in striatum and hippocampus with LM11A-31 treatment (Fig. 3E).

MuHtt expression decreases striatal volume in HD patients and R6/2 mice (1,61,62), and in this study R6/2 mice showed an 18 ± 3% reduction in striatal volume (P ≤ 0.005 versus WT-vehicle), which was unaltered by LM11A-31 (Supplementary Material, Fig. S1A). LM11A-31’s effects on Htt aggregates and striatal volume were not analysed in BACHD mice as these appear at a more advanced ages [≥12 months; (63,64)] than used in this study [9 months; (18)].
Striatal MSNs are enriched in the dopamine signalling protein, 32 kDa dopamine- and cAMP-regulated phosphoprotein (DARPP-32), levels of which are markedly reduced in HD patients and mouse models (18,65–68). MSNs also express p75NTR, which can regulate DARPP-32 expression (12,17,19). Western immunoblotting showed that LM11A-31 ameliorated the deficit in striatal DA RPP-32 levels in R6/2 mice (Fig. 3F). DARPP-32 levels are not significantly reduced in BACHD mice at 10 months of age (64), however, our previous work revealed that at 8 months of age these mice have DARPP-32-containing neurites that show signs of degeneration (18). In this study, DARPP32-containing MSNs of BACHD mice (Fig. 3G–I) had significantly fewer neurites (Fig. 3J) that were shorter (Fig. 3K) and tended to occupy less area (Fig. 3L) than those in WTs. LM11A-31 treatment significantly increased the neurite number and length (Fig. 3J and K), and increased area, though this latter measure was not statistically significant (Fig. 3L).
LM11A-31 improves striatal cholinergic interneuron dendrite structure

Unlike MSNs, striatal interneurons are relatively preserved in HD, particularly the cholinergic sub-type (1, 69–71). While cholinergic interneurons do not decrease in number, they do undergo soma shrinkage and dendrite degeneration (72,73). These cells have been shown to contain Htt aggregates (74,75) and have disrupted electrophysiological properties, acetylcholine release and synaptic plasticity (72,76–79). Cholinergic interneurons contain low p75 NTR levels, which increase in deleterious conditions that contribute to HD striatal degeneration, such as excitotoxicity (80,81), however, whether this increase also occurs in HD is unknown. In R6/2 mice, the area, volume and length of choline acetyltransferase (ChAT)-containing dendrites was reduced by ~40% (Fig. 4A–D) and Sholl analysis revealed that dendritic branching was also decreased relative to WTs (Fig. 4E). LM11A-31 partially prevented all of these muHtt-associated changes in ChAT dendrites (Fig. 4).

Microglial activation is reduced by LM11A-31

The effect of LM11A-31 on striatal inflammation was as assessed using immunostaining for IBA-1, a protein expressed by activated or proliferating microglia (82–84). The striatal and hippocampal area occupied by IBA-1-stained microglia was increased in vehicle-treated R6/2 mice (Fig. 5A–E), as shown previously (18,85,86), as well as in vehicle-treated BACHD mice (Fig. 5F–J). The number of striatal IBA-1-positive cells did not differ between WT mice and 11 week-old R6/2 (58855 ± 3280 versus 57613 ± 7045, respectively, mean ± s.e.m, n = 7-8 mice/group) or 9 month-old BACHD mice (53726 ± 5750 versus 53284 ± 3152, respectively, n = 5-6 mice/group), similar to previous reports in which genotype differences were absent between WTs and 12 month-old BACHD and YAC128, another full-length Htt model (87,88).

Microglia undergo distinct phenotype changes when activated. At rest, their soma are oblong with ramified processes but during the activation process they appear rounded and their processes retract (89). While microglia did not proliferate in YAC128 mice, significant morphological changes occurred (87). Thus, microglial morphology was investigated in the BACHD striatum. Microglial phenotypes were diverse, however soma appeared larger, more round and less branched compared to those in WTs (Fig. 5K). Quantification revealed that microglial processes increased in number in BACHD mice while their

Figure 3. LM11A-31 ameliorates two principal HD neuropathologies: intranuclear Htt aggregates and striatal DARPP-32 deficits. (A–B) Representative photomicrographs showing nuclear Htt immunostaining in striatum (STR) of R6/2 mice treated with Veh (A) or C31 (B). Arrows indicate intranuclear Htt aggregates. Scale bar in B = 20 μm. (C–D) Quantification of the total area occupied by Htt aggregates (C), aggregate number (D) and aggregate size (E) in STR, cortex (CX), and hippocampus (HIPP) of R6/2 mice given Veh or C31 (*P < 0.05, **P = 0.002 vs. R6/2-Veh, one-tailed Student’s t-test; n = 11–12 mice/group). Values in C–E are normalized to the R6/2-Veh group. (F) Representative Western blot for DARPP-32 showing immunobands from striatal homogenates of WT and R6/2 mice treated with Veh or C31 and corresponding densitometric group analyses (**P < 0.001 vs. R6/2-Veh; *P ≤ 0.05; n = 11–12 mice/group, ANOVA with Fisher’s LSD). To facilitate comparisons, non-adjacent bands from the same gel were juxtaposed and separated by spaces to indicate they were not adjacent in the original gel. Tubulin from the same stripped and re-probed gel is also shown. (G–I) DARPP-32 immunostaining in striata of WT-Veh mice and BACHD mice treated with Veh or C31. Scale bar in I = 20 μm. Arrowheads indicate distal ends of neurites. (J–L) Quantification of the number (J), length (K), and area (L) of DARPP-32-stained neurites in STR of WT and BACHD mice (*P < 0.05, **P = 0.005 vs. WT-Veh; †P < 0.05, ††P = 0.005 vs. BACHD-Veh; n = 7–11 mice/group; ANOVA with Fisher’s LSD). Values in (F–L) are normalized to their respective WT-Veh groups. Results are expressed as mean ± s.e.m.
Dendritic spine density was reduced on striatal MSN dendrites (Fig. 5B). LM11A-31 significantly prevented dendritic spine loss in striatum (Fig. 6E–G). Density of apical and basal dendritic spines in BACHD hippocampus was not significantly affected (Fig. 6H–I). However, when apical and basal dendrites were analysed together, a significant decrease in thin and stubby spines was detected (Fig. 6I). As observed in the striatum, LM11A-31 markedly increased hippocampal spine density in both BACHD and WT mice (Fig. 6H–J).

LM11A-31 extends survival and improves motor performance in R6/2 mice

LM11A-31 administered to R6/2 mice beginning at 3.5 weeks of age until mortality did not affect the body weight of WT or R6/2 mice (Supplementary Material, Fig. S1B). Notably, a Kaplan-Meier analysis revealed that LM11A-31 significantly increased the survival of R6/2 mice (Fig. 7A). Lifespan was extended by 20 ± 6% (mean ± s.e.m.; P = 0.005, 2-tailed Mann-Whitney test), with an increase in mean survival from 11.9 ± 0.6 to 14.3 ± 0.7 weeks.

R6/2 mice exhibit a progressive decline in general activity and exploration beginning at 4–6 weeks of age (98–101). LM11A-31 did not improve impaired motor balance and coordination of R6/2 mice on an accelerating rotarod (Fig. 7B) but significantly increased the distance moved, velocity and rearing of 7 week-old R6/2 mice in an activity chamber (Fig. 7C–E) with a tendency to decrease immobility duration (P = 0.055; Fig. 7F). When placed in a novel standard cage, vehicle-treated R6/2 mice perform fewer active behaviours (digging and rearing) but groom more than WTs (Fig. 7G and H). They also cross from the cage periphery to the centre less frequently (Fig. 7I) and have a higher angular velocity (speed of movement direction change) which indicates behavioural abnormalities such as stereotypies (Fig. 7J).

LM11A-31 increased the number of active behaviours performed, decreased grooming, normalized periphery-to-centre transitions and decreased stereotypic behaviour in R6/2 mice (Fig. 7G–J).

LM11A-31 normalizes exploratory activity and improves cognition in R6/2 mice

Automated social phenotyping, which allows assessment of exploratory activity and unsupervised/directed learning, was performed on WT and R6/2 mice at 8–9 weeks of age for 2 weeks (Fig. 8A and B). R6/2-vehicle mice visited corners less frequently than WTs during the first 3 h in the cage suggesting reduced novelty-induced exploratory activity; LM11A-31 eliminated this deficit (Fig. 8C). Mice were required to discover and learn the new location of water bottles (corners versus cage top), with first lick latency indicating exploration and unsupervised learning. R6/2-vehicle mice took 3 times longer to discover the water location than WT-vehicle mice, while R6/2 mice given LM11A-31 located the water significantly faster than those given vehicle

LM11A-31 prevents dendritic spine loss in striatum and hippocampus

Dendritic spines are reduced on MSNs and CA1 pyramidal neurons of HD patients and mouse models, including R6/2 and BACHD mice (18,90–94). In the hippocampus, p75NTR is localized to CA1 dendritic spines (95,96) and its post-synaptic levels are increased in HD mice (19). This increase likely contributes to the spine loss seen in HD as p75NTR negatively affects spine density (97) and decreasing its levels in HD mice ameliorates spine deficits (19). LM11A-31 effects on RhoA signalling (28,29) and restoration of hippocampal LTP in an AD mouse model (24) suggests that the compound might influence spine formation. Dendritic spine density was reduced on striatal MSN dendrites in vehicle-treated R6/2 (Fig. 6A and B) and BACHD (Fig. 6C and D) mice due to a decrease in mushroom-type spines. LM11A-31 alleviated spine deficits in R6/2 mice, and, in BACHD mice and their WT littermates, increased the spine density of each morphological type examined. Spine loss also occurred on apical dendrites of hippocampal CA1 pyramidal neurons in R6/2 mice with a non-significant decrease on basal dendrites (Fig. 6E and F). Morphological analysis of apical and basal dendrites combined showed that the decrease could be attributed to fewer mushroom and thin spines (Fig. 6G). LM11A-31 significantly prevented hippocampal spine decrements in R6/2 mice (Fig. 6E–G). Density of apical and basal dendritic spines in BACHD hippocampus was not significantly affected (Fig. 6H and I). However, when apical and basal dendrites were analysed together, a significant decrease in thin and stubby spines was detected (Fig. 6J). As observed in the striatum, LM11A-31 markedly increased hippocampal spine density in both BACHD and WT mice (Fig. 6H–J).

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which suggests better spatial/place learning and memory. 2 mice performed fewer errors than R6/2-vehicle mice (Fig. 8I), established (Fig. 8A). Again, WT mice and LM11A-31-treated R6/2-mice (Fig. 8H). LM11A-31-treated R6/2 mice made fewer errors on day 1 of this test, while R6/2-vehicle mice made more errors including BACHD mice (99,102–105). Catwalk gait analysis showed that BACHD-vehicle mice had a longer stride length (Fig. 9B and C) and less paw contact with the floor when propelling forward during a step cycle (Fig. 9A). LM11A-31 mitigates motor, psychological and cognitive deficits in BACHD mice

Body weights of vehicle-treated BACHD mice at 2 months of age and throughout the 28 weeks of treatment were significantly greater than WT mice, as previously reported (3,63,101), and were unaffected by LM11A-31 (Supplementary Material, Fig. S2A). When mice were allowed to freely explore an activity chamber at 4 and 5 months of age, vehicle-treated BACHD mice travelled less distance and more slowly (Supplementary Material, Fig. S2B and C) than WT mice and also reared less (Supplementary Material, Fig. S2D). BACHD given LM11A-31 reared more frequently than those given vehicle at 4, but not 5, months of age; their distance travelled and velocity were unaltered. LM11A-31 improved the motor performance of BACHD mice on an accelerating rotarod at 3 months of age (after 1 month of treatment) but this effect was not evident at later ages (Fig. 9A).

Gait disturbances are pronounced and particularly debilitating for HD patients and can also occur in HD animal models including BACHD mice (99,102–105). Catwalk gait analysis showed that BACHD-vehicle mice had a longer stride length (Fig. 9B and C) and less paw contact with the floor when propelling forward during a step than WTs (Fig. 9D), and LM11A-31 normalized these measures (Fig. 9B–D). BACHD mice differ from WTs in inter-paw coordination or the temporal relationship between anchor and target paw placement during a step cycle (phase

(Fig. 8D). WT mice treated with LM11A-31 also had longer lick latencies than vehicle-treated WTs however these groups performed similarly on all other measures during this testing. The above deficits in exploratory activity are most likely related to the investigation of novel stimuli and spatial information learning rather than reduced locomotion since, after the initial 3 h of exposure to the novel cage, WT and R6/2 mice visited corners with a similar frequency that decreased and then plateaued as all experimental groups became familiar with the cage (Fig. 8E). Next, corner doors were closed and the mice had to learn to nosepoke to open them and access water. R6/2-vehicle mice performed fewer nosepokes during the first 10 h and licked less frequently than those given vehicle indicating improved performance on this operant conditioning task.

During Place Learning, mice were water deprived for 16 h and were allowed access to water only after nosepoking in their least preferred corner (Fig. 8A and B). WT mice performed less nosepokes at non-reinforced corners (errors) on day 2 versus day 1 of this test, while R6/2-vehicle mice made more errors (Fig. 8H). LM11A-31-treated R6/2 mice made fewer errors on day 2, similar to WTs. Place learning was repeated after the mice were allowed free access to water and corner preference was re-established (Fig. 8A). Again, WT mice and LM11A-31-treated R6/2 mice performed fewer errors than R6/2-vehicle mice (Fig. 8I), which suggests better spatial/place learning and memory.
dispersion). BACHD mice perform more diagonal, girdle, and ipsilateral phase dispersions than WTs (Fig. 9E). These coordination abnormalities were alleviated in BACHD mice treated with LM11A-31. The average intensity of paw contact with the floor did not differ between BACHD and WT mice (WT-vehicle: 36.7 ± 0.3, n = 17 mice, versus BACHD-vehicle: 36.9 ± 0.4 n = 21 mice, intensity units from 0-255), as shown previously (103), suggesting that, although the transgenics weigh more, their gait abnormalities are unlikely attributable to weight supporting limb adjustments (103). Finally, the order in which BACHD mice place their forelimbs (F) and hind limbs (H) during a step sequence significantly differed from WTs in that they used the footfall pattern cruciate a [right F (RF) - left F (LF) - RH - LH] more frequently and the cruciate b pattern [LF - RF - LH - RH] less (Fig. 9F); alternating step sequences (Aa, Ab) were not significantly affected. The footfall patterns of BACHD mice were normalized by LM11A-31 treatment.

Anxiety-like behaviors are increased in BACHD-vehicle mice as evidenced by their increased time spent in the dark compartment of a light-dark box (Fig. 9G) and less time in the open sections of an elevated zero maze (Fig. 9H), compared to WTs. LM11A-31 alleviated anxiety on both of these tests (Fig. 9G and H).

A passive avoidance task was used to evaluate the effects of LM11A-31 on associative and contextual learning and memory in BACHD mice. No genotype or drug effects on task learning were detected (Fig. 9I) but during retention testing the day after training WT mice took longer to enter the aversive zone than vehicle-treated BACHD mice suggesting that WTs learned the association between this area and the aversive stimulus to a
greater extent. WT mice retained this learned association 7 and 14 days after training indicating that the compound greatly improved memory in BACHD mice.

Discussion

Recent evidence has established p75NTR as an important contributor to the loss of trophic support and synaptic plasticity deficits known to play a major role in HD pathogenesis (4,12,17–19). p75NTR is up-regulated in the HD brain, as it is under conditions of cellular stress and in other pathologies (12,16,19,106). Dysfunctional p75NTR signalling has been shown to negatively impact corticostriatal and hippocampal synaptic plasticity, contributing to cognitive deficits and to a lesser extent motor dysfunction in HD, and has been attributed, in part, to a p75NTR/TrkB imbalance (4,12,15,16,19). Interestingly, a similar imbalance involving p75NTR and TrkA occurs in the cells that primarily degenerate in AD (107). This study showed that a p75NTR/TrkB imbalance also exists in 11–12 week-old R6/2 and 9 month-old BACHD mice.

Although p75NTR functions are complex, with contributions to both pro-survival and cell death signalling (10,21,108), its potential multiplex influences on fundamental deleterious HD-associated signalling make it an enticing target for HD therapeutics. The present study supported this idea by demonstrating that the small molecule p75NTR ligand, LM11A-31, positively influenced many of the intracellular signalling pathways negatively affected by muHtt. Furthermore, LM11A-31 reduced Htt aggregates and alleviated striatal cholinergic interneuron degeneration in R6/2 mice. It also decreased inflammation in striatum and hippocampus and reduced dendritic spine loss in striatal MSNs and hippocampal CA1 pyramidal neurons of R6/2 and BACHD mice. Finally, it improved motor performance and cognition in both HD mouse models. Importantly, LM11A-31 increased the survival rate of R6/2 mice by 20%.

The in vivo engagement of p75NTR by LM11A-31 was supported by its induction of increased levels of the CTF and ICD cleavage products of the receptor. Proteolysis of p75NTR is constitutive but is also regulated by ligand binding. Sequential intra-membrane proteolysis by α- and γ-secretases generates the membrane bound CTF and then the ICD (35–38,109,110). The potential biological significance of p75NTR cleavage products is complex, as they have been shown to influence p75NTR signalling via both cell survival pathways (e.g. p75NTR's potentiation of Trk signalling by increasing activated AKT) and death pathways, depending on the cell type and context, as well as neurite outgrowth (36–38,110). However, the roles of the CTF and ICD in the HD brain are unknown. Of interest, CTF levels were significantly decreased in R6/2 mice which could be caused by reduced extracellular ligand-induced proteolysis of p75NTR due to decreased BDNF levels and/or less constitutive cleavage (4,37,110). The current findings, along with prior in vitro and in vivo studies suggesting direct LM11A-31/p75NTR interactions (23,31,33), indicate that proteolysis is likely triggered directly by compound-receptor interactions, though the possibility of indirect mechanisms remains for further investigation.

LM11A-31 was shown previously to prevent degenerative signalling while triggering trophic signalling in numerous neurodegenerative conditions (21,22). In the present study, similar LM11A-31 effects on p75NTR signalling were observed in the presence of muHtt. LM11A-31 partially to completely normalized JNK, RhoA, NFκB and AKT signalling in the striatum of R6/2 and/or BACHD mice, which could underlie its mitigation of diverse and fundamental HD pathologies (see Fig. 1), including...
Htt aggregation, which may be affected by AKT (111–113) and RhoA/ROCK1 signalling (114–116); DARPP-32 and cholinergic interneuron morphological abnormalities, which may involve JNK (117), RhoA (118,119) and AKT (40,66) pathways; and inflammatory reactions, responsive to NFκB signalling (44–46).

The precise mechanism by which LM11A-31 modulates p75NTR to increase survival and decrease degenerative signalling remains unknown. Though nerve growth factor (NGF) has been shown to provide neuroprotection and improve cognition in an HD mouse model (120), and LM11A-31 has structural and chemical features similar to the NGF loop 1b-turn domain which interacts with p75NTR (22,23), the compound’s activities are distinct from those of NGF. Most notably, previous in vitro studies showed that unlike the NTs, LM11A-31 does not activate the NGF receptor TrkA, or other Trks (23). However, like NGF, in some conditions LM11A-31 recruits signalling adaptors to p75NTR to promote p75NTR-dependent activation of downstream pathways and cell survival. These protective effects are also observed in 3T3 cells that lack Trks. In addition, though the p75NTR ligand proNGF promotes cell death via p75NTR in susceptible cells, LM11A-31 does not have this effect, and in fact antagonizes proNGF-induced apoptotic cell death (23). Furthermore, in a model of neuronal Aβ toxicity, LM11A-31 was protective where NGF was not, and added NGF antagonized the protective effects of the compound (24). Together, these results suggest that LM11A-31 has system/state-dependent effects which may involve agonism of p75NTR-coupled survival signalling and/or antagonism of degenerative signalling. Finally, no activation of TrkB was observed in neurons treated with LM11A-31, suggesting the compound did not promote BDNF secretion (23). Thus, it seems unlikely that the beneficial effects of LM11A-31 seen in this study involve potentiation of NT levels or actions, though this possibility cannot as yet be completely ruled out.

LM11A-31 reduced the size and/or number of intranuclear Htt aggregates in the striatum, cortex and hippocampus suggesting that the ligand mitigates production and/or accumulation of muHtt oligomers. These results could be due to LM11A-31 activation of AKT or reduction in RhoA/ROCK signalling. AKT activation has been reported to decrease muHtt aggregation and toxicity directly by phosphorylating Htt or indirectly
by promoting proteasomal degradation of Htt via increasing arfaptin2 activity (40,111,112). Inhibiting the ROCK pathway enhances muHtt degradation and aggregation while improving motor function in R6/2 mice (52,53,114–116). The aggregate load reduction has also been associated with improved cognition and/or motor performance in HD mice (5,18,121–125). Thus, LM11A-31’s effects on muHtt metabolism may contribute to its restorative effects on motor performance and cognition.

LM11A-31 also prevented spine morphology abnormalities and loss in R6/2 and BACHD mice and, in the latter model, elevated spine density to levels greater than that observed in vehicle-treated WT mice. Post-synaptic p75NTR levels are elevated in hippocampus of HD mice (19) which likely contributes to spine loss as p75NTR negatively affects spine density (97) and decreasing levels of the receptor in HD mice mitigates spine deficits (19). While LM11A-31 does not affect p75NTR levels, it activates AKT and PAK signalling, which can positively regulate spine formation, morphology and/or function (126–129), while decreasing RhoA-associated signalling which can negatively affect spines (119,130). LM11A-31 also prevented the decline in striatal PSD-95 levels in R6/2 mice. Taken together, these results suggest that LM11A-31 can reduce p75NTR-associated synaptic dysfunction seen in HD (19). The positive effects of LM11A-31 on synaptic-related signalling /proteins and spine density provide...
substrate mechanisms for its beneficial effect on cognition in R6/2 and BACHD mice.

While LM11A-31 largely normalized p75NTR signalling in the striatum, it only partially recovered motor behaviour. It mildly improved locomotion and exploratory activity but did not affect the rotarod performance of R6/2 mice, and, in BACHD mice, general locomotor activity and motor learning/balance were ameliorated by LM11A-31 in early but not later stages of treatment. Incomplete motor recovery is consistent with the partial reversal of DARPP-32 deficits shown in this study, and the lack of significant improvement in rotarod performance and corticostriatal synaptic plasticity seen in the HdhQ111 knock-in HD mouse model when p75NTR expression is decreased (19). Thus, other signalling pathways and extra-striatal brain areas involved in motor function are likely not affected by LM11A-31, at least with the dosing protocol used in this study. Future investigations will examine whether other LM11A-31 dosing paradigms will be more effective against motor deficits and neurodegeneration.

LM11A-31 reduced the stereotypic behaviours displayed by R6/2 mice. Behavioural inflexibility and stereotypes, including cognitive impairments related to attentional set shifting or choreiform movements in HD patients, are associated with striatal dopamine imbalance and disrupted corticostriatal communication and thalamostriatal inputs to cholinergic interneurons (131–135). Thus, the reduction in repetitive behaviours, including grooming, seen with LM11A-31 treatment may arise from the compound’s partial restorative effects on DARPP-32 levels and prevention of cholinergic interneuron degeneration.

Second only to chorea, gait abnormalities are the most prominent motor dysfunction exhibited by HD patients and can be distinguished in pre-symptomatic gene mutation carriers (105,136–138). Recuperative effects of LM11A-31 were seen on aspects of impaired gait in BACHD mice at 9 months of age, including stride length, step sequence and brake/propulsion transitions. These results suggest that, despite the relatively small effects of LM11A-31 on locomotion, the compound does alleviate gait disturbances, a major quality of life factor for HD patients because of loss of independence and fall risk (137–139), which could be easily and reliably used as a clinical trial outcome measure (136).

In conclusion, these studies demonstrate that LM11A-31, when administered once per day for 5–6 days per week, significantly reduced motor, cognitive, psychiatric and neuropathological abnormalities in two HD mouse models. Taken together, these results provide validation that a small molecule ligand p75NTR, a receptor critically positioned in survival and neurodegenerative signalling, may serve as an effective HD therapeutic. LM11A-31 has successfully completed Phase I safety and pharmacokinetic clinical studies in normal subjects and is therefore a viable lead candidate for HD clinical testing.

Materials and Methods
Study design
This study aimed to establish p75NTR as a feasible therapeutic target for HD by determining the effectiveness of a small molecule p75NTR ligand, LM11A-31, on multiple HD-related signalling, neuropathological and behavioural endpoints in both male R6/2 and BACHD mice. R6/2 mice are particularly beneficial for testing therapeutics because they develop HD symptoms rapidly and reliably; BACHD mice better represent the genetic component of HD and have a slower disease progression which allows targeting of early degenerative mechanisms (101). These experiments used a 2 × 2 study design [WT/transgenic X vehicle/LM11A-31] with the random group assignment. Group size needed to obtain statistical significance was determined based on previously published studies using these mice (18,101,140). LM11A-31 was dissolved in sterile water and given to experimental groups after 4 h of fasting at 50 mg/kg (10 ml/kg) via oral gavage once daily 5–6 days/week. Vehicle control groups received water in the same manner. This dose was used based on brain concentrations (23,32) and biological effects determined in previous in vitro and in vivo studies (23,24,26,32,34).

Mouse brain half-life of LM11A-31 following a single oral-gavage dose of 50 mg/kg is 3–4 h (32). R6/2 mice and their WT littermates were treated in multiple cohorts with dosing started at 4 weeks of age. Cohort 1 of R6/2 mice was dosed throughout their lifespan to determine the effects of LM11A-31 on survivability (n = 20–24/group). Cohorts 2 and 3 were dosed for ~7 weeks. Cohort 2 mice received rotarod testing and one brain hemisphere was used for immunohistochemistry and the other for Western immunoblotting (n = 18–22/group). Cohort 3 mice were tested in an activity chamber, then the InteliCage, followed by the novel home cage and brains were used for Golgi staining (n = 10–13/group). BACHD mice were treated from 2 to 9 months of age in 2 cohorts (total n = 20–22/group) that received all behaviour tests. Cohort 1 brains were used for modified Golgi staining and, for Cohort 2, one brain hemisphere was used for immunostaining and the other for Western immunoblotting. Immunostaining, behaviour testing, and histological and quantitative analyses were conducted by experimenters that were blind to the treatment and genotype conditions.

p75NTR ligand
LM11A-31 [2-amino-3-methyl-pentanoic acid (2-morpholin-4-yl-ethyl)-amide] is a water soluble isoleucine derivative identified via in silico screening for compounds corresponding to the NGF loop 1 β-turn domain that interacts with p75NTR (22,23). Its chemical structure, pharmacokinetics and pharmacodynamics have been detailed previously in published reports from our laboratories (23,24,26,32). LM11A-31 successfully completed Phase 1 clinical trials evaluating safety and pharmacokinetics in healthy individuals (Longo et al., 2015, personal communication). LM11A-31 in a sulphate salt form [(2R,3S)-2-amino-3-methyl-N-(2-morpholinooethyl) pentanamide] was used in this study and was custom manufactured by Ricerca Biosciences.

Mice and genotyping
All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals using protocols approved by the Institutional Animal Care and Use Committee at Stanford University. These protocols included efforts to minimize animal suffering and numbers used. Mice were group-housed (3–5 mice/cage), except aggressors if fighting occurred, and received cotton nestlets, paper tubes and rodent chow ad libitum. Tail DNA was used for genotyping via real-time PCR and CAG repeat number measurement via ABI GeneMapper 4.0 by Laragen Inc. For R6/2 mice, breeding pairs were purchased from Jackson Laboratories (female hemizygous ovarian transplant.
Mice that had an average of 125 used in this study all had 98 CAG repeats. BACHD mice are a bacterial artificial chromosome mediated transgenic model that expresses full-length human muHtt with 97–98 CAG repeats (63). BACHD mice used in this study all had 98 CAG repeats.

**Behaviour testing**

Behaviour tests were chosen for each mouse model based on the severity of HD phenotypes and/or the existence of published reports for the test in that model. To evaluate the effects of LM11A-31, behaviour tests that had previously shown significant genotype differences were used (101,103,141,142). Because R6/2 mice have severe symptoms, are prone to stress-induced seizures, and have a short lifespan, the number of behaviour tests that can be performed in a cohort is limited compared to the BACHD mice. R6/2 mice received the following behaviour tests at the ages denoted during the light phase of the light/dark cycle: activity chamber (7 weeks), accelerating rotarod (10 weeks), Intelligence (8–10 weeks), and novel home cage (10 weeks). BACHD mice received the following behaviour tests at the ages denoted during the dark phase of the light/dark cycle: accelerating rotarod (3, 5 and 7 months), activity chamber (4 and 5 months), light-dark box (4 and 5 months), elevated zero maze (7 months), Catwalk (9 months), and passive avoidance (9 months); tests were separated by at least a week.

Behaviour testing procedures will be described briefly here and in more detail in the Supplementary Materials. Both R6/2 and BACHD mice underwent accelerating rotarod (4 to 40 rpm over 5 min) and activity chamber testing, as described previously (18). IntelliCage (New Behaviour AG) testing involved automated recording of home cage behaviours and exploratory activity of mice living in social groups (n = 13–16 mice/cage). R6/2 mice and their WT littermates (n = 10–13/group) were moved to Intelligenetics for 2 weeks starting at 8 weeks of age. They were implanted subcutaneously with RFID-transponders under isoflurane anaesthesia for individual tracking. Testing paradigms used were similar to those used previously in R6/2 mice (141,142). Mice explored the cage for 3 days with all 4 corner doors open and water available from each corner. Corner doors were shut for the next 2 days and the mice must learn to nosepoke in the door vicinity to open them and receive water (Nosepoke Learning); the preferred drinking corner for each mouse was determined. After 16 h of water deprivation, all corner doors were closed for 2 days and mice only received water via a nosepoke at their least preferred corner (Place Learning 1); correct nosepokes were paired with LED light illumination. This learning process was repeated and mice were again only permitted to drink from their least preferred corner (Place Learning 2). One week after mice were removed from the IntelliCage, a subset was allowed to explore a novel standard home cage for 10 min. Their behaviour was recorded by an overhead camera and then analysed with Ethovision XT software v8.5 (Noldus). Gait abnormalities were assessed in BACHD mice, similar to that shown previously (103), using the video-based CatWalk XT system (v9.1, Noldus). After training mice to traverse the walkway, test sessions of three consecutive runs were conducted and averaged. Anxiety-like behaviour was evaluated in BACHD mice using a light-dark box and an elevated zero maze. In the light-dark box, mice were allowed 15 min of exploration in a chamber with two compartments; one dimly lit (10 lux), the other brightly lit (800 lux). Total time spent in each compartment was digitally recorded and analysed using tracking software (Med Associates Inc.). For the elevated zero maze, BACHD mice were allowed 5 min of exploration of a circular walkway that had two portions enclosed with walls and two equal portions without walls; time spent in each section of the maze was analysed. The passive avoidance test was conducted in a two compartment chamber (Gemini™ Avoidance System, SD Instruments): one light and one dark. Tactile cues were added to the floor of the light compartment. Mice were allowed to explore the apparatus and, when they entered the dark chamber, received a mild foot shock. Testing for retention of the pairing of the aversive stimulus with the preferred compartment occurred 1 day later and was expressed as latency to enter the dark chamber. Extinction of the learned association was assessed 7 and 14 days later.

**Western immunoblotting**

One hour post-injection with LM11A-31 or vehicle, R6/2 and BACHD mice along with their respective WT littermates (n = 8–20 mice/group) were deeply anaesthetized with avertin and their brains were removed rapidly. Striatum was dissected from the brains and flash frozen at −80 °C. Brain tissue was prepared for Western blotting, as described previously (18). Briefly, tissue homogenates were prepared in RIPA lysis buffer containing protease and phosphatase inhibitors. Protein samples from each genotype and treatment group were electrophoresed through a 26 well NuPAGE 4–12% Bis-Tris Gel with MOPS SDS running buffer (Invitrogen) and transferred to polyvinylidene difluoride membranes. Membranes were probed using the following antibodies: p75NTR (Promega), TrkB (Millipore); phospho(p)-AKT (Ser473) and AKT (Cell Signalling); p-JNK (Thr183/Tyr185) and JNK (Cell Signalling); IkB-α (Santa Cruz Biotechnology); ROCK1 (Cell Signalling); PTEN (Cell Signalling); pPAK1 (Thr423)/PAK2 (Thr402; Epitomics); and DARPP-32 (Epitomics). All blots were also stripped and reprobed with α-tubulin monoclonal antibody (Sigma) as a loading control. Immunoreactive bands were manually outlined and densities were measured using Un-Scan-It gel software (v6.1, Silk Scientific). The densities of phospho-protein-immunoreactive bands were expressed as a fraction of the band for the total protein and all other bands were expressed as a fraction of tubulin in the same lane after reprobing. Samples were run 4 to 6 separate times per mouse and data was normalized to the WT-vehicle group of that gel then averaged. In the Western blot figures, some non-adjacent bands were moved together for comparison purposes; these were separated by spaces.

**Immunohistochemistry procedures and quantification**

Mice were deeply anaesthetized with avertin and transcardially perfused with saline solution. One brain hemisphere was used for Golgi staining and the other was immersion-fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4), cryoprotected in 30% sucrose/PB, and sectioned (40 μm, coronal) using a freezing microtome. Free-floating sections were processed for immunocytochemical localization of Htt (1:200; clone EM48, Millipore), ChAT (1:800; Millipore), DARPP-32 (1:1,000; Millipore), or IBA-1 (1:1,000;
WAKO) using procedures described previously (18). Immunostaining in striatum was examined at rostral to mid-caudal levels [+1.18 to +0.02 mm relative to Bregma,(143)] with sampling fields placed ventral to and abutting the corpus callosum. For the analysis of nuclear Htt staining, the primary motor cortex (+0.98 to +0.86 mm relative to Bregma) and dorsal hippocampus (~1.7 to ~2.3 mm) were also examined (143). In this latter region of the hippocampus, IBA-1 immunostaining was also quantified. Images were acquired with a Zeiss AxioImager M2 microscope, AxioCam Hrc camera and Axiovision (Zeiss) or MicroLucida (MBF Biosciences) software. If immunostaining was performed in multiple sets, quantifications were normalized to the WT-vehicle group of that staining set and cohort. Intracranial Htt accumulation was evaluated by manually tracing immunoreactive nuclei and intranuclear aggregates in the striatum (sample field 250 × 250 μm), cortex [sample field (250 × 250 μm) in cortical layers 4/5], and hippocampus [sample field (125 × 25 μm) in CA1 pyramidal layer] in one section per mouse while viewing with a 100X objective using NeuroLucida v11.07 (MBF) image analysis software. Striatal volume was determined and set. Cells were counted in a 100 μm × 100 μm counting frame inside a 200 μm × 200 μm grid using a 40X oil objective. Gundersen m = 1 coefficient of error for cell counts was ≤0.05.

**Modified Golgi staining**

Brains were immersed in modified Golgi-Cox staining solution (purchased from Drs. Deqiang Jing and Francis Lee at Cornell University) and processed as described previously (18). Dendritic spine density was determined by manually tracing Golgi-stained MSNs in the dorsal striatum and CA1 pyramidal cells in the dorsal hippocampus while viewing and adjusting focus at 100X using NeuroLucida. Neurons (3–5/mouse) that minimally overlapped adjacent neurons were selected. For the striatum, all branches from one dendritic tree were traced per neuron and for the hippocampus, 3 basal and 3 apical dendrite segments (>100 μm) were traced. Dendritic branch orders 3 and higher were analysed, as these are the most affected in HD mice (93,144). Spine morphology, including mushroom, thin and stubby, was also recorded using criteria defined elsewhere (145).

**Statistical Analysis**

Statistical significance was determined via a one-way analysis of variance (ANOVA) with a Fisher’s LSD post hoc test and/or a Student’s t-test for paired comparisons or Mann-Whitney for non-parametric tests using GraphPad Prism v6. A repeated measures (RM) ANOVA was used for body weight. Values that were two standard deviations from the mean (criteria determined a priori) were removed as statistical outliers. Results are expressed as group mean ± standard error of the mean (s.e.m.) and statistical significance was set at P ≤ 0.05.

**Supplementary Material**

Supplementary Material is available at HMG online

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Conflict of Interest statement F.M.L. and S.M.M. are listed as inventors on patents relating to LM11A-31 which are assigned to the University of North Carolina, University of California (UC), San Francisco and the Dept. of Veterans Affairs (VA). F.M.L. and S.M.M. are entitled to royalties distributed by UC and the VA per their standard agreements. F.M.L. is a principal of, and has a financial interest in PharmatrophiX, a company focused on the development of small molecule ligands for neurotrophin receptors which has licensed several of these patents.

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