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Increased motor neuron resilience by small molecule compounds that regulate IGF-II expression

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ABSTRACT

The selective vulnerability of motor neurons in amyotrophic lateral sclerosis (ALS) is evident by sparing of a few subpopulations during this fast progressing and debilitating degenerative disease. By studying the gene expression profile of resilient vs. vulnerable motor neuron populations we can gain insight in what biomolecules and pathways may contribute to the resilience and vulnerability. Several genes have been found to be differentially expressed in the vulnerable motor neurons of the cervical spinal cord as compared to the spared motor neurons in CNIII/IV. One gene that is differentially expressed and present at higher levels in less vulnerable motor neurons is insulin-like growth factor II (IGF-II). The motor neuron protective effect of IGF-II has been demonstrated both in vitro and in SOD1 transgenic mice. Here, we have screened a library of small molecule compounds and identified inducers of IGF-II mRNA and protein expression. Several identified compounds significantly protected motor neurons from glutamate excitotoxicity in vitro. One of the compounds, vardenafil, resulted in a complete motor neuron protection, an effect that was reversed by blocking receptors of IGF-II. When administered to naïve rats vardenafil was present in the cerebrospinal fluid and increased IGF-II mRNA expression in the spinal cord. When administered to SOD1 transgenic mice, there was a significant delay in motor symptom onset and prolonged survival. Vardenafil also increased IGF-II mRNA and protein levels in motor neurons derived from healthy subject and ALS patient iPSCs, activated a human IGF-II promoter and improved survival of ALS-patient derived motor neurons in culture. Our findings suggest that modulation of genes differentially expressed in vulnerable and resilient motor neurons may be a useful therapeutic approach for motor neuron disease.

1. Introduction

ALS is a fast progressing, fatal neurodegenerative disorder of familial or sporadic etiology, characterized by the proceeding loss of somatic motor neurons (MNs) and degeneration of a fraction of cortical neurons that projects to the spinal cord. To date, there is no effective therapy and the only FDA approved drug for ALS, riluzole, extends life by only a few months. Several genes have been implicated in ALS, for example mutations in superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (TDP-43) and Fused in Sarcoma (FUS) (Renton et al., 2014). The chromosome 9p21–linked hexanucleotide repeat expansion in C9ORF72 accounts for about 40% of familial ALS cases and about 7% of sporadic ALS cases (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Majounie et al., 2012), thus a significant fraction of ALS patients.

Although most somatic MNs are affected by the disease, a few subfractions are spared from degeneration. As an example, the oculomotor/trochlear complex and the sacral MNs are relatively healthy compared to cranial nerve XII and spinal MNs, which eventually degenerate. The factors contributing to this selective and predictable vulnerability of different MN populations in ALS are unknown. However, studying the genetic, cell biological and physiological differences between the resilient and vulnerable MN populations may provide unique insights and drug discovery opportunities. Based on this, differential gene expression in MNs of the normal rat was previously studied. MNs in nuclei that are affected were compared to MNs of resilient sub populations by use of laser capture microdissection and gene microarray (Hedlund et al., 2010). The comprehensive gene expression analysis revealed innate physiological properties of these

Abbreviations: ALS, amyotrophic lateral sclerosis; ChAT, choline acetyltransferase; FTD, frontotemporal dementia; IGF-II, insulin-like growth factor II; IGF-IIR, IGF-II/cation-independent mannose-6-phosphate receptor; IGF-IR, IGF-I receptor; isl-1/2, islet 1 and 2; iPSC, induced pluripotent stem cells; JB1, IGF-I peptide analog; MN, motor neuron; VDFL, vardenafil hydrochloride; PDC, L-*trans*-Pyrrolidine-2,4-dicarboxylic acid; PDE5i, phosphodiesterase 5 inhibitor; SOD1, superoxide dismutase 1; SSRI, selective serotonin re-uptake inhibitor; TDFL, tadalafil; SDFL, sildenafil

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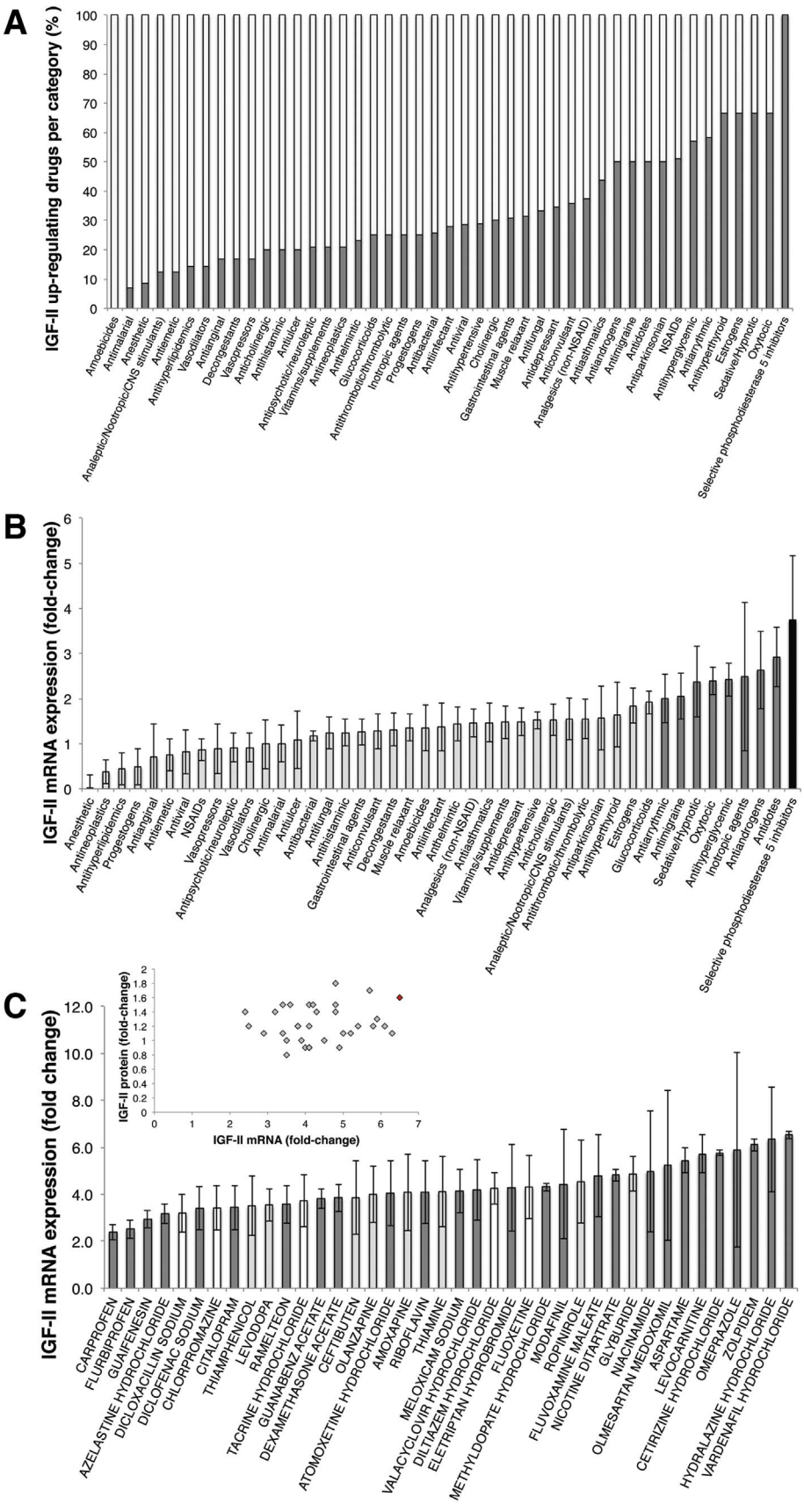

Fig. 1. Summary of pharmaceutical-induced IGF-II up-regulation.

(a) Pharmaceuticals from several different treatment categories drive IGF-II mRNA expression in primary spinal cord cultures. The graph shows percentage of pharmaceuticals within each treatment category that induce an > 2-fold up-regulation in IGF-II mRNA expression. Each treatment category shown contains at least 3 pharmaceutical compounds. (b) Average IGF-II mRNA expression increase obtained by different pharmaceutical treatment categories. The dark grey columns indicate drug categories for which the average IGF-II mRNA expression increase was ≥ 2-fold. The selective PDE5is (black column) induced the highest average expression increase. Fold mRNA up-regulation was obtained by quantitative PCR and using the ΔΔCT-method (baseline control: vehicle treated, housekeeping gene: 18S). (c) IGF-II up-regulation by selected pharmaceuticals. 38 of the highest IGF-II expression-inducing pharmaceuticals were selected for additional in vitro assays based on their safety profiles, potential for CNS permeability and fold IGF-II up-regulation. Inset illustrates the correlation between IGF-II mRNA expression and level of IGF-II protein expression for these pharmaceuticals. Most of the IGF-II mRNA up-regulating compounds resulted in an increase in IGF-II protein. The PDE5i VDFL induced the highest IGF-II mRNA expression increase (6.5 ± 0.2 fold), which translated to a 1.6-fold increase in IGF-II protein expression (red diamond, inset). See supplemental Table 1, S1 for a complete list of IGF-II up-regulating pharmaceuticals (bars in b and c indicate average ± SEM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

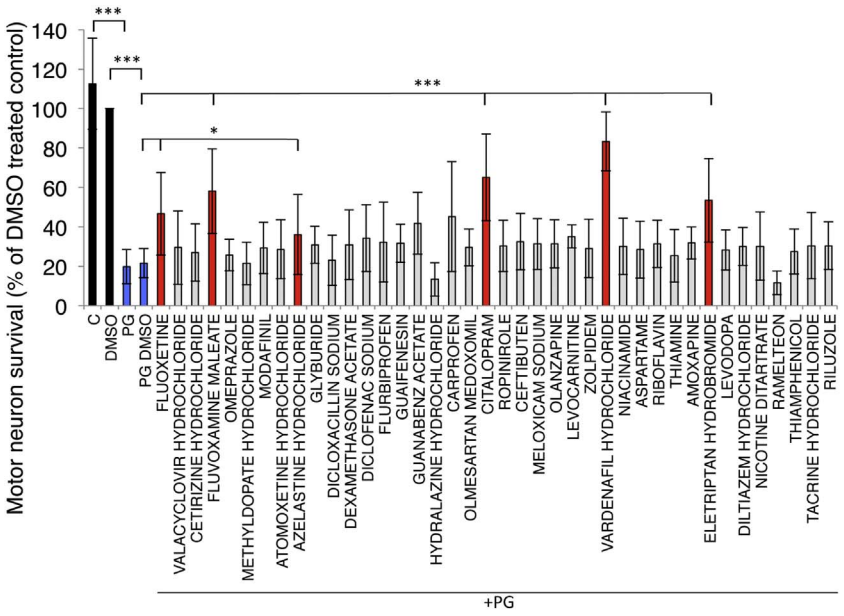

Fig. 2. Effect of selected IGF-II up-regulating drugs on progressive glutamate toxicity in primary spinal cord cultures.
The PDE5i VDFL (vardenafil hydrochloride), the SSRIs fluvoxamine maleate, fluoxetine and citalopram, the anti-migraine drug eletriptan hydrobromide and the H1 antihistamine azelastine hydrochloride significantly improved survival of MNs exposed to progressive glutamate toxicity (red bars). Drug-treatment regimen (doses and number of additions) were optimized for each drug and ranged between 1 and 10 μM applied 1–3 times throughout the progressive toxicity protocol. Data is presented as percent surviving MNs as compared to no-glutamate treated, DMSO-treated control. The FDA approved pharmaceutical Riluzole, which mildly improves survival-time of ALS patients does not increase IGF-II mRNA levels and was included as a control (bars indicate average ± SEM, $n = 4$, $^{***}P < 0.001$, $^{*}P < 0.05$, 1-way ANOVA, Bonferroni's Multiple comparison, C: untreated control, PG: PDC glutamate uptake inhibitor + glutamate).

different MNs that may explain their differential vulnerability to degeneration. One of the key findings was that higher insulin-like growth factor II (IGF-II) levels correspond to resilience (Hedlund et al., 2010). This has recently also been confirmed in both human control and ALS patient tissues (Allodi et al., 2016) and a recent study found a significant reduction of IGF-II protein in cerebrospinal fluid (CSF) from ALS patients compared to controls and suggested it's use as a biomarker (Chen et al., 2016). Furthermore, the MN protective effect by IGF-II has been confirmed both in in vitro glutamate toxicity assays using recombinant IGF-II and by gene delivery in $SOD1^{G93A}$ mutant ALS model mice (Hedlund et al., 2010; Allodi et al., 2016).

In the CNS, IGF-II confers neurotrophic properties, and is together with IGF-I, insulin and their receptors involved in energy homeostasis, neuronal plasticity and cognitive function (Fernandez & Torres-Aleman, 2012). In the present work we sought to identify pharmaceutical compounds that increase IGF-II expression in MNs both in vitro and in vivo and has the potential to increase the resilience of MNs to ALS-associated degeneration. We show proof of the concept that relative vulnerabilities of MNs can be changed by targeting differentially expressed genes. We utilized regulatory approved pharmaceuticals, which have the advantage of a fast translation into an ALS patient population compared to small molecule compounds that have not been studied in clinical trials for safety in humans. In our pre-clinical screen, we have identified a widely used and extensively clinically tested phosphodiesterase 5 inhibitor (PDE5i) (ClinicalTrials.gov), vardenafil hydrochloride (VDFL) that modulates IGF-II expression and appears to be a suitable pharmaceutical for additional investigation and potential translation to a clinical application for MN disease.

2. Results

2.1. Identification of small molecule compounds that drive igf-II mRNA and protein expression in primary spinal cord cultures

In order to identify small molecules that increase IGF-II expression and could be used for fast translation to a patient population, we utilized a library of 1040 pharmaceutical compounds. The library contained pharmaceuticals from over 90 different treatment categories. Each of the drugs was added at a fix concentration (10 μM) to primary spinal cord cultures from E12 mice. Inducers of IGF-II mRNA expression were identified by qRT-PCR 24 h after drug addition. Approximately 10% of the 1040 compounds gave a 3-fold increase in IGF-II mRNA expression. Activators were among many different groups of drugs, including PDE5is, antidepressants, antibacterial, antihypertensive, antiparkinsonian, antihistamine, antimigraine, NSAIDs, antidiabetics, estrogens and antiarrhythmics (Fig. 1 and Table 1, S1). Glucocorticoids are known IGF-II promoter activators (Dell et al., 1997) and were also identified in our screen. Among the antibiotics there were activators in the groups aminoglycosides, antitubercular, cephalosporins, fluoroquinolones, lincosamides, macrolides, penicillins, quinolones, sulfonamides and tetracyclines. No IGF-II up-regulators were found among choramphenicols, nitrofurans, glycopeptides and polymyxins. IGF-II inducers were found also among various subcategories of antihypertensive pharmaceuticals (Table 1 S1). Fig. 1a shows the percentage of pharmaceuticals within each treatment category that up-regulates IGF-II mRNA in vitro and Fig. 1b depicts the average fold-change in IGF-II mRNA for the various drug categories. In the group selective PDE5is all of the pharmaceuticals increased IGF-II mRNA expression. One of the most potent activators identified in the library was the PDE5i VDFL (Fig. 1c, Table 1, S1). VDFL induced a 6.5 ± 0.2 fold increase in IGF-II mRNA expression. The other PDE5is in the library, sildenafil (SDFL) and tadalafil (TDFL) induced a 2.3-fold and 2.4-fold increase in IGF-II mRNA levels, respectively. Pentoxifylline, a general PDEi previously investigated in a clinical trial for ALS without clinical benefit (Meininger et al., 2006), did not induce and increase in IGF-II mRNA. In order to narrow down the candidate drugs for additional in vitro testing, 38 drugs were selected based on fold IGF-II up-regulation, likelihood to cross the blood-brain-barrier and their known clinical safety profile (Table 2, S2). IGF-II protein up-regulation was confirmed by ELISA of cell supernatant for the majority of these drugs (Fig. 1c, inset).

2.2. IGF-II up-regulating drugs reduce excitotoxic motor neuron death in vitro in primary spinal cord cultures

It is known that recombinant IGF-II protein protects spinal MNs from excitotoxicity (Hedlund et al., 2010). In order to determine the MN protective potential of the 38 selected candidate drugs, a progressive glutamate toxicity cell culture paradigm was applied. In this protocol, adapted from Hedlund et al., (2010), 50–80% of the MNs degenerate by the end of the protocol (Fig. 2 and 3 a, b). The glutamate uptake inhibitor L-*trans*-Pyrrolidine-2,4-dicarboxylic acid (PDC, EAAT1-5 inhibitor (Hedlund et al., 2010)) blocks glutamate uptake by astrocytes in the culture, thus mimicking an in vivo scenario of reduced

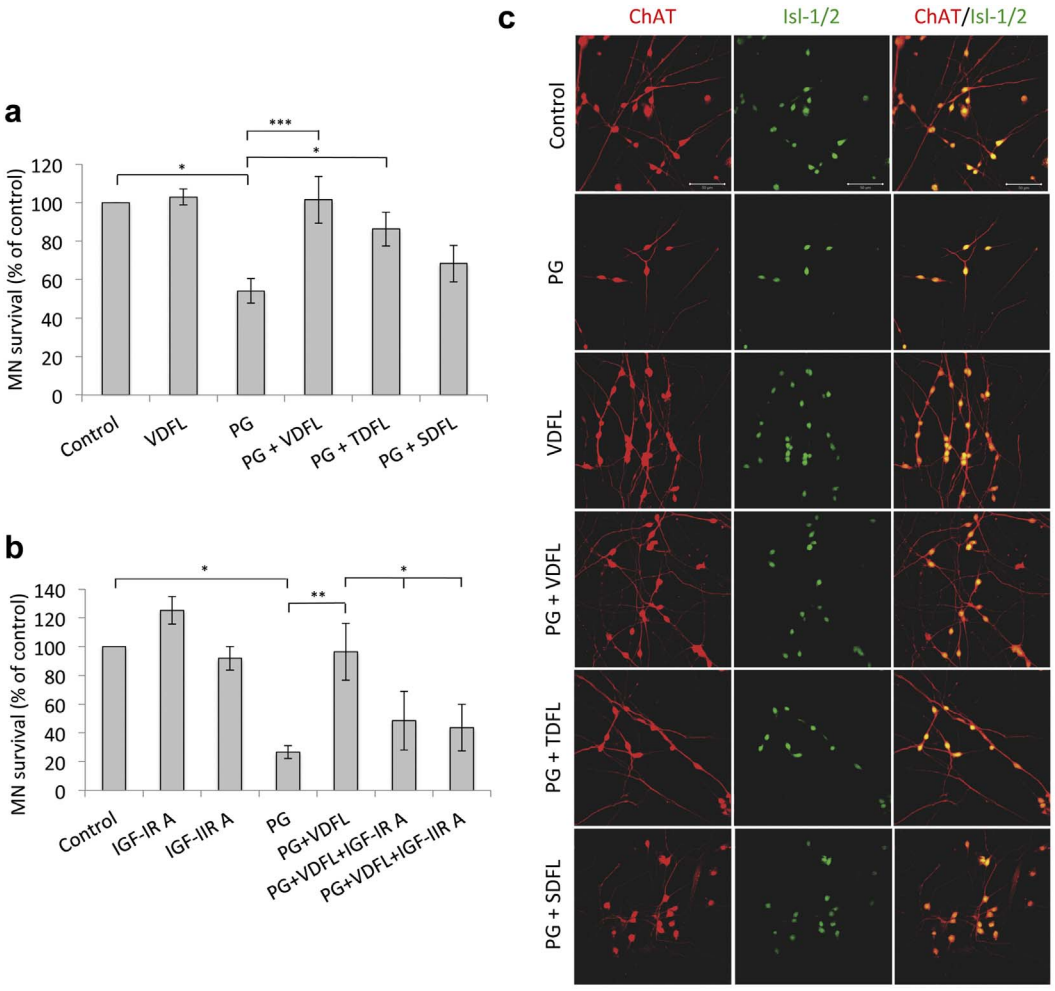

Fig. 3. Effect of PDE5is and IGF receptor antagonists on progressive glutamate toxicity in primary spinal cord cultures.
(a) The PDE5is VDFL and TDFL significantly improve MN survival against progressive glutamate toxicity. SDFL a PDE5i that has a higher clinical ED_{50} than VDFL and TDFL did not result in significant protection of MNs with the same dosing regimen ($n = 3$). (b) The MN protective effect of VDFL was blocked both by an IGF-IR antagonist (JB1) and an IGF-II receptor (IGF-IIR) antibody, suggesting involvement of the IGF-II pathway in the MN protective effect of VDFL. Neither of the antagonists alone resulted in any decrease in MN survival ($n = 4$–8). (c) Immunocytochemistry of representative cultures used for quantification of MN survival. MNs were defined as cells positive for both ChAT (red) and isl-1/2 (green) and normalized to total number of cells (Fig. S3; scale bar 50 μm). Data are shown as average ± SEM (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, 1-way ANOVA, Bonferroni's Multiple comparison, PG: PDC glutamate uptake inhibitor + glutamate, VDFL: vardenafil, TDFL: tadalafil, SDFL: sildenafil).

astrocytic glutamate transport (Lin et al., 1998). The MN content in the untreated control cultures was 11% ± 3% as determined by co-staining for choline acetyltransferase (ChAT) and islet 1 and 2 (isl-1/2). 99% of the isl-1/2 positive ChAT positive cells were also Hb9 positive. MN loss was determined as previously described (Hedlund et al., 2010). Using the optimal dose of each pharmaceutical (ranging from one 1 μM addition to a series of three additions of 1–10 μM) the effects on MN survival was determined (Fig. 2). VDFL gave an almost complete protection against the progressive glutamate/PDC induced toxicity, resulting in 83.3 ± 8% surviving MNs as compared to 21.5 ± 4% for DMSO treated controls ($P < 0.001$). In addition, partial protection was observed with a few selective serotonin re-uptake inhibitors (SSRIs); fluvoxamine maleate (58.1 ± 11%, $P < 0.001$), fluoxetine (46.6 ± 11%, $P < 0.05$), citalopram (65.1 ± 11%, $P < 0.001$), the anti-migraine drug eletriptan hydrobromide (53.5 ± 11%, $P < 0.001$) and the H1 antihistamine azelastine HCl (36.1 ± 10%, $P < 0.05$).

2.3. Effect of VDFL-related PDE5 inhibitors on excitotoxic motor neuron death in vitro

The PDE5is sildenafil (SDFL) and tadalafil (TDFL) both result in an up-regulation of IGF-II in primary spinal cord cultures (Fig. 1a, Table 1, S1). When applied in the progressive glutamate toxicity assay described above, TDFL conferred a significant MN protection, which resulted in 86.3 ± 9% surviving MNs. SDFL did not significantly improve MN survival at the concentration tested, although there was a trend towards increased survival (68.3 ± 9%, Figs. 3a, c, S3).

2.4. Involvement of the IGF-II pathway in VDFL mediated motor neuron protection in vitro in primary spinal cord cultures

To determine whether IGF-II signaling is involved in the VDFL-mediated MN protection we used an IGF-I peptide analog (JB1), which acts as an antagonist of the IGF-I receptor (IGF-IR) and an IGF-II/cation-independent mannose-6-phosphate receptor (IGF-IIR) antibody. These antagonists were added to the cultures 24 h after VDFL treatment in the progressive glutamate toxicity protocol. The antagonists alone did not contribute to any noticeable toxicity (Fig. 3b). The MN survival with JB1 was 125.2 ± 10% and with the IGF-IIR antibody 91.8 ± 8% at baseline as compared to vehicle treated control. The progressive glutamate toxicity protocol resulted in 26.7 ± 4% of surviving MNs in these cultures. Pre-treatment with VDFL 24 h before glutamate and PDC addition prevented this MN loss (96.4 ± 20% surviving MNs). When

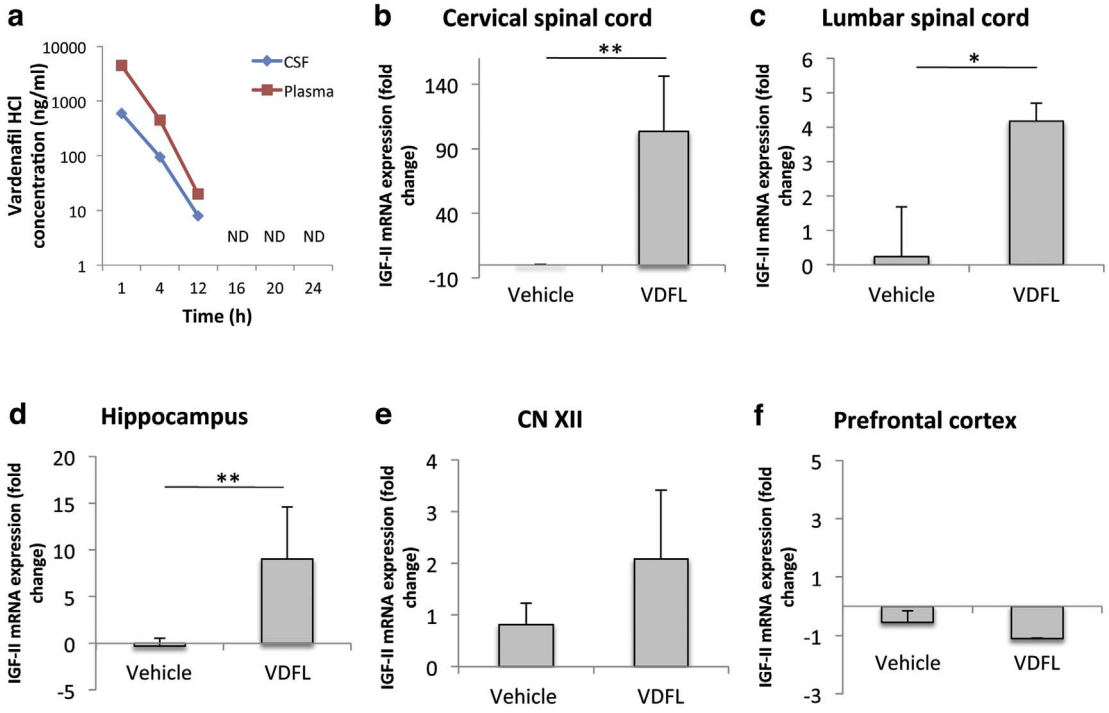

Fig. 4. IGF-II is up-regulated in the CNS following gavage administration of VDFL in normal rats.
(a) VDFL passes the blood-brain-barrier in normal rat after per oral gavage administration and is present in CSF at about 15% of plasma concentrations. Data shown is from 7 days of daily administrations of 8 mg/kg/day in water vehicle. (b) VDFL increases IGF-II expression the cervical spinal cord ($P = 0.0022$). (c) VDFL increases IGF-II expression the lumbar spinal cord ($P = 0.048$). (d) VDFL increases IGF-II expression in the hippocampus ($P = 0.0036$). (e) There is no significant change in IGF-II mRNA levels in CNXII after treatment with VDFL. (f) There is no significant change in IGF-II mRNA levels in the prefrontal cortex after treatment with VDFL. Data are shown as average ± SEM ($n = 6$; $^*P < 0.05$, $^{**}P < 0.01$; unpaired, two-sided *t*-test (c); Mann-Whitney (b,d); VDFL: vardenafil; ND: not detected; CSF: cerebrospinal fluid).

JB1 or the IGF-IIR antibody were added 24 h after VDFL, the MN survival decreased to 48.5 ± 20% and 43.7 ± 16%, respectively, suggesting that IGF-I and IGF-II receptor activation is involved in the VDFL-mediated MN protection.

2.5. VDFL passes the blood-brain-barrier after gavage administration in naïve rats

In order for VDFL to have an effect on IGF-II expression in the spinal cord target region, the pharmaceutical compound must pass the blood-brain-barrier. Although it has previously been shown that VDFL passes the blood-brain-barrier (Reneerkens et al., 2012), it has not been shown what the levels in CSF are with our dosing regimen. To determine VDFL levels in the CSF we administered VDFL (8/mg/kg/day) in water vehicle by gavage to rats for 7 days. Blood and CSF samples were collected for LC-MS/MS at 1, 4, 12, and 24 h (Charles River, Wilmington, MA) after the final dose. VDFL was detected in both plasma and CSF at 1 h (15% of plasma levels), 4 h (29% of plasma levels) and 12 h (42% of plasma levels, although only detected in one of the rats) (Fig. 4a). The highest concentration detected (C_{max}) was 2710 ng/ml in plasma and 404 ng/ml in CSF at 1 h after the final dose. The t1/2 was 1.46 h in plasma and 1.78 h in CSF. The lowest quantification limit was 5 ng/ml. VDFL was not detected in blood or plasma 24 h after the last dose.

2.6. VDFL increases IGF-II mRNA expression in the spinal cord of naïve rats and mice

When VDFL (8 mg/kg) was administered daily for 2 weeks by gavage, a significant increase in IGF-II mRNA was observed in the cervical spinal cord of naïve rats (Fig. 4b). The average fold-increase in IGF-II mRNA in the cervical spinal cord was 103.4 ± 42.5 in VDFL treated rats ($P = 0.002$; Mann-Whitney test). The fold-change was quite variable between different rats, ranging from a 3-fold to a 252-fold up-regulation. There was also a significant up-regulation of IGF-II mRNA in the lumbar spinal cord after daily administration for 2 weeks by gavage (Fig. 4c). The up-regulation was less pronounced and less variable between rats than for the cervical region, averaging at a 4.2 ± 0.5-fold up-regulation ($P = 0.0413$, two-sided *t*-test). The range was between a 2.2-fold and 5.2-fold increase. Alternative dosing regimens (once a day for 3 days, once a day for 1 week, every third day for 1 week or every third day for 2 weeks) did not result in a significant up-regulation of IGF-II in any region. In addition, a significant but variable up-regulation of IGF-II was observed in the hippocampus (9.0 ± 5.6-fold, $P = 0.0036$, Mann-Whitney test, Fig. 4d). There was no up-regulation in CNXII or the prefrontal cortex (Fig. 4e, f). In mice, VDFL (10 mg/kg/day for 14 days) administration induced a smaller, compared to what was observed in rats, but significant up-regulation of IGF-II mRNA in the cervical spinal cord. The up-regulation was 2.4 ± 0.5-fold in VDFL treated mice as compared to water vehicle treated control mice (two-tailed *t*-test, $P = 0.0247$, Fig. 5a).

2.7. Chronic VDFL administration delays ALS motor symptom onset and prolongs survival of $SOD1^{G93A}$ ALS model mice

Pre-symptomatic VDFL treatment starting at 5 weeks of age significantly postponed symptomatic onset in $SOD1^{G93A}$ ALS model mice by 11 days as compared to water vehicle treated control mice. The onset occurred at 127 ± 4 days in control treated mice and at 138 ± 3 days in VDFL treated mice. Onset was determined as a change of neuroscore from 0 to 0.5 (two-tailed *t*-test, $P = 0.0423$, Fig. 5b). VDFL treatment also increased survival time of $SOD1^{G93A}$ ALS model mice significantly. VDFL treated mice had an increased median survival of 6 days as compared to water vehicle treated control mice (Mantel-Cox log-rank test, $\chi^2 = 4.382$, $P = 0.0363$).

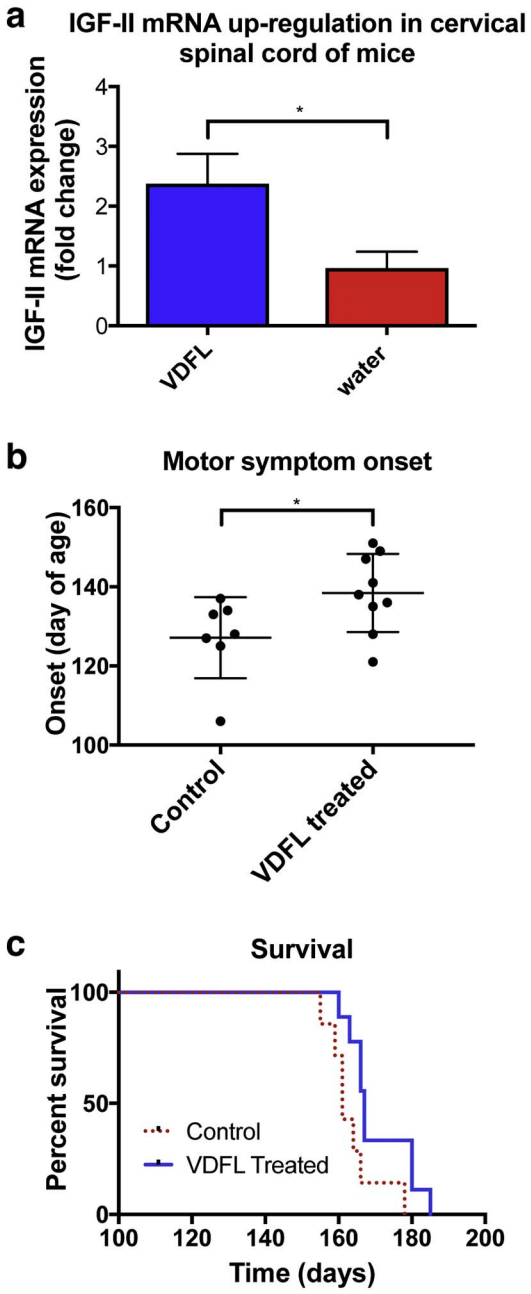

Fig. 5. VDFL induces up-regulation of IGF-II mRNA in mouse spinal cord, delays motor symptom onset and prolongs survival of SOD1^{G93A} ALS model mice.
(a) VDFL induces and up-regulation of IGF-II mRNA in the cervical spinal cord in mice as compared to water vehicle treated control mice. Data are shown as average ± SEM (*$P < 0.05$, n = 6 VDFL treated and n = 7 controls; unpaired, two-tailed *t*-test, $P = 0.0247$). (b) Pre-symptomatic VDFL treatment starting at 5 weeks of age significantly postpones symptomatic onset in SOD1^{G93A} ALS model mice by 11 days as determined by a change of neuroscore from 0 to 0.5 (n = 7 vehicle treated and n = 9 VDFL treated mice; unpaired, two-tailed *t*-test, $P = 0.0423$). (c) VDFL treatment significantly increases survival time of SOD1^{G93A} ALS model mice. VDFL treated mice had an increased median survival of 6 days (n = 7 vehicle treated and n = 9 VDFL treated mice; Mantel-Cox log-rank test, $\chi^2 = 4.382$, $P = 0.0363$).

2.8. VDFL induces activation of the human P4 IGF-II promoter

Because of differential expression and regulation of IGF-II at the fetal stage compared to adult stage and because of differences in the IGF-II promoters between rodents and human (Rotwein & Hall, 1990; Sussenbach et al., 1993) we constructed a human IGF-II reporter line in order to determine the potential effect on adult human IGF-II regulation. The human P4 IGF-II promoter was cloned from human DNA and inserted into a promoter-less vector upstream of the luciferase gene (Fig. 6a). We generated a stable cell line by transfection into a human neuroblastoma line that harbors the necessary regulatory elements for IGF-II expression but has a relatively low intrinsic IGF-II expression (El-Badry et al., 1991). Addition of VDFL or the known P4 activator hydrocortisone to the cells induced a dose-dependent increase in luminescence (Fig. 6b), indicative of IGF-II promoter activation.

2.9. VDFL increases IGF-II mRNA expression in motor neurons derived from human induced pluripotent stem cells

In order to be a suitable potential therapeutic in ALS, VDFL should have effects on human MNs. Therefore, human induced pluripotent stem cells (iPSCs) were utilized to determine the effects of VDFL on IGF-II expression in human MN cultures. IPSCs from healthy subjects were differentiated to MNs using an EB based differentiation protocol (Fasano et al., 2009; Ding et al., 2013). The presence of MNs were confirmed by co-staining of ChAT and isl-1/2 or BIII-tubulin and isl-1/2 (Fig. 6c). The total neuronal content in the culture was 55.7 ± 14% for the HS line and 41.2 ± 2% for the C9orf72 line. The MN content as defined by Isl-1/2 and BIII tubulin double positive cells was 13.8 ± 2% in the HS cultures and 14.9 ± 2% in the C9orf72 cultures (Fig. 6d). The MNs also expressed glutamate receptor 2 and the NMDA receptor NR2B (Fig. 6e, f). Up-regulation of IGF-II was determined 24 h after VDFL addition by qPCR and demonstrated a significant increase in IGF-II mRNA in VDFL (1 μM) treated cultures as compared to DMSO treated control (2.2-fold up-regulation in both HS and C9orf72 iPSC derived MNs, $P < 0.05$, Fig. 6g, h). There was no significant up-regulation with 0.1 μM VDFL. There was also a corresponding increase in IGF-II protein secreted into the culture media of the VDFL treated C9orf72 iPSC derived MNs (Fig. 6i).

2.10. VDFL improves survival of motor neurons derived from ALS patient iPSCs

In C9orf72 repeat expansion MN cultures we observed a loss of MNs when the cultures were kept one month after the end of the differentiation protocol (DIV60 compared to DIV30, $P < 0.0035$). When adding VDFL to the culture media every other day, the loss of MNs was not observed ($P < 0.033$ Fig. 5j). In healthy control MN cultures, which did not show a significant change in MN number with time, VDFL did not have an effect on the MN numbers, indicating that VDFL does not induce proliferation of MNs in these cultures (Fig. S4). This suggests that the effect observed in the C9orf72 repeat expansion cultures is likely due to prevention of degeneration rather than cell proliferation.

3. Discussion

There is a relative vulnerability among MN populations. Gene expression analysis suggests that MN intrinsic expression of different genes may contribute to their vulnerability or resilience (Hedlund et al., 2010; Allodi et al., 2016; Chung et al., 2005; Brockington et al., 2013). Such analysis has revealed several genes that are differentially regulated. In efforts to determine if modulation of differentially expressed genes can be used as a therapeutic approach we chose to study modulation of the differentially expressed gene IGF-II. We, and others, have previously shown that IGF-II is expressed at lower levels in MNs that are vulnerable to ALS-associated degeneration as compared to resilient MNs (Hedlund et al., 2010; Allodi et al., 2016). IGF-II is up-regulated in the protected MN population in cranial nerve III/IV as compared to the vulnerable MNs in the cervical spinal cord. Furthermore, recombinant IGF-II protein protected MNs in primary spinal cord cultures from excitotoxic degeneration (Hedlund et al., 2010). With this background, we initiated a screen to identify pharmaceutical compounds that can

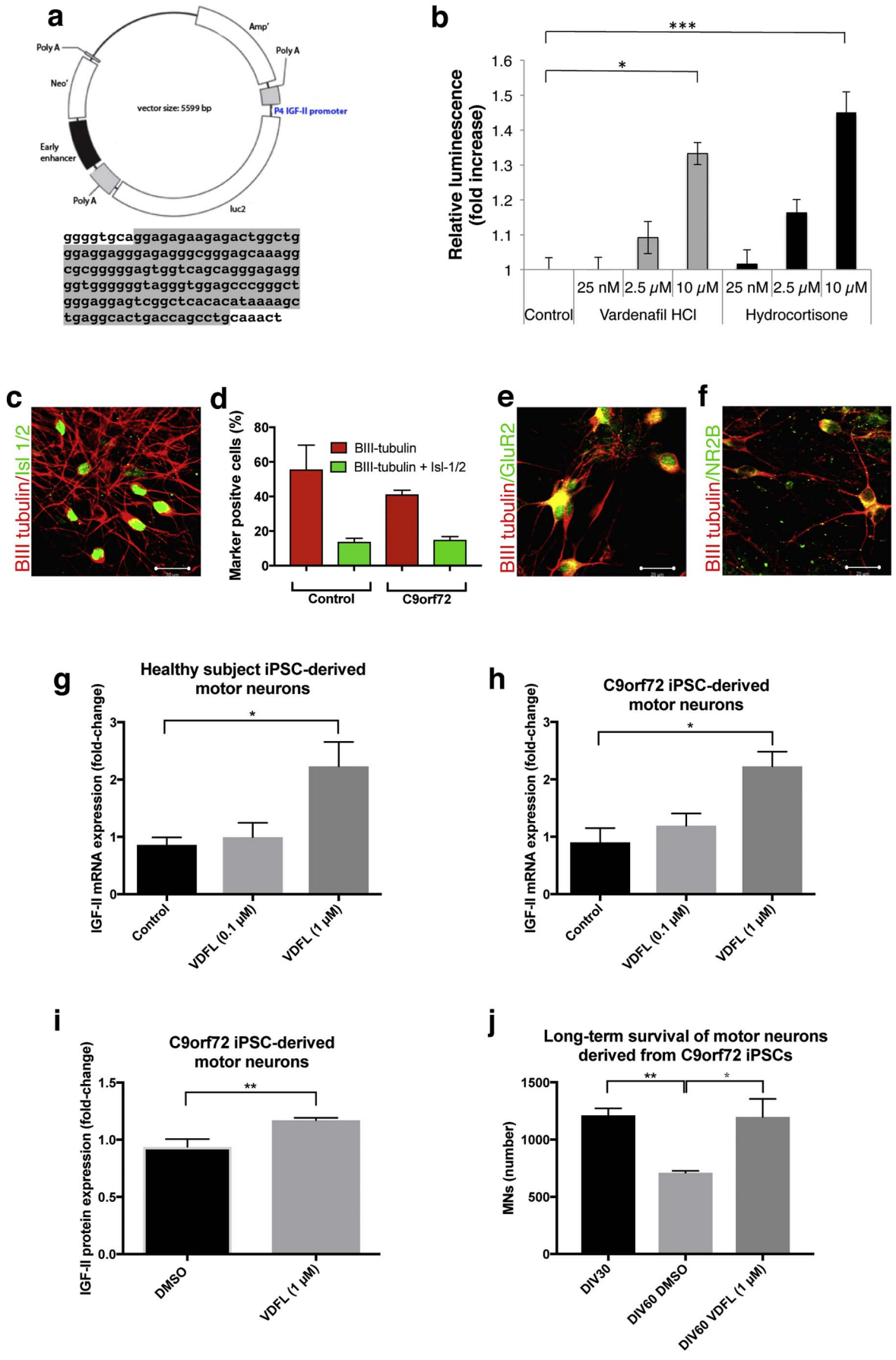

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Fig. 6. VDFL can activate the human P4 IGF-II promoter in vitro, increase IGF-II expression in human MN cultures and improve survival of C9orf72 MNs.
(a) The human P4 IGF-II promoter (see sequence) was cloned into a promoter less luciferase reporter vector. A stable reporter cell-line was generated by transfecting human neuroblastoma cells (SKNFI) and applying G418 antibiotics for selection of transfected cells. (b) Using the cell line described in (a) VDFL activated the P4 IGF-II promoter in a dose-dependent manner (control: DMSO vehicle treated cells). Hydrocortisone is a known activator of the P4 IGF-II promoter and was included as a positive control (n = 3; * P < 0.5, *** $P < 0.001$, 1-way ANOVA, Bonferroni's Multiple comparison). (c) Representative MN culture differentiated from human iPSCs (BIII-tubulin: red, Isl-1/2: green). (d) Quantification of BIII-tubulin and BIII-tubulin/Isl-1/2 positive cells in MN cultures as a percentage of total cells (identified by Hoechst nuclear stain). (e) MNs expressed the glutamate receptor GluR2 (BIII-tubulin: red, GluR2: green). (f) MNs expressed the NMDA receptor NR2B (BIII-tubulin: red, NR2B: green). (g) VDFL increases IGF-II mRNA expression in MNs from healthy subject iPSCs (n = 4–8, * P < 0.05, 1-way ANOVA, Tukey's Multiple comparison). (h) VDFL increases IGF-II mRNA expression in MNs from C9orf72 ALS patient iPSCs (n = 4–7, * P < 0.05, 1-way ANOVA, Tukey's Multiple comparison). (i) Administration of VDFL increases IGF-II protein levels in the media of MN cultures from C9orf72 ALS patient iPSCs (n = 8, ** P < 0.01, $P = 0.0055$) unpaired two-tailed t-test). (j) There was a reduction of MNs in cultures differentiated from C9orf72 repeat expansion iPSCs 30 days after the end of the differentiation protocol (DIV60) as compared to DIV30. When VDFL was added to the culture media the MN loss was not observed. Data are shown as average ± SEM (n = 4, ** $P < 0.035$ as compared to DIV30, * $P < 0.033$ as compared to DIV60 DMSO control condition; DIV: day in vitro, 1-way ANOVA, Tukey's Multiple comparison). Scale bar is 20 μM (c, e, f).

increase IGF-II expression.

3.1. Identification of pharmaceuticals that increase the expression of IGF-II

Pharmaceuticals that are FDA approved for other indications have undergone extensive safety testing, thus identified candidate drugs could be used for fast translation into MN disease patient populations. We identified pharmaceuticals from many different drug categories that induce an increase in IGF-II expression in primary spinal cord cultures in vitro. We found that approximately 10% of the compounds investigated induced a three-fold or higher up-regulation in IGF-II mRNA levels and several more compounds induced a two-fold increase. We selected 38 pharmaceuticals based on known safety profile, potential for blood-brain-barrier permeability and fold-change in IGF-II mRNA up-regulation. We found that for most of these candidate drugs the mRNA expression increase was followed by an increase in secreted IGF-II protein. The PDE5i VDFL was one of the compounds that gave rise to the highest increase in IGF-II mRNA expression. The other two PDE5is included in the screen, SDFL and TDFL also increased IGF-II expression, albeit less than VDFL at the concentration investigated. PDE5 inhibition specifically results in increased levels of cyclic guanosine monophosphate (cGMP). The current FDA approved indications for PDE5is, erectile dysfunction (Anon, 1998) (Levitra®, Viagra® and Cialis®) and pulmonary hypertension (Watanabe et al., 2002) (Revatio®, Adcirca®) were developed on the basis of PDE5 smooth muscle localization, however, PDE5s are widely expressed in a variety of other tissues, such as brain, kidney, lung, and platelets (Lin, 2004).

3.2. Protection against progressive glutamate excitotoxicity by IGF-II up-regulating compounds

In order to identify candidate pharmaceuticals with the potential to confer MN protection we utilized a progressive glutamate toxicity protocol originally developed in Hedlund et al. (Hedlund et al., 2010) and further optimized in this study. The advantage of this progressive protocol is a gradual death of MNs over the course of multiple low-doses of glutamate, a scenario resembling of the progressive course of ALS-related MN degeneration. In addition, the use of an astrocytic EAAT1-5 inhibitor also may mimic the clinical scenario in ALS, where decreased astrocytic glutamate uptake has been observed due to the loss of EAAT2, which in turn may increase the toxicity of glutamate on MNs (Lin et al., 1998; Rothstein et al., 1996). We identified 6 pharmaceuticals that improved MN survival in primary spinal cord cultures in vitro. Of those, VDFL gave the highest level of protection. The MN protective potentials of the PDE5is TDFL and SDFL were also investigated. We found that TDFL also resulted in MN protection. Of the other candidate compounds that induced IGF-II expression up-regulation there was also a MN protection observed with a few different SSRIs: fluvoxamine maleate, fluoxetine and citalopram as well as with the anti-migraine drug eletriptan hydrobromide and the H1 antihistamine azelastine HCl. Both SSRIs and eletriptan hydrobromide provide additional promising opportunities for repurposing, given their blood-brain-barrier permeability.

3.3. VDFL-mediated motor neuron protection involves the IGF-II pathway

IGF-II binds to both the IGF-IR and the IGF-IIR. The IGF-IIRs are located both intracellularly (cation independent mannose 6-phosphate receptors) and extracellularly. Intracellular IGF-IIRs facilitate trafficking between the trans-Golgi network, endosomes and lysosomes (Ghosh et al., 2003) whereas extracellular IGF-IIRs sequester local IGF-II. The affinity is higher for the IGF-IIR but IGF-II activates tyrosine-kinase signaling through the IGF-IR as well. To determine whether IGF signaling is involved in the VDFL-mediated MN protection we used an IGF-I peptide analog (JB1), which acts as an antagonist of the IGF-IR and an IGF-IIR antibody. The MN protective effect of VDFL was blocked by both of these compounds, showing that both IGF-I and IGF-II receptor activation is involved in the VDFL-mediated MN protection. Interestingly, IGF-II is mainly thought of as signaling through the IGF-IR but the IGF-IIR appears to be important for the IGF-II-related cognitive enhancement, as such is abolished by administration of the IGF-IIR antibody (Chen et al., 2011). The exact mechanism for how VDFL-induced IGF-II expression improves MN survival and potential effect remains to be elucidated. We can also not rule out that other biomolecules may be affected by VDFL and contribute to the MN protective effect.

3.4. Motor neuron protection by PDE5 inhibitors may come from both an intrinsic up-regulation of IGF-II in motor neurons and increased neurotrophic support by release from surrounding cells

Pre-clinical studies in ALS-model mice suggest that the initiation of disease is intrinsic to MNs (Boillee et al., 2006; Jaarsma et al., 2008), while progression is caused by glial cells (Hedlund et al., 2010; Boillee et al., 2006; Di Giorgio et al., 2008; Nagai et al., 2007). Although we hypothesize that intrinsic up-regulation of IGF-II in MNs would contribute to increased resilience and protection against ALS-mediated MN degeneration, up-regulation of IGF-II in surrounding cells may also contribute a protective neurotrophic environment, since IGF-II is secreted into the extracellular space. This is also supported by the observation that recombinant IGF-II added to primary spinal cord cultures protects against glutamate mediated MN death (Hedlund et al., 2010). In the analysis of up-regulation, we did not distinguish between up-regulation in MNs and surrounding spinal cord interneurons and glial cells, however we did observe an increase in IGF-II protein in the media of VDFL treated cultures. It is therefore possible that the MN protection observed by PDE5 inhibitors is a combined effect of intrinsic up-regulation in MNs and an increase in IGF-II secreted by surrounding cells.

3.5. The IGF-II up-regulating effect of VDFL in murine and human cells

IGF-II is the predominant insulin-like growth factor in adult humans (reviewed (Daughaday & Rotwein, 1989)). The P4 promoter appears to be relevant for IGF-II gene expression in the adult brain (Zhan et al., 1998), therefore, one important finding was that VDFL is an activator of the human P4 IGF-II promoter. The expression-regulation of IGF-II has shown to be relatively complex and species specific (Constancia et al., 2002; DeChiara et al., 1991; Wilkins et al., 2016; Ye et al., 2015).

Despite this, VDFL induced an increase in IGF-II mRNA and protein expression in both mouse spinal cord cultures, human healthy subject iPSC-derived MN cultures and C9orf72 patient iPSC-derived MN cultures. Furthermore, we confirmed that VDFL passes the blood-brain barrier and is present in the CSF after gavage administration in rats and observed an increase in IGF-II mRNA expression in both the cervical and lumbar spinal cord regions and in the hippocampus. The local up-regulation in the spinal cord region is important since this is a region where IGF-II levels are intrinsically lower as compared to other MN sub population regions (Hedlund et al., 2010). Since IGF-II is secreted from cells and exogenous recombinant IGF-II protein can confer MN protection when added to cultures (Hedlund et al., 2010) a local increase if IGF-II in the extracellular space surrounding the vulnerable MNs may be important for MN protection. Interestingly, the VDFL-mediated up-regulation of IGF-II appeared to be region specific, as no up-regulation was observed in the prefrontal cortex or CNXII. Because of a drift in the phenotype of SOD1 transgenic rats observed by the breeder (Taconic) and the ALS association, the in vivo efficacy study of VDFL was performed in ALS model mice. VDFL also induced a significant up-regulation of IGF-II mRNA locally in the mouse cervical spinal cord region after chronic daily treatment. The VDFL-treated mice showed delayed disease onset and improved survival as compared to vehicle treated control mice. Given that IGF-II does not appear to play as important of a role in adult mice (DeChiara et al., 1991) as compared to adult human, the effect observed in this mouse model may indeed be more profound in human.

3.6. Mechanism(s) of PDE5is in IGF-II up-regulation and motor neuron protection

The tested PDE5 inhibitors VDFL, SDFL and TDFL all gave an up-regulation in IGF-II mRNA expression in primary spinal cord cells but only the highest inducers, VDFL and TDFL induced a significant protection of MN. Our data indicate that the protection is through an IGF-II dependent mechanism, since the effect was blocked by IGF receptor antagonists and since the compound that gave the highest IGF-II up-regulation, VDFL, also gave the highest MN protection. However, the potency in inhibiting PDE5 is also the strongest for VDFL (Sáenz de Tejada et al., 2002) so we cannot rule out that there is an additional or synergistic mechanism. There is some evidence that increased cGMP levels can increase mRNA expression, DNA binding and transcriptional activation of Egr-1 in neurons (Esteve et al., 2001; Pilz et al., 1995; Thiriet et al., 1997). Egr-1 is a transcription factor that is upstream of IGF-II expression and one of several activators of the P4 IGF-II promoter (Hyun et al., 1994), thus this may be one mechanism for the increased IGF-II expression by PDE5 inhibitors. Although no prior studies, that we know of, have shown IGF-II up-regulation as a result of PDE5i treatment, there is additional evidence of neuroprotection by PDE5is. For example, VDFL had neuroprotective properties in a 3-nitropropionic acid (3NP) model of Huntington's disease (Puerta et al., 2010) and also against 3,4-methylenedioxyamphetaine-induced neurotoxicity (Puerta et al., 2009). In CNS, PDE5 inhibitors increase concentrations of cGMP in the cortex, hippocampus and striatum (Puerta et al., 2009; Zhang et al., 2002; Marte et al., 2008), which may contribute to the protective mechanism (Charriaut-Marlangue et al., 2014). Furthermore, PDE5 inhibitors have been shown to increase brain levels of the neurotrophin brain-derived neurotrophic factor (BDNF) (Puerta et al., 2010). Additional PDEis (e.g. PDE7i, PDE10Ai, PDE4i and PDE1bi) have been implicated as potential therapeutic targets in Parkinson's Disease (Morales-Garcia et al., 2011) and neuropsychiatric disorders (Nishi & Snyder, 2010). This further supports the potential of PDE5is as therapeutic potentials for neurodegenerative disorders and highlights the multiple mechanisms (separate or in conjunction) by which neuroprotection may occur.

3.7. IGF-II expression increase may benefit cognitive function in certain ALS patient populations and the effect may be potentiated by PDE5 inhibition

Various degrees of cognitive impairment can be seen in up to 50% of ALS patients, around 15% of which meet the criteria for frontotemporal dementia (FTD) (Ringholz et al., 2005). Reversely, it is estimated that about 15% of FTD patients develop impaired motor function associated with MN loss (Lomen-Hoerth et al., 2002). With VDFL administration, we observed an improved survival of C9ORF72 ALS patient iPSC-derived MNs. In addition to contributing to a significant proportion of ALS cases, the C9ORF72 repeat expansion is the underlying cause of 25% of FTD cases and contributes to the majority of overlap between ALS and FTD cases (Majounie et al., 2012). Rodent studies have provided evidence for an important role for hippocampal IGF-II in brain plasticity, learning and memory (Chen et al., 2011; Schmeisser et al., 2012). Moreover, administration of IGF-II protein to the hippocampus in rats, leads to increased memory retention and persistence, and prevents forgetting (Chen et al., 2011). Several studies have shown that PDE5 is improve cognitive performance in rodents (Reneerkens et al., 2012; Prickaerts et al., 2002a; Rutten et al., 2009; Cuadrado-Tejedor et al., 2011; Puzzo et al., 2008; Puzzo et al., 2009). In this study, we show that VDFL increases hippocampal IGF-II mRNA expression after repeated gavage administration in naïve rats. Although a direct link between IGF-II protein and/or signaling and the observed positive cognitive effects of PDE5is (Reneerkens et al., 2012; Rutten et al., 2009; Cuadrado-Tejedor et al., 2011; Puzzo et al., 2008; Puzzo et al., 2009; Baek et al., 2011; Orejana et al., 2012; Prickaerts et al., 2002b) has not previously been demonstrated, our data suggest that the effect of PDE5is on cognition could at least partly be explained by an up-regulation of IGF-II in the hippocampus. Taken together this suggests a potentially dual pharmacological benefit for patients with MN disease and cognitive decline. Further studies using additional C9orf72 patient iPSC lines are required to determine the importance of these findings for ALS and ALS/FTD patients.

3.8. IGF signaling as a therapeutic approach for patients with ALS

Consistent with the IGF system being involved in MN protection it has been shown that adeno-associated viral delivery of IGF-I to ALS model SOD1 transgenic mice delays disease progression and extends survival time (Kaspar et al., 2003). Although IGF-II gene-delivery or up-regulation has never been tested in clinical trials, recombinant IGF-I has been administered subcutaneously in clinical trials with variable outcome (Borasio et al., 1998; Lai et al., 1997; Sorenson et al., 2008). One reason for the variable outcomes with recombinant IGF-I may be inadequate bioavailability locally in the spinal cord following subcutaneous administration as well as treatment initiation at a late stage of the disease where a majority of MNs already have degenerated. Drug-induced local increases of IGFs intrinsically or in the vicinity of the vulnerable motor neuron nuclei, as obtained by VDFL or targeted viral delivery of IGFs (Allodi et al., 2016; Kaspar et al., 2003) may confer a higher level of protection. Furthermore, if biomarkers were available to detect patients in early stages of ALS, treatment could be initiated earlier and patients may have better outcomes.

In conclusion, we have identified a pharmaceutical compound that modulates expression of IGF-II, a gene differentially expressed in resilient and vulnerable MNs. Our work supports the hypothesis that genes that are differentially expressed can be targeted by pharmaceuticals to increase MN resilience. The compound identified in this study, VDFL HCl, is an FDA approved widely used compound. We observed an increase in IGF-II expression in cultured MNs from both mice and human as well as in the spinal cord and hippocampus of mice and rat. Administration of VDFL to cultured murine MNs protects against glutamate-induced toxicity through a mechanism that involves IGF signaling. VDFL also improves survival of cultured human MNs derived from ALS patient iPSCs and activates a relevant human IGF-II promoter.

Although further studies are required to fully understand the complete mechanism, the data presented demonstrate that VDFL may be a promising pharmaceutical candidate for further evaluation as a therapeutic in degenerative MN disease. The extensive safety studies and the fact that PDE5is are already used for chronic daily treatment, could allow for fast translation into a MN disease patient population. Given that MN disease is a heterogenous condition with many different genetic and sporadic etiologies, further pre-clinical studies are required to identify specific patient populations that may benefit from use. Although we here show that modulation of IGF-II may be beneficial in MN disease, additional differentially expressed genes may also be useful therapeutic targets.

4. Materials and methods

4.1. Animal procedures

Female adult Sprague Dawley rats and CD-1 timed-pregnant mice were purchased from Charles River (Wilmington, MA). C57BL/6 J WT mice and B6·Cg-Tg(SOD1*G93A)1Gur/J transgenic mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were genotyped prior to use in the study. All animal procedures were performed in accordance with the National Institute of Health guidelines and were approved by the Animal Care and Use Committee at McLean Hospital, Harvard Medical School. Animals were housed according to standard conditions, with access to food and water ad libitum and a dark/light cycle of 12 h.

4.2. Primary spinal cord cultures

Timed-pregnant CD-1 mice were obtained from Charles River Laboratories, Wilmington, MA. Mice were euthanized by cervical dislocation and embryonic day (E) 12 embryos were collected. Spinal cords were dissected from decapitated embryos and collected into HBSS on ice. The cells were dissociated by incubating in trypsin EDTA (0.05%, ThermoFisher, Waltham, MA) at 37 °C for 10 min with intermittent agitation followed by gentle trituration using a blunted glass Pasteur pipette. The cells were plated onto 96-well plates or coverslips coated with poly-L-ornithine (15%) and mouse laminin (1 μg/ml, both from Sigma-Aldrich, St Louis, MO). For mRNA up-regulation they were seeded at 100.000 cells per well in a 96 well plate. For glutamate toxicity experiments and imaging they were seeded at 65.000–130.000/cm^2. They were cultured in Neurobasal media containing 10% fetal bovine serum, B27 supplement, 500 mM glutamine, 25 mM β-mercaptoethanol, penicillin–streptomycin (all from ThermoFisher, Waltham, MA) for 3 days prior to compound addition for mRNA up-regulation studies and for 6 days prior to start of glutamate toxicity assays as previously described (Hedlund et al., 2010).

4.3. iPSC cultures

All cell cultures were maintained at 37 °C and 5% CO2. iPSCs were propagated on Matrigel (Corning, New York, NY) in mTeSR1 media (Stem Cell Technologies, Vancouver, Canada) and passaged by dispase (1 mg/ml, ThermoFisher, Waltham, MA). The C9orf72 cell line was the 19f clone generated and described in Kiskinis et al., (2014) and Wainger et al., (2014). The HS cell lines and clones used were the 2131 and 2135 lines derived and characterized in Cooper et al., (2012) and a line derived from Coriell repository number ND34769/ND34811 subject population control fibroblasts. The donor for those fibroblasts is a female and was 61 years of age at the time of donation of the fibroblasts. The reprogramming method for this line was VSV-G encapsulated retroviruses (MLV-based) encoding oct4, klf4, sox2 and c-myc. The line was generated at the Harvard Stem Cell Institute as part of the PDiPS Cell Consortium. Pluripotency markers were adequately detected by immunocytochemistry and qPCR and the line differentiated in vitro to all three germ layers. The karyotype (G-banding) was normal.

4.4. Differentiation of MNs from human iPSCs

iPSCs were differentiated to MNs using an EB based protocol (Fasano et al., 2009; Ding et al., 2013). To initiate differentiation, iPSCs were dissociated by dispase (1 mg/ml, ThermoFisher, Waltham, MA) washed in PBS and DMEM/F12 and replated onto ultra-low attachment culture dishes in dual smad media (50/50 DMEM/F12/Neurobasal media, NEAA, L-glutamine, N2 supplement, B27 supplement (all from ThermoFisher, Waltham, MA), Dorsomorphin (1 μM, Stemgent, Lexington, MA), SB431542 (10 μM, Stemgent, Lexington, MA)), ROCK inhibitor Y-27632 (5 μM, Sigma, St Louis, MO) and bFGF (10 ng/ml, ThermoFisher, Waltham, MA). On day in vitro (DIV) 3 media was changed to dual smad media and on DIV5 to dual smad media plus retinoic acid (RA, 1 μM, Sigma, St Louis, MO). On DIV7-13 the EBs were differentiated in MN differentiation media (50/50 DMEM/F12/Neurobasal media, NEAA, L-glutamine, N2 supplement, B27 supplement (all from ThermoFisher, Waltham, MA), RA (1 μM, Sigma, St Louis, MO), purmorphamine (1 μM, Stemgent, Lexington, MA) and sonhic hedgehog agonist (SAG, 1 μM, Enzo Lifesciences, Farmingdale, NY)) with media changes every other day. At DIV14 the EBs were incubated with Accutase for 10 min. at 37°C prior to dissociation and plating onto poly-L-ornithine (15%) and mouse laminin (1 μg/ml, both from Sigma-Aldrich, St Louis, MO) coated plates. The cells were cultured in MN differentiation media supplemented with DAPT (2.5 μM, Bristol, UK), BDNF and GDNF (each 10 ng/ml, PeproTech, Rocky Hill, NJ) from DIV14–23. On DIV23 the cells were dissociated and plated onto poly-L-ornithine (15%) and mouse laminin (1 μg/ml, Sigma-Aldrich, St Louis, MO) coated 96-well imaging plates (Corning, Corning NY) at a density of 25.000 cells per well in MN maturation media (50/50 DMEM/F12/Neurobasal media, NEAA, L-glutamine, N2 supplement, B27 supplement, sodium pyruvate (all from ThermoFisher, Waltham, MA), DAPT (2.5 μM, Bristol, UK), BDNF and GDNF (each 10 ng/ml, PeproTech, Rocky Hill, NJ) and ascorbic acid (200 μM, Sigma-Aldrich, St Louis, MO)).

4.5. VDFL pharmacokinetic studies and liquid chromatography/mass spectrometry (LC-MS/MS)

Pharmacokinetic test including LC-MS/MS of VDFL HCl was performed by Charles River Laboratories (Wilmington, MA). 16 female Sprague Dawley rats, approximately 250 g at dose initiation, were dosed at 8 mg/kg/day once a day for 7 days by gavage administration. Exact dosing for each animal (ml/kg) was based on each animal's individual body weight recorded on the morning of dose administration. VDFL (ChemPacific, Baltimore, MD) was dissolved in sterile distilled water at a concentration of 1 mg/ml. Blood samples (2 × 1 ml) were collected into K_2EDTA by cardiac puncture on day 7 at time-points 1, 4, 12 and 24 h post-dose. Maximum obtainable volume of CSF was collected at the same time points. 4 animals were sacrificed at each scheduled time-point. Samples were kept on ice prior to processing and centrifuged at 2200 × *g* for 10 min at 4°C. Plasma and CSF samples were stored at − 70 °C until submitted for analysis. Dose formulation (standards), plasma and CSF were transferred on dry ice for LC-MS/MS analysis. Samples were prepared for LC-MS/MS by acetonitrile protein precipitation. Matrix calibration standards were prepared at 1–10,000 ng/ml in plasma or artificial CSF. LC conditions: Thermo Transduced Allegra Pumps with a Leap CTC PAL autosampler and Supelco Discovery C18, 5u, 50 × 2.1 mm column were used. The flow rate was 0.6 ml/min. The mobile phases were formic acid (0.1%) in A) water or B) acetronile. The injection volume was 5 μl. A 1.0 min gradient was utilized going from 5% to 98% of mobile phase B for a total run time of 2.8 min. MS conditions: An ABSciex Qtrap 5500 instrument was used with Multiple Reaction Monitoring (MRM). The dwell-time was 35 ms, the transition ion (*m/z*) parent/product was 489.257/

151.278 and the ionization mode was APCI +.

4.6. In vitro and in vivo drug-treatment

In the drug-screen to identify compounds that increase IGF-II expression in mouse spinal cord cultures, the US Drug Collection (MicroSource Discovery Systems Inc., Gaylordsville, CT), a library of 1040 compounds, was used. All compounds were added to spinal cord cultures in 96-well plates at a final concentration of 10 μM. Compound addition was performed in triplicate on cultures from three different dissections and the average fold-change was determined. Cells were lysed for mRNA isolation 24 h after compound addition. For in vivo administration, VDFL (ChemPacific, Baltimore, MD) solutions of 2 mg/ml were prepared in ddH_2O for oral gavage. Rats were given 8 mg/kg/day over two weeks. Mice were given 10 mg/kg/day over two weeks in the IGF-II mRNA up-regulation study and starting at 5 weeks of age and continuing to the end-point in the ALS model onset and survival study. For in vitro administration of VDFL to human MN cultures a stock solution of 10 mM was made in DMSO and further dilutions were made in culture media for a final 0.1–1 μM concentration. Cells were lysed for mRNA extraction 24 h after VDFL addition. See below for compound treatment in glutamate toxicity assay and C9orf72 MN survival study.

4.7. Quantitative real-time PCR

mRNA was isolated from mouse and human MN cultures using a kit (TurboCapture 96 mRNA kit, QIAGEN, Valencia, CA) according to manufacturer's instructions for adherent cells. mRNA from rat tissue was isolated using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions, including the DNAse treatment step. mRNA and cDNA concentration and purity was assessed using a NanoDrop (ThermoFisher, Waltham, MA) with a ratio of absorbance at 260 nm over 280 nm of at least 1.8 for cDNA and at least 2.0 for mRNA. cDNA was prepared from mRNA using the Superscript III first-strand synthesis system (ThermoFisher, Waltham, MA). qPCRs were run with TaqMan gene expression assays on a StepOnePlus Real-Time PCR system (Applied Biosystems/ThermoFisher, Waltham, MA). For mouse cultures the following primer assays were used: Mm00439565_g1 (IGF-II) and 4333760F (18S); For rat tissue the following primer assays were used: Rn01454518_m1 (IGF-II), Rn00667869_m1 (Beta-actin), Rn01775763_g1 (GAPDH), Rn01527840_m1 (hprt1); For human cells the following primer assays were used: Hs04188276_m1 (IGF-II), Hs03929097_g1 (GAPDH), Hs99999903_m1 (beta actin), Hs01003267_m1 (hprt1). Relative quantification was performed, analyzing changes in IGF-II gene expression normalized to a housekeeping gene. Housekeeping genes were validated and only genes that were stable between samples (i.e. not changing with drug-treatment) were selected. The efficiencies for the housekeeping gene and the IGF-II amplifications were relatively similar and CTs not larger than 10 amplicons apart. Fold-changes were calculated using the $2^{-\Delta\Delta}$CT method. A pooled control sample containing cDNA from all eight of the vehicle treated control samples per plate was used for $2^{-\Delta\Delta}$CT calculations and all controls were also run individually for statistical analysis of data.

4.8. ELISA assay

ELISA assays (mouse IGF-II DuoSet eliza, R&D systems, Minneapolis, MN and human IGF-II ELISA kit, Uscn Life Science, Hubei, China) were performed according to manufacturer's instructions to detect secreted IGF-II protein. For mouse IGF-II up-regulation ELISA was performed on embryonic spinal cord cultures treated with the 38 drugs that gave the highest increase in IGF-II mRNA expression and DMSO control. For human ELISA, MN cultures were treated with VDFL or DMSO control. Media supernatants were collected 72 h after drug treatment.

4.9. In vitro glutamate toxicity

Primary spinal cord cultures were exposed to glutamate toxicity 6 days after plating. Prior to applying the drugs in the progressive glutamate toxicity protocol, the drug-dosing (both doses and number of additions) was optimized for each pharmaceutical in order to avoid toxicities. The doses used in the progressive glutamate toxicity protocol ranged between 1 and 10 μM and were applied 1–3 times throughout the experiment. The first dose was given 72 h prior to initiating glutamate toxicity for all the drugs. For VDFL, TDFL and SDFL, the dosing was the following: 72 h and 24 h prior to glutamate toxicity, cultures were treated with VDFL, SDFL or TDFL (10 μM and 2.5 μM respectively). 24 h prior to the last addition, VDFL, SDFL or TDFL (1 μM) was added as a booster. Glutamate (50 μM, L-Glutamic acid monosodium salt hydrate, Sigma-Aldrich, St Louis, MO) and the glutamate re-uptake inhibitor, L-*trans*-2,4-PDC, (10 μM, Tocris, Bristol UK) were added on two occasions, 48 h apart. Media was not changed in the cultures after the initial VDFL addition in order for secreted IGF-II protein to remain in the culture media. The IGF-I peptide analog JB1, (20 ng/μl, Bachem Biosciences, Torrance, CA), which acts as an antagonist of the IGF-IR and an IGF-IIR antibody (5 ng/μl, anti-IGF2R, R&D Systems, Minneapolis, MN) were added to the cultures 24 h after VDFL treatment to determine IGF-II pathway involvement. These doses are previously proven to be successful in blocking IGF-IR and IGF-IIR activity (Chen et al., 2011).

4.10. Long-term survival of MNs differentiated from C9orf72 ALS patient derived iPSCs

MNs differentiated from iPSCs were kept in culture for 30 days post the end of the differentiation protocol in MN maturation media (see above). Cultures were treated with VDFL (1 μM) or DMSO (vehicle) every other day and subject to only partial weekly media changes. On DIV60 media was collected for ELISA analysis of IGF-II protein content prior to paraformaldehyde fixation of the cells (see below).

4.11. Immunocytochemistry and imaging

Cells were fixed in paraformaldehyde (PFA, 4%). Prior to staining, the cells were blocked and permeabilized using normal donkey serum (10%, Jackson ImmunoResearch Laboratories, West Grove, PA) with Triton X-100 (0.1%, ThermoFisher, Waltham, MA) in PBS for 1 h. Primary antibodies were incubated overnight at 4 °C in blocking solution. The following dilutions were used: βIII-tubulin (TUJ1) - 1:1000 (Covance, Princeton, NJ), isl-1/2 1:100 (40.2D6-c, 39.4D5-c, DSHB, Iowa City, IA), ChAT - 1:500, GluR2 1:500, NR2B 1:100, GFAP 1:500 (all from Millipore, Billerica, MA). Samples were washed 3 times with 0.1% T_PBS and incubated with the secondary antibodies at 1:500 dilution in blocking solution for 2 h at room temperature (donkey anti-mouse/rabbit Alexa Fluor 488/568, ThermoFisher, Waltham, MA). Lastly, a nuclear stain was used (Hoechst 33,258, Polysciences, Inc., Warrington, PA). Stained 96-well imaging plates containing mouse embryonic cultures were imaged on an automated imaging system (InCell Analyzer Imaging system, GE Healthcare, UK). Quantitative analysis was performed on 4 different cultures in triplicate. At least nine images per well were acquired at 20 × magnification using autofocus setting. Images were analyzed in an automatically and unbiased way using the IN Cell Workstation Software (GE Healthcare, UK). The total number of cells, isl-1/2–expressing cells, and isl-1/2 and ChAT-co-expressing cells were quantified using multitarget analysis. Individual cells were identified as having a nuclear area (identified by Hoechst) larger than 25 mm^2 with top-hat segmentation. Isl-1/2 expressing cells were identified as stain within the nucleus and ChAT-expressing cells were identified as exhibiting staining in an area extending outside of the nucleus. Stained 96-well imaging plates containing human iPSC derived MN cultures were imaged on an ArrayScanTM High-Content

Systems (ThermoFisher, Waltham, MA). Quantitative analysis was performed in triplicate. At least four images per well were acquired with autofocus at 20 × magnification. Images were analyzed in a standardized, unbiased way using Columbus 2.5 Image Data Storage and Analysis System (PerkinElmer, Waltham, MA). The total number of cells, isl-1/2–expressing cells, and isl-1/2 and ChAT or βIII-tubulin co-expressing cells were quantified. Isl-1/2 expressing cells were identified as stain within the nucleus and ChAT and/or βIII-tubulin-expressing cells were quantified with cytoplasmic identifier. High resolution images of MNs were acquired using a LSM510 confocal microscope (Carl Zeiss, Peabody, MA) using a 40 × oil objective.

4.12. Symptomatic onset and survival study in $SOD1^{G93A}$ ALS model mice

VDFL (10 mg/kg/day) or water vehicle was administered to WT and ALS $SOD1^{G93A}$ transgenic mice daily by gavage starting at 5 weeks of age. ALS $SOD1^{G93A}$ transgenic mice were initially monitored bi-weekly for weight loss and changes in neuroscore. The scoring was performed by investigators blinded to the treatment groups according to the following protocol: 0.5: Hind leg trembling during tail suspension; 1: Partial collapse of one or both hind legs' extension towards lateral midline during tail suspension; 1.5: Full collapse of one hind leg extension towards lateral midline during tail suspension; 2: Full collapse of both hind legs' extension towards lateral midline during tail suspension. When animals approached the expected age of symptomatic onset (100 days of age) they were monitored by daily weight measurements, and neuroscore. After disease initiation (3 consecutive neuroscores of 0.5) they were monitored bi-daily, and if needed more frequently. Study end-point was determined to be when an animal could not right itself in 30 s after being placed on either of its' sides (Scott et al., 2008).

4.13. IGF-II promoter activation studies

The human P4 IGF-II promoter (see Fig. 6a for sequence) was cloned from human DNA and inserted into a promoter-less vector (PGL4.17[*luc2*/Neo], Promega, Madison, WI) containing the luciferase reporter gene *luc2 (Photinus pyralis)*. The vectors with and without constructs were transfected into a cholinergic human neuroblastoma line, SKNFI (ATCC, Manassas, VA), which has endogenous low-level expression of IGF-II, using calcium phosphate co-precipitation (Calcium phosphate Transfection Kit, ThermoFisher, Waltham, MA) according to manufacturer's instructions. The line was propagated under aminoglycoside antibiotic (G-418) selection pressure in growth media (Dulbecco's Modified Eagle's Medium, 0.1 mM Non-Essential Amino Acids and 10% fetal bovine serum) for 2 weeks prior to propagation of clonal lines. Clonal lines were established by seeding cells at low density in 96-well plates in growth media. Individual clones were further propagated under selection pressure. For determination of P4 promoter activation, cells were plated in white opaque 96-well plates. Twenty-four hours after plating, VDFL or DMSO control were added to the cells. Luciferase luminescence was measured 72 h after compound addition using the Steadylite plus Reporter Gene Assay System according to manufacturer's instructions and an EnVision™ multilabel plate reader (Perkin Elmer. Waltham, MA). The cell-line containing the vector without promoter insert was used as a control.

4.14. Statistics

Statistical analyses were performed with GraphPad Prism 6 software (Graph Pad, La Jolla, CA). For multiple comparisons of a single data set ordinary one –way analysis of variance (ANOVA) was used. For post-hoc analyses Tukey's or Bonferroni's multiple comparison tests were used where appropriate as indicated in figure legends. For in vivo IGF-II up-regulation in VDFL treated and vehicle treated rats and mice two-tailed, unpaired Student's *t*-test or Mann-Whitney (when normal distribution was not apparent) was used to compare the two groups in respective region as indicated in the figure legend. For IGF-II protein up-regulation in VDFL or vehicle treated cultures, unpaired Student's *t*-test was used to compare the two conditions. For disease onset unpaired Student's *t*-test was used to determine significant difference and the survival studies were analyzed using Mantel-Cox log-rank test. Experiments were performed in triplicate at a minimum and as indicated in the figure legends. The results are expressed as average ± SEM as indicated in the figures.

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