CDNF rescues motor neurons in models of amyotrophic lateral sclerosis by targeting endoplasmic reticulum stress

Francesca De Lorenzo,[1,2] Patrick Lüningschrör,[3] Jinhan Nam,[1,2] Liam Beckett,[1,2] Federica Pilotto,[4] Emilia Galli,[1] Päivi Lindholm,[1] Cora Rüdt von Collenberg,[3] Simon Tii Mungwa,[3] Sibylle Jablonka,[3] Julia Kauder,[5] Nadine Thau-Habermann,[5] Susanne Petri,[5] Dan Lindholm,[6,7] Smita Saxena,[4] Michael Sendtner,[3] Mart Saarma[1,†] and Merja H. Voutilainen[1,2,†]

[†]**These authors contributed equally to this work.**

Abstract

Amyotrophic lateral sclerosis is a progressive neurodegenerative disease that affects motor neurons (MNs) in the spinal cord, brainstem, and motor cortex, leading to paralysis and eventually to death within 3 to 5 years of symptom onset. To date, no cure or effective therapy is available. The role of chronic endoplasmic reticulum (ER) stress in the pathophysiology of amyotrophic lateral sclerosis, as well as a potential drug target, has received increasing attention.

Here, we investigated the mode of action and therapeutic effect of the ER-resident protein cerebral dopamine neurotrophic factor (CDNF) in three preclinical models of amyotrophic lateral sclerosis, exhibiting different disease development and etiology: (i) the conditional choline acetyltransferase (ChAT)-tTA/TRE-hTDP43-M337V rat model previously described, (ii) the widely used SOD1-G93A mouse model, and (iii) a novel slow-progressive TDP43-M337V mouse model. To specifically analyse the ER stress response in MNs, we used three main methods: (i) primary culture of MNs derived from E13 days embryos, (ii) immunohistochemical analyses of spinal cord sections with ChAT as spinal MNs marker, and (iii) qPCR analyses of lumbar MNs isolated via laser microdissection.

We show that intracerebroventricular administration of CDNF significantly halts the progression of the disease and improves motor behavior in TDP43-M337V and SOD1-G93A rodent models of amyotrophic lateral sclerosis. CDNF rescues motor neurons *in vitro* and *in vivo* from ER stress-associated cell death and its beneficial effect is independent of genetic disease etiology.

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Notably, CDNF regulates the unfolded protein response (UPR) initiated by transducers IRE1α, PERK, and ATF6, thereby enhancing MN survival.

Thus, CDNF holds great promise for the design of new rational treatments for amyotrophic lateral sclerosis.

Author affiliations:

1 Institute of Biotechnology, HiLIFE, P.O. Box 56, Viikki Biocenter 2, University of Helsinki, FIN-00014, Helsinki, Finland

2 Division of Pharmacology and Drug therapy, Faculty of Pharmacy, P.O. Box 56, Viikki Biocenter 2, University of Helsinki, FIN-00014, Helsinki, Finland

3 Institute of Clinical Neurobiology, University Hospital Würzburg, 97078 Würzburg, Germany

4 Department of Neurology, Inselspital University Hospital, University of Bern, CH-3010 Bern, Switzerland

5 Department of Neurology, Hannover Medical School, 30625 Hannover, Germany

6 Medicum, Department of Biochemistry and Developmental Biology, Faculty of Medicine, University of Helsinki, Helsinki, Finland

7 Minerva Foundation Institute for Medical Research, Helsinki, Finland

Correspondence to: Merja Voutilainen

Division of Pharmacology and Drug therapy, Faculty of Pharmacy, P.O. Box 56, Viikki Biocenter 2, University of Helsinki, FIN-00014, Helsinki, Finland

E-mail: merja.h.voutilainen@helsinki.fi

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Abbreviations: ABC = avidin-biotin complex; ATF6 = activating transcription factor 6; BDNF = brain-derived neurotrophic factor; CDNF = cerebral dopamine neurotrophic factor; ChAT = choline acetyltransferase; CNTF = ciliary neurotrophic factor; C9orf72 = chromosome 9 open reading frame 72; DAB = 3'-diaminobenzidine; ER = endoplasmic reticulum; HRP = horseradish peroxidase; i.c.v. = intracerebroventricular; iPSC = induced pluripotent stem cells; IRE1 = inositol-requiring enzyme 1; MANF = mesencephalic astrocyte-derived neurotrophic factor; MN = motor neuron; PDI = protein disulfide isomerase; PERK = protein kinase RNA-like endoplasmic reticulum kinase; SOD1 = superoxide dismutase 1; TDP43 = TAR DNA-binding protein 43; TM = tunicamycin; TP = thapsigargin; UPR = unfolded protein response; UT = untreated; WT = wild type

Introduction

Amyotrophic lateral sclerosis is a progressive motor disorder characterized by the dysfunction and death of motor neurons (MNs) in the spinal cord, brainstem, and motor cortex, which results in the atrophy of skeletal muscles and paralysis. Any cure or disease-modifying therapy is currently lacking. Amyotrophic lateral sclerosis patients die within 3 to 5 years of diagnosis and respiratory failure is the most common cause of death.[1] To date, the etiology of amyotrophic lateral sclerosis remains mostly unknown: only about 5-10% of the cases are familial, while the remaining 90-95% of the cases occur sporadically, indicating the influence of multiple factors in its pathogenesis. Accumulation of misfolded and aggregated proteins leads to endoplasmic reticulum (ER) stress, which eventually activates the unfolded protein response (UPR). The UPR is a physiological signaling cascade that suppresses protein translation, degrades misfolded proteins, and facilitates protein folding. In mammalian cells, the UPR consists of three pathways, initiated by transmembrane ER sensors at the ER membrane: inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). At onset, the UPR is protective, but, under prolonged ER stress conditions, the UPR promotes apoptotic pathways.[2]

Substantial evidence supports the involvement of chronic ER stress in the pathophysiology of MN degeneration in amyotrophic lateral sclerosis, both in patients and animal models. UPR

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markers were found to be upregulated in the spinal cord of sporadic and familial amyotrophic lateral sclerosis patients[3-6] and increased amount of protein disulfide isomerase (PDI) was detected in the cerebrospinal fluid of sporadic patients.[3] Transcriptional analysis of MNs derived from induced pluripotent stem cells (iPSCs) of patients revealed that UPR alterations were conserved among MNs harbouring SOD1 or C9ORF72 mutations, emphasizing the susceptibility of MNs to ER stress.[7-9]

Fast-fatigable MNs in the SOD1 transgenic mice were shown to be intrinsically more sensitive to ER stress[10] and an analogous upregulation of UPR markers was also reported in other studies on this mouse model.[11,12] Interestingly, a recent work revealed that, under chronic ER stress conditions, also wild type (WT) SOD1 protein tends to aggregate in mice, forming abnormal species.[13] Similarly, pharmacological induction of ER stress causes TDP43 to accumulate and, *vice versa*, overexpression of mutated TDP43 triggers ER stress.[14] Genetic ablation of the ER chaperone SIL-1 in the SOD1-G93A mouse model enhanced ER stress and consequently exacerbated the disease progression, emphasizing the importance of ER homeostasis in amyotrophic lateral sclerosis pathophysiology.[15] Furthermore, translocation of FUS protein from the nucleus to the cytoplasm has been linked to ER stress.[16,17]

Additionally, mutations in genes encoding ER proteins have been linked to familial and sporadic cases of amyotrophic lateral sclerosis. A missense mutation E102Q in the ER chaperone Sigma-1 receptor gene has been reported in a few familial cases.[18] A total of nine PDIA1 missense variants and seven PDIA3 missense variants for the gene encoding PDI were identified in sporadic patients.[19]

Compounds interfering specifically with the PERK/p-eIF2alpha pathway or the knockdown of its downstream effector ATF4 partially alleviate the disease phenotype in the SOD1-G93A rodent model, providing neuroprotection and delaying disease progression.[10,20-22] Inhibiting the IRE1α - XBP1 arm of UPR signalling since embryonic stage revealed a beneficial role in the disease pathophysiology, due to an enhanced clearance of mutant SOD1 aggregates by autophagy pathways.[4] Moreover, targeting ER folding capacity and homeostasis by overexpression of SIL-1 in the SOD1-G93A mouse model proved to be neuroprotective and prolonged survival.[15]

Until now, only inhibitors of individual UPR pathways have been described, but due to the double-edged sword nature of UPR, molecules modulating all three pathways are needed.

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Despite efforts to find drugs targeting ER stress to treat protein-misfolding and aggregation disorders, its value as a pharmacological target warrants further exploration.

Cerebral dopamine neurotrophic factor (CDNF) is an ER-resident protein expressed in the central nervous system mostly in neurons and peripheral tissues, including skeletal muscles, the target tissue of MNs.[23] By virtue of its C-terminal signal KTEL, which resembles the canonical Lys-Asp-Glu-Leu (KDEL) sequence for ER retention, CDNF-protein is primarily located in the ER lumen and secretion of mouse CDNF is regulated by the ER-resident proteins GRP78 and KDEL-R1.[24] The analysis of CDNF knockout mice, as well as closely related mesencephalic astrocyte-derived neurotrophic factor (MANF 59% sequence homology with CDNF) knockout mice, revealed an increase in the UPR response in several tissues in the absence of CDNF and/or MANF,[25-27] hinting to a role of CDNF in regulating the ER stress response. When overexpressed or microinjected into neurons, CDNF rescues cells from ER stress-induced apoptosis.[23] In a recent paper from our group, we showed that deleting the ER retention signal KTEL significantly reduced the neuroprotective effect of CDNF, suggesting that a reduced ER localization caused a loss in the anti-apoptotic activity of CDNF.[28] CDNF´s ability to rescue neurons from apoptosis was also blocked by IRE1a and PERK inhibitors. CDNF protects and restores dopamine neurons in rodent and non-human primate models of Parkinson's disease.[23,29-31] CDNF was tested in Phase I-II trial on Parkinson's patients and the study met primary endpoints of safety and tolerability at 12 months (Clinical trial number NCT03295786).[32]

In order to determine the efficacy and potential of CDNF as a drug candidate for amyotrophic lateral sclerosis, we treated three independent rodent models: (i) a severe fast progressing TDP43- M337V rat model of acute induction of TDP-43-M337V toxicity, previously described by Huang *et al.*[33]; (ii) the well-characterized SOD1-G93A mouse model; (iii) a novel chronic TDP43-M337V expressing mouse model. Albeit being very different from each other, all three models exhibited signs of ER stress and death of MNs. Our results reveal that CDNF markedly delays the pathological phenotype and rescues MNs from ER stress-induced cell death in all three models, indicating that ER stress is an important component of the pathophysiology of amyotrophic lateral sclerosis, irrespective of its genetic etiology. Moreover, our data provide evidence that CDNF-mediated inhibition of ER stress at diseases stages has potential as a therapeutic intervention for this disorder.

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Materials and methods

Study design

Being aware of the drawbacks of available amyotrophic lateral sclerosis pre-clinical models, we used three different models: (i) the conditional choline acetyltransferase (ChAT)-tTA/TRE-hTDP43-M337V rat model described by *Huang et al*.,[33] (ii) the SOD1-G93A mouse model, and (iii) a slow-progressive TDP43-M337V mouse model. We monitored disease progression and evaluated motor behavior through a battery of different motor test. To evaluate the ER stress response specifically in MNs, which are the most affected cell-type in amyotrophic lateral sclerosis, we utilized three main methods: (i) primary culture of MNs derived from E13 embryos, (ii) immunohistochemical analyses of spinal cord sections with ChAT as spinal MNs marker, and (iii) qPCR analyses of lumbar MNs isolated via laser microdissection. All the specific methods and characteristics of the animal models are described in the supplementary materials.

Data availability

All raw data are available upon request.

Results

CDNF treatment rescues motor neurons and improves motor behavior in a TDP43-M337V rat model

To investigate the potential neuroprotective effect of CDNF in amyotrophic lateral sclerosis, we started administering CDNF in the acute ChAT-tTA/TRE-hTDP43-M337V rat model introduced by Huang *et al*..[33] In this preclinical model, the expression of human mutated TDP43 protein was dependent on the choline acetyltransferase (ChAT) promoter via a Tet-regulatory system. The transgene was kept inactive until adult age by administration of doxycycline (Dox) in drinking water (50 mg/ml). At the age of 60 days, Dox was completely withdrawn from the water and expression of TDP43-M337V protein became evident in the spinal cord MNs of the rats already

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three days later. In the original study, the model showed a severe disease development, as the rats exhibited signs of motor impairment within a week and reached paralysis about one week after the onset of the disease phenotype. At this stage, over 60% of spinal MNs were lost in the transgenic rats, and within 20 days all the animals had reached the end point.[33] As the progression of the disease was very fast and, therefore, the therapeutic window was narrow, we decided to implement the protocol and opted for a partial withdrawal of Dox, instead of a complete one. Starting from 60 days of age, rats were administrated Dox in drinking water at a concentration of 10 mg/ml (**Fig. 1A**). The partial withdrawal of Dox-induced a slower progression of the disease (**Fig. 1B**). A continuous infusion of 6 µg/day of CDNF or phosphate-buffered saline (PBS) as vehicle in the brain lateral ventricle of TDP43-M337V and WT littermates was achieved by using Alzet minipumps. The minipumps, connected through a catheter to a cannula directly infusing in the cerebral ventricle (i.c.v.), were implanted one week before the activation of the transgene and continued to administer CDNF or the vehicle for 28 days. After the partial withdrawal of Dox, the rats were monitored for weight and motor behavioral changes for three weeks (**Supplementary Fig. 1, Fig. 1B**). Upon transgene activation, no difference in the rotarod performance was detected between the TDP43-M337V rats and the tTA littermates (used as control) until day 10. The ability to run on the rotating rod decreased substantially in the transgenic rats from day 10 onwards and major signs of hind limb stiffness and gait impairment were detected by day 20 (**Fig. 1B)**. A continuous i.c.v. administration of CDNF significantly ameliorated the motor performance of treated TDP43-M337V, which was comparable to tTA littermates. No difference over time in the latency to fall was detected in tTA rats (**Fig. 1B**). Moreover, we verified whether the improvement in the motor performance of CDNF treated rats compared to PBS treated ones correlated with the number of MNs in the lumbar spinal cord. Indeed, we found that the transgenic rats that received PBS lost about 50% of the lumbar spinal MNs but treatment with CDNF significantly increased the number of surviving MNs in the same area to about 75% in comparison to the tTA controls (**Fig. 1C-D**).

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CDNF attenuates the ER stress response in the TDP43-M337V *in vitro* and *in vivo*

Next, we sought to identify whether the protective effect of CDNF lay in its ability to reduce the ER stress response, as previous evidence has hinted to a possible role of CDNF in the ER homeostasis.[28,34] Thus, we examined the effect of CDNF *in vitro* on MNs isolated from embryonic day 13 (E13) mice. Neurons were pharmacologically stressed with thapsigargin (TP) to induce ER stress, and then treated with CDNF. 24 hours after TP treatment, the survival of MNs was reduced to about 30% and CDNF treatment was able to improve the rate of MNs survival to 60% (**Fig. 2A)**. To verify whether this positive outcome was due to a specific attenuation of the UPR pathways, we extended our dataset including new treatment groups where CDNF was administrated in combination with PERK inhibitor GSK2606414, IRE1 ribonuclease (RNase) inhibitor 4μ8C or kinase-inhibiting RNase attenuator 6 (KIRA6). We hypothesized that inhibition of the UPR pathways would block CDNF survival-promoting activity. Three different concentrations of the inhibitors were used, and we observed that the beneficial effect of CDNF was inversely correlated with the increasing concentrations of GSK2606414, 4μ8C, and KIRA6. The highest concentration of each inhibitor significantly blocked CDNF action (**Fig. 2A)**. Survival assays with all three inhibitors in absence of TP were also performed to exclude any potential toxic effect of the inhibitors themselves. The survival of MNs was evaluated either in the presence or absence of established neurotrophic factors BDNF[35] and CNTF,[36] which have been shown to increase MNs survival *in vitro*. Inhibiting IRE1 or PERK pathways did not have any effects on naive cells, neither beneficial nor toxic and did not influence CNTF and BDNF survival promoting effect (**Fig. 2B**). Furthermore, we analysed the effect of CDNF treatment on the modulation of each UPR pathway in TP-stressed WT TDP43 and mutated TDP43-M337V expressing neurons. To address whether CDNF acts on the IRE-1 branch of the UPR, GFP-tagged IRE-1 was expressed in MNs via lentiviral transduction and MNs were treated with TP after 5 days. As previously described, we observed a clustering of IRE1 upon ER stress induction.[37] Therefore, we used the number of IRE1 clusters per cell and the percentage of cells with GFP-positive clusters as a readout for IRE1 activation. We observed that some TDP43-M337V-expressing cells already showed clustering of IRE1 without TP treatment when cultured without CDNF, while in WT cells the activation of IRE1 was only detectable upon treatment

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with TP. CDNF was able to reduce IRE1 clustering in all the aforementioned conditions (**Fig. 2C and E-F**). Similarly, WT and TDP43-M337V MNs were transduced with ATF6-GFP lentiviral vectors to evaluate the translocation of ATF6 protein from the cytoplasm to the nucleus of neurons. A partial translocation of ATF6 was found in both TP-treated WT and TDP43-M337V MNs and it was reduced by CDNF administration (**Fig. 2D and G**). The partial translocation of ATF6 most likely reflects the fact that only a subset of ATF6-EGFP is cleaved and translocates to the nucleus. Finally, TDP43 and mutated TDP43-M337V expressing neurons were cultured with or without CDNF and pharmacologically stressed with TP or tunicamycin (TM) to induce ER stress. The protein levels of phosphorylated eIF2α (p-eIF2α) and CHOP (gene *Ddit3),* as well as the mRNA levels of *Chop*, were decreased by CDNF treatment (**Fig. 2H-K, Supplementary Fig. 2A-B**). Taken together, these data indicate that CDNF attenuates ER stress in this model. The levels of phosphorylated TDP43 and the total levels of the protein did not change upon CDNF treatment (**Supplementary Fig. 2C-D**). To further investigate the neuroprotective mechanism behind CDNF action in the ChAT-tTA/TRE-hTDP43-M337V rat model, we sacrificed the rats 21 days after the transgene activation and collected the spinal cord for immunohistochemical analyses. We found that the levels of ER chaperone GRP78 (*alias* BiP, gene *Hspa5*) and p-eIF2α were upregulated in the vehicle-treated TDP43-M337V rats compared to tTA controls (**Fig. 3A-F**). This increase in the ER stress response in this model was not previously described in the literature and strengthens the hypothesis of the importance of ER stress in the pathophysiology of amyotrophic lateral sclerosis. Staining of the ER-marker GRP78 was found from the perinuclear area to the cell membrane. It has been previously shown that the ER in neurons forms a highly dynamic network that can reach out to the cell surface, entering axons and presynaptic terminals, and make direct contact with the plasma membrane.[38-41] This may explain while the staining for GRP78 is not restricted to the perinuclear area, as in many other cell types. Upon treatment with CDNF, the expression of GRP78 and the phosphorylation of p-eIF2α were significantly reduced in the ventral horn of the lumbar spinal cord and, specifically, in the $ChAT^+$ MNs (**Fig. 3A-F**).

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A single i.c.v. CDNF injection improves survival and rescues motor neurons in SOD1-G93A mice

To determine whether the effect of CDNF would be specific only to the TDP43 model or if it could be applied to any model affected by ER stress, we treated SOD1-G93A mice with CDNF. In this well-characterized amyotrophic lateral sclerosis model, chronic ER stress response in the corresponding MNs has been previously reported.[10,11] To this end, we utilized a single injection of CDNF, which in Parkinson's disease animal models effectively counteracted dopamine neuron degeneration.[23] As proof-of-principle, we first confirmed that CDNF and ^{125}I-labelled CDNF injected in the brain lateral ventricle efficiently diffuse to different areas of the brain, including cortex, striatum, and substantia nigra, and to the lumbar spinal cord (**Supplementary Fig. 3**). Moreover, we found that CDNF specifically co-localizes with lumbar MNs (**Supplementary Fig. 4**). 13 weeks SOD1-G93A mice (early disease stage) and WT littermates of 13 weeks of age received a single i.c.v. injection of 10 µg of human CDNF or PBS as vehicle and were examined twice a week to follow changes in disease indications and motor behavior until the final stage of paralysis (experimental timeline in **Fig. 4A**). At the time of treatment, SOD1-G93A mice displayed measurable tremors in the hind limbs. Upon a single CDNF injection, SOD1- G93A mice developed gait impairment and paralysis significantly more slowly than PBS-treated mutant littermates **(Supplementary Fig. 5A-B**), and, in females but not in males, we also observed a slower decrease in body weight (**Supplementary Fig. 5C-D**). The median survival time in females was 148 days for CDNF-treated mice and 140 days for PBS-treated mice, with an increase of 8 days. In males, the median survival was 140.5 days for the CDNF-treated mice and 132 days for the PBS-treated mice, with an increase of 8.5 days (**Fig. 4B-C**). Along with this, their balance and motor behavior performance were significantly ameliorated by CDNF treatment. In the accelerating rotarod, CDNF-treated female and male mice showed an increased latency to fall compared to vehicle-treated mice. No statistical differences were found in CDNF or vehicle-injected WT littermates over time (**Fig. 4D-E**). One week after CDNF or PBS injection, the CDNF group showed an increased ability to run on the smallest 8 mm rod in the multiple static rods experimental paradigm for females and on the 21 and 11 mm rods for males (**Fig. 4F-G**), compared to the PBS group. These sex differences in the experimental tasks are in accordance with previous observations that males develop a major

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disease phenotype approximately one week earlier than female littermates.[42,43] In the open field, SOD1-G93A mice exhibited fewer rearings compared to WT mice, which is associated with less strength in the hind limb muscles, and CDNF treatment increased the number of rearings compared to PBS controls at 16 weeks (**Fig. 4H**). Moreover, immunohistochemical analyses of the lumbar spinal cord revealed a significantly higher number of MNs present in the CDNF-treated compared to PBS-treated mice, which correlated with the behavioral improvement (**Fig. 4I and Supplementary Fig. 6**).

CDNF increases survival of SOD1-G93A embryonic motor neurons by regulating all three UPR pathways

We further investigated whether CDNF modulates ER stress and survival of SOD1-G93A-derived embryonic MNs *in vitro* and whether its effect is limited to only one branch of the UPR or is extended to all three as in the TDP43-M337V expressing MNs. MNs from E13 WT and SOD1-G93A mouse embryos, which depend on trophic support for their survival *in vitro*, were cultured either in the presence of BDNF or CNTF or only with CDNF. Interestingly, CDNF alone had a significant effect on the survival of the MNs as compared to the untreated conditions, though not to the same degree as observed with the combination of BDNF and CNTF **(Fig. 5A)**. No difference in the morphology of cultured MNs was observed between any of the aforementioned culture conditions (**Supplementary Fig. 7**). SOD1-G93A mutant MNs were obtained from the same SOD1-G93A mouse model used in the *in vivo* study, which develops first signs of a pathological phenotype at about three months after birth. However, when ER stress was induced in cultured MNs by TP treatment, these embryonic SOD1-G93A mutant MNs appeared significantly more sensitive compared to MNs from WT littermates, supporting previous observations made with this mouse model.[10] This sensitivity was seen despite the continuous presence of BDNF and CNTF in culture, but when CDNF was added to ER stressed SOD1-G93A mutant MNs, survival was rescued to the levels observed in WT MNs (**Fig. 5B**). The protective effect of CDNF in SOD1-G93A MNs from TP-induced ER-stress occurred in a dose-dependent manner and at a concentration of 20 ng/ml or higher the survival of SOD1-G93A MNs was comparable to that of WT cells (**Fig. 5C**). Next, we analysed whether CDNF, when compared to BDNF/CNTF, can modulate the signaling pathways of the UPR in cultured MNs treated with either TP or TM. We observed a partial translocation of ATF6 protein from the

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cytoplasm to the nucleus of the neurons upon treatment with TP or TM. However, the addition of CDNF significantly reduced ATF6 translocation in SOD1-G93A treated with TP or TM (**Fig. 5D-E**). Furthermore, CDNF diminished the splicing levels of *xbp1s* transcripts observed after TP or TM treatment, particularly in SOD1-G93A MNs, suggesting an inhibition of the IRE1α-linked pathway (**Fig. 5F**). Additionally, CDNF treatment significantly decreased p-eIF2α and CHOP, activated downstream of the PERK signaling pathway, in TP and in TM treated SOD1-G93A MNs (**Fig. 5G-I, Supplementary Fig. 8**). Collectively, these results show that SOD1-G93A MNs are already at embryonic stage intrinsically more sensitive to ER stress and that CDNF efficiently counteracts the ER stress-induced cell death by reducing the activity of all three major UPR signaling pathways.

CDNF attenuates all three UPR branches initiated by PERK, IRE1α, and ATF6 *in vivo*

To investigate the mechanisms involved in the *in vivo* action of CDNF in the SOD1-G93A model, we analysed the UPR signaling pathways in lumbar MNs isolated via laser microdissection (**Fig. 6A**) at 13 and 17 weeks, i.e. 5 days after CDNF i.c.v. injections (**Fig. 6B-F**). RNA was isolated from micro-dissected neurons and the mRNA levels of UPR markers were analysed. Interestingly, at 13 weeks we only found an upregulation of *Atf4* and *Chop* transcripts in PBS-treated SOD1-G93A mice compared to PBS-treated WT littermates (significance marked with # in **Fig. 6B-F**). As both molecules are downstream effectors of the PERK pathway, which blocks general protein translation initiation in stressed cells, these data suggest that this branch of UPR signaling is the first to be activated in lumbar MNs of SOD1-G93A mice. Notably, upon CDNF treatment, *Atf4* mRNA was decreased to WT levels (**Fig. 6C**). In accordance with previous results,[10,11,44] at 17 weeks of age the PERK, IRE-1α, and ATF6 pathways were all upregulated in SOD1-G93A mice compared to WT littermates, and in particular the mRNA levels of *Chop*, which has a prominent role in apoptosis, increased more than 4-fold. CDNF treatment was able to attenuate markers from all three UPR branches (**Fig. 6B-F)**. Furthermore, fluorescence staining revealed that the levels of the ER chaperone GRP78 and the phosphorylation of eIF2α were increased in lumbar MNs of 17 weeks old SOD1-G93A mice, but these were efficiently reduced by CDNF administration, resulting in expression levels comparable to WT mice (**Fig. 6G-J**). Finally, we investigated whether CDNF treatment would

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affect the levels of mutant SOD1 protein and its clearance using its conformation-specific antibody B8H10 (MM070, Medimabs, Canada): a trend in the reduction of mutant SOD1 protein in CDNF-treated mice was found at 17 weeks (**Supplementary Fig. 9A-B)**. We did not find any difference in mutated SOD1 between CDNF and PBS-treated mice at the transcriptional level (**Supplementary Fig. 9C)**.

Endogenous CDNF levels change with disease progression in SOD1-G93A mice

We next evaluated the endogenous levels of CDNF in non-treated WT and SOD1-G93A mice at different disease stages, from the pre-pathological stage of 1 month to the endpoint of approximately 5 months, collected lumbar spinal cord, motor cortex, and skeletal gastrocnemius muscle samples and analysed protein levels by ELISA and mRNA levels by qPCR. We found that levels of CDNF change during SOD1-G93A lifespan. CDNF was upregulated before the appearance of a disease phenotype in the spinal cord and later in the motor cortex at the mRNA level. On the contrary, CDNF protein levels in skeletal muscle were significantly decreased at the onset of the disease phenotype stage and onwards (2, 3 and 5 months of age, **Supplementary Fig. 10**), which might correlate with reduced activity of paralyzed muscles.

CDNF reduces the UPR response in a slow progressive TDP43-M337V mouse model before disease onset

Since enhanced sensitivity to ER stress in SOD1-G93A MNs is detectable starting from embryonic stages **(Fig. 5B)** and long before the appearance of first impairment signs in the corresponding mouse model **(Fig. 4)**, we sought to explore the protective effect of CDNF in a third and new model of amyotrophic lateral sclerosis, which displayed a slow progressive development of the disease. In this newly generated transgenic mouse model, the murine Tdp43-M337V coding sequence N-terminally tagged to HA was expressed under the control of the human Ubiquitin C promoter (**Supplementary Fig. 11**). Staining with an HA antibody confirmed the expression of the transgene in every cell type within the spinal cord, including MNs, as shown by co-staining against ChAT (**Supplementary Fig. 11A-B**). We did not detect any TDP43 positive inclusions in these mice nor c-terminal cleavage products (**Supplementary Fig. 11B, F**). Transgenic animals were born without any obvious phenotype, but showed a

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progressive, late-onset MNs loss, as determined by Nissl staining of lumbar spinal cord sections (**Supplementary Fig. 11C-D**). On the transcriptional level, we did not detect any silencing of the endogenous TDP43 (**Supplementary Fig. 11E-F**), most likely due to the mild expression of the transgene (**Supplementary Fig. 11E-F**). Aged mice showed a remodeling towards reduced number of motor units, as demonstrated by elevated amplitude of single motor unit potentials (**Supplementary Fig. 11G**). When challenged on a rocking and accelerating rotarod, we also observed an impaired motor performance in aged transgenic mice (**Supplementary Fig. 11H**). In contrast to previous reported TDP43 models with overexpression of the transgene,[45,46] this newly generated mouse model showed a moderate expression of the transgene, as shown by Western blot analysis (**Sup. Fig. 11E-F**). This is in agreement with two recent studies, describing the phenotype of new mouse models in which the mutation in the TDP43 gene was introduced into the endogenous locus and where only a mild motor phenotype with late onset was observed.[47,48] Signs of an enhanced ER stress were present long before an altered behavior was detected in this mouse model (**Fig. 7A**). To test the effect of CDNF, TDP43-M337V mice received a single i.c.v. injection of 10 µg of CDNF at 6 weeks of age and the ER stress response was analysed at 6 months of age in the spinal cord and the motor cortex of the mice (**Fig. 7B**). An exacerbation of the UPR response was found in the transgenic mice compared to the WT littermates. As expected, this upregulation was not as strong as the one detected in the SOD1-G93A at late-stage. However, treatment with CDNF had a similar effect in reducing the ER stress response, with a significant decrease in *Chop* and *Xbp1t* mRNA and an equivalent trend in most of the other genes (**Fig. 7C-H**). Moreover, the mRNA levels of the UPR markers were downregulated in the full lysate of motor cortex upon treatment with CDNF (**Fig. 7I**). Finally, we wanted to evaluate whether CDNF can improve the motor behavior and the number of surviving lumbar MNs in aged TDP43-M337V mice. Therefore, we injected 10 µg of CDNF or vehicle i.c.v. to 18 months old TDP43-M337V and age-matched WT controls. The mice were followed for 5 weeks, and weight changes and motor performance were analysed weekly (experimental timeline in **Fig. 8A**). While no significance difference in weight changes was observed (**Supplementary Fig. 12**), TDP43-M337V mice were performing worse on the rotarod compared to WT mice when challenged by rocking and reverse acceleration (**Fig. 8B-E**). CDNF-treated female TDP43-M337V mice were significantly performing better at 2, 3 and 4 weeks upon the drug administration, while an amelioration of the motor behavior in male TDP43-M337V mice was

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observed at 2 and 5 weeks after injection (**Fig. 8D-E**). No difference in WT mice was found upon CDNF or PBS treatment (**Fig. 8B-C**). It should be noticed that male mice performed overall worse than female mice due to their higher weight (**Fig. 8B-E**). The mice were sacrificed 5 weeks after CDNF/vehicle injection and the number of lumbar MNs was assessed. We observed a reduced number of MNs in the spinal cord of TDP43-M337V mice compared to WT mice, but CDNF administration increased the number of surviving MNs (**Fig. 8F-G**). These results are in line with the effects observed in the TDP43-M337V rat and in the SOD1-G93A mouse and demonstrate that CDNF improves MNs survival and actively suppresses ER stress in amyotrophic lateral sclerosis preclinical models independently of the disease etiology.

Discussion

Amyotrophic lateral sclerosis is a complex and multifactorial disease.[49,50] Several studies have indicated that ER stress plays a pivotal role in the pathophysiology of the disease,[3-19] thus it became an interesting target for therapeutic intervention. In this study, we explored the role of CDNF as a modulator of the ER stress response in amyotrophic lateral sclerosis and show that CDNF is the first compound that can attenuate all the three UPR pathways activated by IRE1α, PERK, and ATF6 in MNs *in vitro* and *in vivo*. Moreover, we present evidence that the activity of IRE1a and PERK pathways are required for the antiapoptotic effect of CDNF in MNs. This is remarkable, as previous compounds have been shown to interfere only with the PERK/p-eIF2α pathway, in particular inhibiting the de-phosphorylation of eIF2α[10,20,21] or by suppressing the activity of the dual leucine zipper kinase (DLK).[51] Small molecule allosteric inhibitors for IRE1α oligomerization, KIRAs, have also been described: KIRA6 is a specific IRE-1α kinase inhibitor, whereas KIRA8 blocks IRE-1α RNase activity. They have been shown to reduce beta-cell ER stress and death, and thus ameliorate diabetes in mouse models.[52,53] We have previously discussed that all three pathways are chronically active in amyotrophic lateral sclerosis and that the UPR is a finely intertwined network. Thus, targeting only one of the pathways is not sufficient and limits the efficacy of these compounds, whereas CDNF has a broader therapeutic scope than any single pathway-specific inhibitor. Furthermore, it has also been reported that persistent translation inhibition by p-eIF2α, which is accentuated by drugs as salubrinal, mediates neurodegeneration in a model of prion disease, which is another protein misfolding

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disorder that shares several pathogenic mechanisms with amyotrophic lateral sclerosis.[54] Treatment with guanabenz, which was shown to be protective in female SOD1-G93A mice by Jiang *et al.*,[21] led to an opposite effect in another study, with an exacerbation of the disease phenotype especially in male mice.[55] These results suggest that guanabenz, especially if administrated systemically, may have a detrimental effect because of a sustained block of protein translation, acceleration of apoptosis, and also because of possible side effects due to the activity of the drug as an agonist of the α2-adrenergic receptor.[55] On the other hand, a complete restoration of protein translation by PERK inhibitor GSK2606414, albeit being neuroprotective in a prion model, was found to be highly toxic for pancreatic cells, where UPR activation is essential for physiological activity.[56] These reports suggest the need for a drug candidate with the ability to attenuate the whole UPR network in affected neurons, without side effects. Interestingly, we found that CDNF has very little effect *in vitro* on healthy MNs but has a strong protective effect on injured and ER stressed-MNs. Broad toxicity assays for CDNF have been already carried out in non-human primates and CDNF was successfully tested in phase I-II clinical trial in Parkinson's disease patients.[57] Importantly, the long-lasting downregulation of UPR markers in the SOD1-G93A and TDP43 mouse models by CDNF, even after a single injection of CDNF, indicates that the protective effect is maintained for a long time period, highlighting its potential therapeutic value.

Chronically activated UPR pathways eventually lead to neuronal dysfunction and finally to CHOP-mediated cell death.[58] CDNF significantly reduced the levels of CHOP, and we postulate that CDNF inhibits CHOP-mediated apoptosis, therefore enhancing MNs survival. CDNF was also shown to activate the PI3K-Akt pathway in dopamine neurons.[34] In addition to the regulation of ER stress and UPR and anti-apoptotic activity, CDNF may have some other interesting properties. Analyzing the phenotype of CDNF-deficient mice we found that an age-dependent loss of enteric neurons occurs selectively in the submucosal but not in the myenteric plexus. This neuronal loss is a consequence not of increased apoptosis but of neurodegeneration and autophagy, suggesting a role for CDNF in the regulation of autophagy.[59] Autophagy has lately emerged as an essential protective mechanism during ER stress. These two organelle systems are dynamically interconnected, and several studies have established that ER stress can either stimulate or inhibit autophagy. In fact, the signaling pathways mediated via IRE1α, PERK, ATF6, and Ca2+ are necessary for the activation of ER stress-mediated autophagy.[60,61]

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Moreover, our data on reduced mutated SOD1-G93A at the protein, but not mRNA level, suggest that the decrease may be the effect of improved clearance of the mutated protein. Therefore, previous and current results do not exclude the possibility that CDNF may exert its protective effect through other pathways in addition to the regulation of the chronic ER stress response, such as autophagy, activation of pro-survival pathways, or preventing the synthesis and release of pro-inflammatory cytokines.[62] These aspects should be further explored also in the context of MNs and amyotrophic lateral sclerosis disease.

The direct mechanism of action of CDNF in reducing the ER stress response is still under investigation. We have previously shown that CDNF can interact with the main ER chaperon GRP78.[28] In the current study we have shown that IRE1a and PERK inhibitors block CDNF ability to promote the survival of MNs, but do not inhibit the action of BDNF or CNTF. These data strongly suggest that the activity of PERK and IRE1α pathways is essential for the survival-promoting activity of CDNF. Whether CDNF directly interacts with PERK and IRE1a or this interaction is facilitated by GRP78 is still unclear. It was recently discovered that MANF reduces the UPR signaling by decreasing IRE1α oligomerization and IRE1α phosphorylation. MANF also interacts with other UPR receptors PERK and ATF6, but with lower affinity.[63] In the light of these results, we favor the hypothesis that CDNF may directly interact with the UPR sensors PERK and IRE1a. These problems require, however, a thorough independent investigation.

Due to the genetically variegated and multifactorial nature of amyotrophic lateral sclerosis, creating an animal model that would properly mimic all the pathophysiological events involved in the development and progression of the disease has revealed as a complex task.[64] Therefore, being aware of the advantages and disadvantages of available pre-clinical model, we sought to investigate the therapeutic effect of CDNF and in three rodent models with different etiology and disease development, harboring mutations in either SOD1 or TDP43. Albeit their differences, all three models exhibit signs of ER stress and death of MNs. Accumulation of misfolded/unfolded proteins in the ER lumen is one determinant for the initiation of the ER stress; however, ER stress, and consequent UPR, is induced by a plethora of other stressors, including calcium buffering, inflammation, proteostasis impairment, altered neuronal activity, mitochondrial dysfunction, aging, etc. Notably, ER stress can be triggered by depletion of ER calcium, and impaired calcium homeostasis has been well documented in most familial forms of amyotrophic lateral sclerosis.[65-67] Mutated SOD1-G93A and TDP43, albeit being mostly found in the

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cytoplasm, interfere with some of these processes. As an example, mutant SOD1 protein has been shown to be a strong inducer of the ubiquitin-proteasome system (UPS).[12,68] In addition, TDP43 accumulation within the rough ER membrane has been documented in amyotrophic lateral sclerosis patients.[6]

To implement our study on CDNF's role in the regulation of the UPR response, we used embryonic cultured MNs harboring SOD1-G93A and TDP43-M337V mutations. As there was only a modest UPR activation in untreated SOD1-G93A and TDP43-M337V MNs, we induced ER stress pharmacologically using either TP or TM. The modest UPR activation can be explained with the fact that cultured MNs are derived from embryonic mice and not adult mice. Albeit ER stress signs have been found in SOD1-G93A mice[10] and TDP43-M337V mice (**Fig. 7**) at a pre-symptomatic stage, these data refer to adult mice. We reckon that it will take time for the mutations to cause a chronic ER stress response, and therefore the UPR response is low at the embryonic level. Moreover, MNs cultures are highly enriched and do not contain activated microglia or immune cells, which provide pro-apoptotic stimuli and usually contribute to the pathophysiology of neurodegeneration. The reduction in the UPR response in isolated MNs demonstrate the direct effect of CDNF on MNs and exclude the possibility of indirect effect that are mediated through other cell types.

Administration of CDNF via the i.c.v. route attenuated the ER stress response, increased the number of surviving MNs and improved the motor behavior in the mouse SOD1-G93A and in both the mouse and rat TDP43-M337V models, irrespective of the disease etiology. We believe that this enhances the predictive value that CDNF could also be beneficial in patients with amyotrophic lateral sclerosis. To the best of our knowledge, no previous drug candidate has been reported to have a protective effect in this severe and fast progressive TDP43 rat model. In a recent report, it was shown that riluzole had no therapeutic efficacy on the behavioral deficits, nor on the neuropathological features in this model.[69] Furthermore, CDNF showed a beneficial effect in the SOD1-G93A mice when treated at 3 months of age, i.e., after disease signs onset. This is a clinically relevant time point for initiation of therapy in patients, as usually they are not diagnosed before disease onset. SOD1-G93A mice treated daily with riluzole in drinking water, starting approximately 40 days before the onset of a disease phenotype, showed improved survival by 13 days,[70] whereas no increase in survival was observed after edaravone intraperitoneal administration.[71] Additionally, the evidence from our novel TDP43-M337V

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mouse model that ER stress is already present before the appearance of a pathological phenotype suggests that CDNF is also a strong candidate for the prevention of disease initiation in amyotrophic lateral sclerosis cases with familial monogenic origin.

Finally, in this study we demonstrate that endogenous levels of CDNF change during disease progression, being upregulated at pre-disease stages and decreasing at later stages. In particular, the decreased levels of CDNF in muscle compared to WT might correlate with paralysis and reduced activity of non-functional muscle. Furthermore, the decrease in the endogenous levels of CDNF with disease progression suggests that the administration of exogenous protein at this stage should have a therapeutic effect, which is supported by the results described here.

In conclusion, CDNF rescues MNs *in vitro* and *in vivo* by regulating the ER stress response, a key player in the pathophysiology of amyotrophic lateral sclerosis. Hence, we propose that CDNF is a promising ER stress regulator for injured MNs that warrants further study as a drug candidate in amyotrophic lateral sclerosis.

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Competing interests

Päivi Lindholm, Mart Saarma and Merja H Voutilainen are inventors of the CDNF-patent, which is owned by Herantis Pharma Plc. M Saarma is a shareholder of Herantis Pharma Plc.

Supplementary material

Supplementary material is available at *Brain* online.

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Figure legends

Figure 1 Continuous i.c.v infusion of CDNF improves motor behavior and protects spinal MNs in the ChAT-tTA/TRE-TDP43-M337V rat model. (**A**) Experimental design: upon reaching adult age, Alzet minipumps connected to a catheter were implanted in ChAT-tTA/TRE-TDP43-M337V and tTA rats to infuse CDNF (6 µg/day) or PBS. One week later, the activation of transgene was induced by partial withdrawal of Dox. Rats were monitored for weight and motor behavior changes until day 21 from transgene induction, when the rats were perfused. **(B)** Latency to fall of PBS/CDNF-treated transgenic and tTA littermates recorded three times per week. BL=baseline. (**C**) Quantification and comparison of the number of MNs in the lumbar (L) spinal cord, area L3-L5. (**D**) Representative images of Nissl and $ChAT^+$ MNs in the lumbar spinal cord. Scale bar 500 µm. Mean ± SEM, *n*=4-9/group in **B-D**. **P*<0.05, ***P*<0.01, *****P*<0.0001, repeated measures ANOVA followed by Tukey post-hoc test in **B** and one-way ANOVA followed by Tukey post-hoc test in **C**.

Figure 2 CDNF treatment decreases UPR markers expression in TDP43-M337V expressing MNs. (A) Effect of CDNF alone or in combination with different concentrations of 4µ8C, KIRA6 or GSK2606414 on MNs survival after 24h of thapsigargin (5 nM) treatment. Mean ± SEM of at least 3 different experiments. (**B**) Percentage of MNs survival 5 days after treatment with different concentrations of IRE1 inhibitors 4µ8C and KIRA6 and PERK inhibitor GSK2606414. The effect of the inhibitors was evaluated either in presence or absence of trophic factor CNTF and BDNF. Mean ± SEM of 3 different experiments (**C**) Effect of CDNF on the clustering of IRE1-3FH5GFP in MNs treated with TP. (**D**) Effect of CDNF on ATF6 translocation from cytoplasm to nucleus in MNs treated with TP. (**E**) Quantification of the number of IRE1-3FH5GFP per cell in **C**. (**F**) Quantification of the percentage of cells with IRE1-3FH5GFP clusters in **C**. Mean ± SEM of 5 different experiments in **C, E** & **F**. At least 15 cells per experiment were analysed. (**G**) Quantification of **D**. Mean ± SEM of at least 45 cells from

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three different experiment. (**H-J**) Protein expression of UPR markers phosphorylated eIF2α and CHOP in the MNs expressing TDP43 WT and TDP43-M337V, which were previously stressed with TP and TM. (**K**) Representative blots of p-eIF2α and CHOP. Mean ± SEM of 5 different experiments in **H-J**. **P*<0.05, ***P*<0.01, ****P*<0.001; one way ANOVA followed by Tukey post-hoc test in **A-B**; two-way ANOVA followed by Sidak post-hoc test in **E-J.**

Figure 3 CDNF administration attenuates the expression of UPR markers in the spinal cord of ChAT-tTA/TRE-TDP43-M337V rats at 21 days after transgene activation. (**A**) Representative fluorescence images of ChAT (Green) and GRP78 (Red) protein expression in PBS/CDNF-treated ChAT-tTA/TRE-TDP43-M337V compared to PBS/CDNF-treated tTA rats. Scale bar 50 µm. (**B**) Quantification of the pixel value of GRP78 in the lumbar ventral horn area. (**C**) Quantification of the pixel value of GRP78 in the $ChAT^+$ MNs. Mean ± SEM, *n*=5-9/group. (**D**) Representative fluorescence images of ChAT (Green) and p-eIF2α (Red) protein expression in PBS/CDNF-treated ChAT-tTA/TRE-TDP43-M337V compared to PBS/CDNF-treated tTA rats. Scale bar 50 µm. (**E**) Quantification of the pixel value of p-eIF2α in the lumbar ventral horn area. (**F**) Quantification of the pixel value of p-eIF2α in the $ChAT^+$ MNs. Mean ± SEM, *n*=5-9/group; **P*<0.05, ****P*<0.001, *****P*<0.0001; one-way ANOVA followed by Tukey post-hoc test in **B**, **C**, **E** and **F**.

Figure 4 A single i.c.v. injection of CDNF halts disease progression, ameliorates motor behavior, and improves the survival of mice and spinal MNs in the SOD1-G93A mouse model. (**A**) SOD1 mice with early disease signs and WT littermates were injected with 10 ug of CDNF or vehicle at 13 weeks of age. Animals were monitored twice per week for weight changes, disease development, and motor performance. (**B-C)** Survival of SOD1-G93A mice upon a single injection of CDNF or PBS treatment. The median survival time in females was 148 days for CDNF-treated mice and 140 days for PBS-treated mice. Therefore, there was an increase of 8 days. In the males, the median survival was 140.5 days for the CDNF-treated mice and 132 days for the PBS-treated mice. The increase was 8.5 days. (**D-E**) Latency to fall of CDNF/PBS treated-SOD1-G93A and WT littermates measured every week. (**F-G)** Travel time of CDNF or PBS-treated SOD1-G93A mice along suspended rods of decreasing diameters (27 to

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8 mm) one week after treatment. (**H)** Number of rearings of CDNF/PBS treated-SOD1-G93A and WT littermates in the open field paradigm measured at 1 and 3 weeks after injection. (**I**) Quantification and comparison of the number of MNs in the lumbar spinal cord after CDNF or vehicle treatment. Mean ± SEM, *n*=10-12/group in **B-C**, *n*=18-20 for females, *n*=14-15 for males, *n*=20 for WT in **D-G**, *n*=11/group in **H**, *n*=3 for WT and *n*=5 for SOD1 in **I**. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, log-rank test in **B-C**, repeated measures ANOVA followed by Tukey post-hoc test in **D-E** and **H**; unpaired t-test in **F-G;** one-way ANOVA followed by Tukey post-hoc in **I**.

Figure 5 CDNF protein administration increases the survival of SOD1-G93A mouse-derived embryonic MNs and reduces the expression of UPR markers after toxin treatment. (**A**) Survival of WT and SOD1-G93A embryonic MNs upon CDNF or CNTF and BDNF treatment. (**B**) Effect of CDNF administration on WT and SOD1 MNs survival upon 5 nM thapsigargin (TP) treatment or no treatment. (**C**) Dose-response curve of CDNF administration upon TP treatment on WT and SOD1-G93A MNs survival. (**D)** Effect of CDNF on ATF6 translocation from cytoplasm to nucleus in MNs treated with TP or tunicamycin (TM). (**E**) Quantification of **D,** each dot representing the ratio of an individual cell. (**F**) mRNA expression of UPR markers *Xbp1s* in the MNs treated as in Figure **B**. (**G-H**) Expression levels of eIF2α phosphorylation and CHOP protein, respectively, in the MNs treated as in Figure **D**. (**I**) Representative blots for the quantification in **G** and **H**. Mean ± SEM of 3 independent experiments in **A**, **B**, **C** (18-33 cells) and **F**, 4 and 7 experiments in **G-I**. **P*<0.05, ***P*<0.01, ****P*<0.001, two-way ANOVA followed by Bonferroni post-hoc test in **B** and **F**, and Sidak´s post-hoc test in **C, E, G** and **H**. TG=transgenic.

Figure 6 A single i.c.v. injection of CDNF decreases the expression of UPR markers in the lumbar spinal cord of SOD1-G93A animals at 17 weeks. (**A**) Representative pictures of a lumbar spinal cord section before and after the MNs dissection and mRNA level of *ChAT*, marker of spinal cord MNs, in the putative MNs area and in a control region where no MNs should be present (marked with *). (**B-F**) mRNA expression of UPR markers *Atf6*, *Atf4*, *Chop*, *Xbp1t* and *Xbp1s* in microdissected lumbar MNs after CDNF/PBS injection in 13 and 17 weeks

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SOD1-G93A mice and WT littermates. (**G**) Expression of GRP78 protein in lumbar MNs after CDNF/PBS treatment in 17 weeks SOD1-G93A mice and WT littermates. (**H**) Quantification of **G**. (**I**) Expression of p-eIF2α protein in lumbar MNs after CDNF/PBS treatment in 17 weeks SOD1-G93A mice and WT littermates. (**J**) Quantification of **I**. Mean ± SEM, *n*=6-9/group in **B-F**, *n*=3-5/group in **G-J**. **P*<0.05, ***P*<0.01, *****P*<0.0001, $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, two-way ANOVA followed by Bonferroni post-hoc test in **B-F**, one-way ANOVA followed by Tukey post-hoc test in **H** and **J.**

Figure 7 CDNF treatment decreases UPR markers expression in a novel TDP43-M337V mouse model with ER stress pathology. (**A**) Schematic representation of the appearance of ER stress and MNs loss in the novel TDP43-M337V mouse model. (**B**) TDP43-M337V mice were injected at 6 weeks of age and UPR markers were analysed by qPCR at 6 months. (**C-H**) mRNA expression of UPR markers *Atf6, Atf4, Chop, Xbp1t, Xbp1s* and *Grp78* in micro-dissected lumbar MNs from 6 months TDP43-M337V mice treated with CDNF or vehicle at 6 weeks of age. (**I**) mRNA expression of UPR markers *Atf6, Chop, Xbp1s* and *Grp78* in total lysates of motor cortex from 6 months TDP43-M337V mice treated with CDNF or vehicle at 6 weeks of age; results are presented as fold change increase compared to naïve control. Mean ± SEM, *n*=5/6/group in **C-H**, *n*=5/group in **I**. **P*<0.05, ***P*<0.01; two-way ANOVA followed by Bonferroni post-hoc test in **C-H**; unpaired t-test in **I**.

Figure 8 CDNF rescues MNs and improve motor performance in 18 months old TDP43-M377V mice. (**A**) Experimental timeline: injected mice were monitored for weight changes and motor performance once per week. The mice were sacrificed 5 weeks after CDNF/PBS administration and the spinal cord was collected for immunohistochemistry. (**B-E**) Latency to fall in a rotarod test with rocking and reverse acceleration in WT females (**B**), WT males (**C**), TDP43-M337V females (**D**), and TDP43-M337V males (**E**). (**F**) Quantification of the number of Chat and $Nissl^{+}$ in the lumbar (L) spinal cord, area L3-L5, of WT and TDP43-M337V mice. (**G**) Representative images of Nissl and $ChAT^{+}$ MNs in the lumbar spinal cords. Scale bar 500 µm. Mean ± SEM, *n*=8/group for females, *n*=5-6/group for males, *n*=4-6/group for WT in **B-E**, *n*=13-14/group for TDP43-M337V mice and *n*=9-10 for WT mice in **F**. **P*<0.05, ***P*<0.01,

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***P<0.001, ****P<0.0001, Kruskal-Wallis test followed by Dunn's Multiple Comparisons test in **B**-**E**, one-way ANOVA followed by Tukey post-hoc test in **F.**

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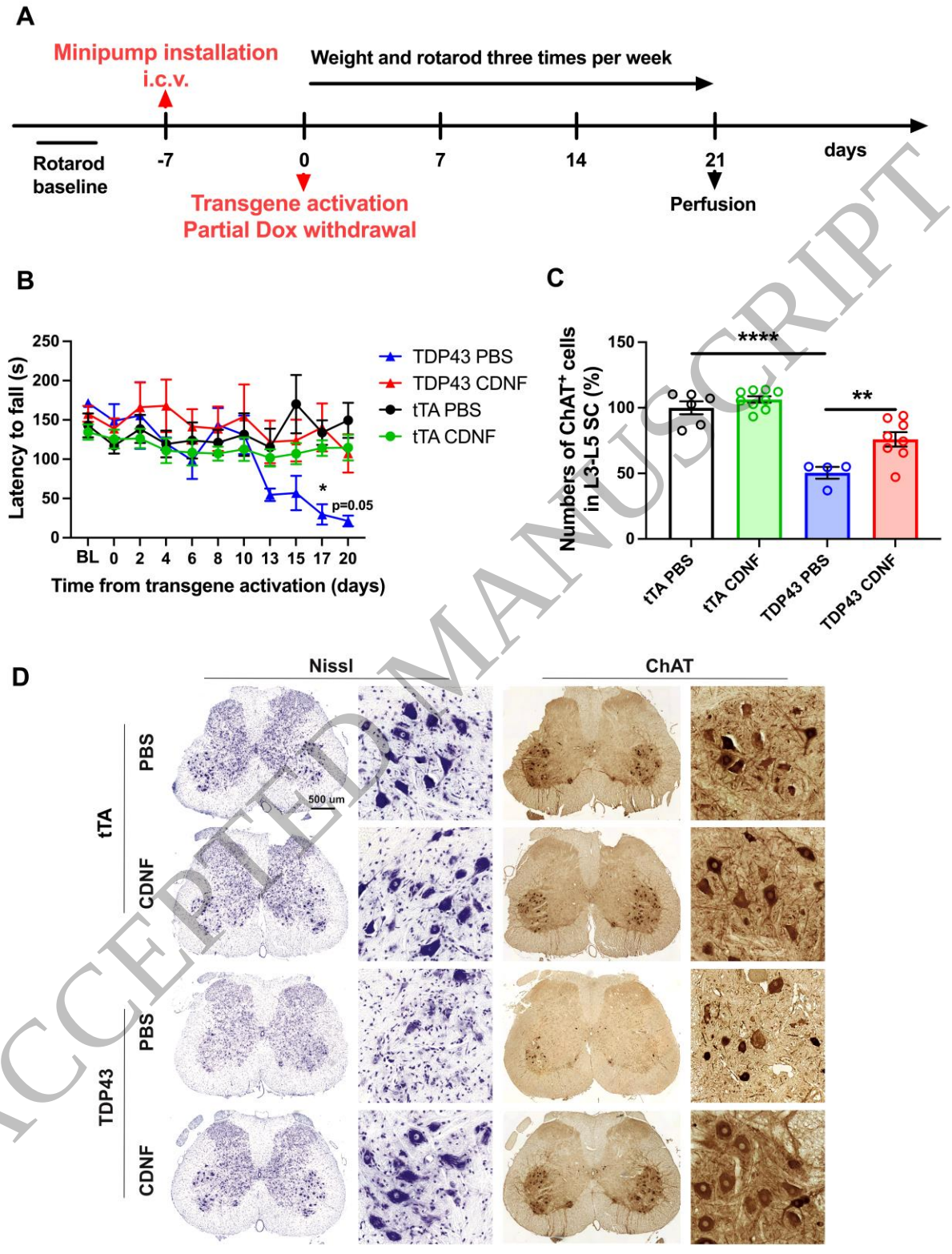

Figure 1
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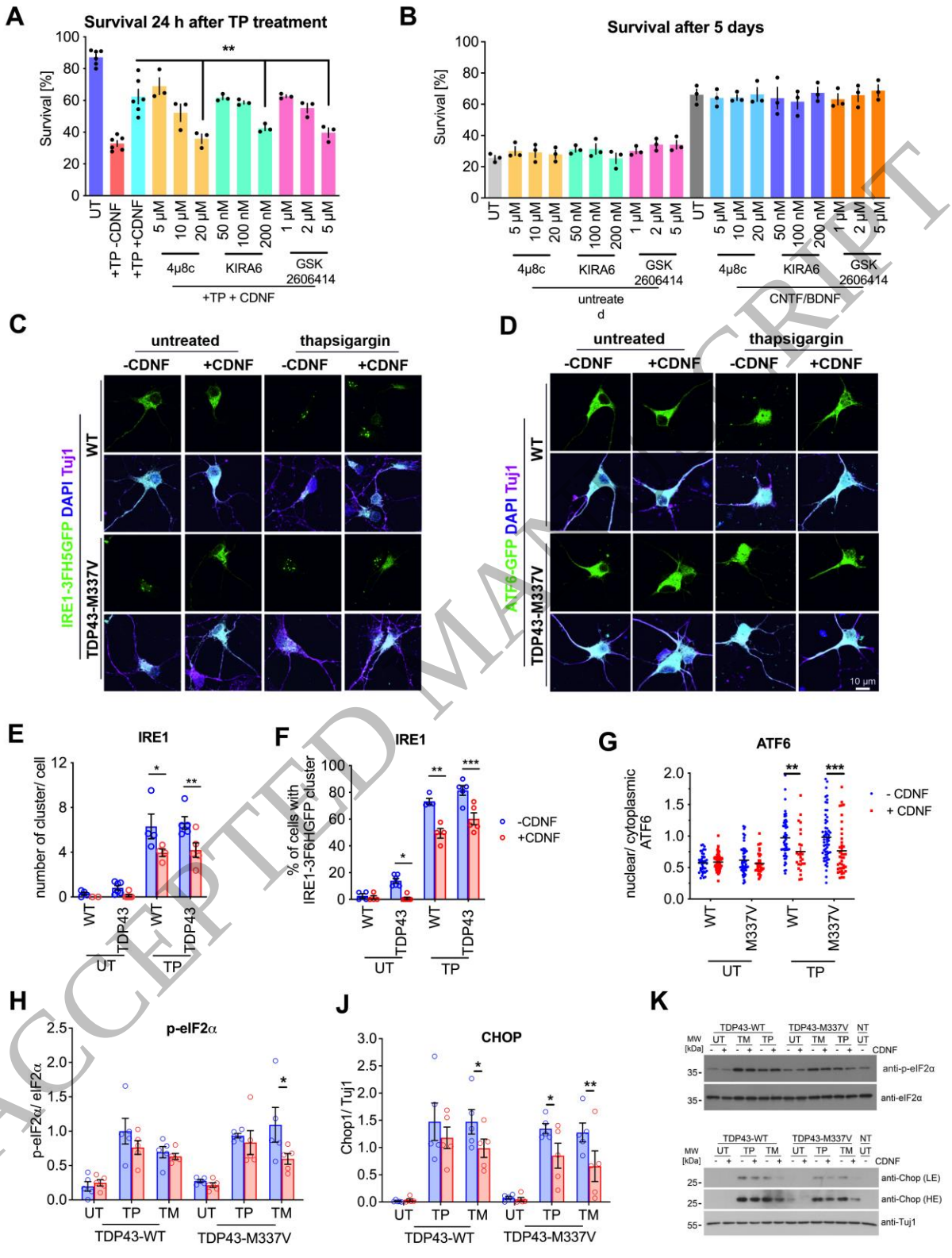

Figure 2
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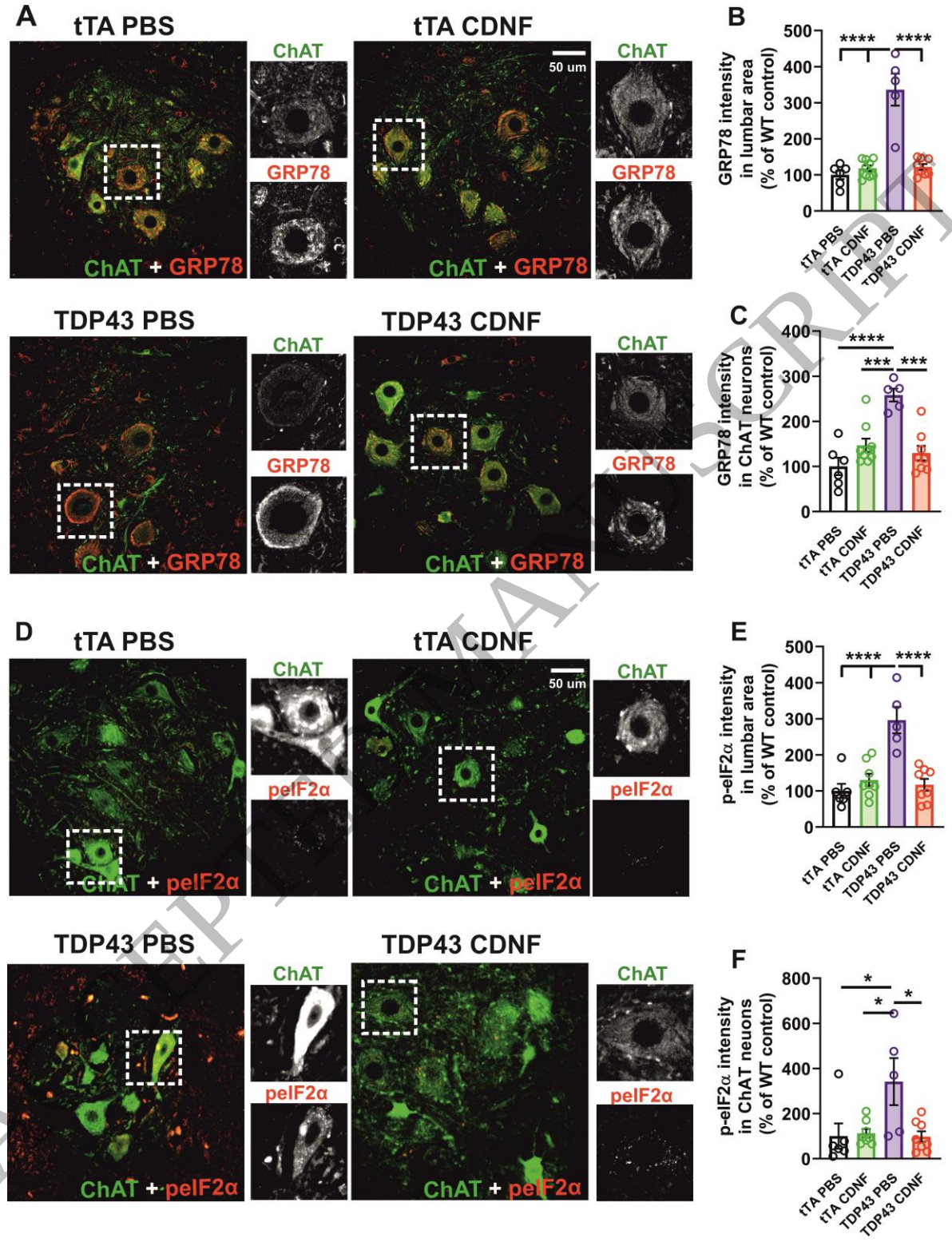

Figure 3
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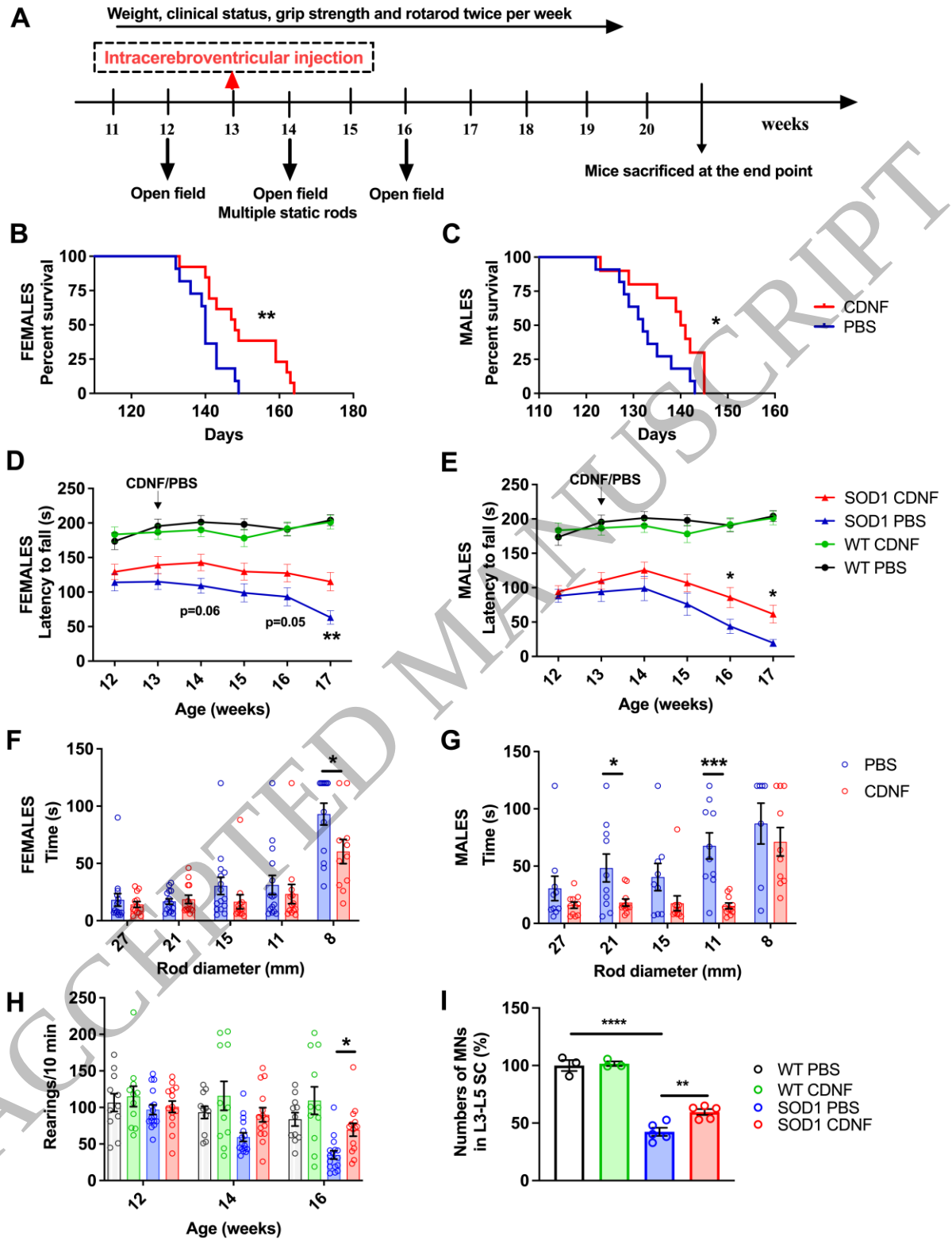

Figure 4
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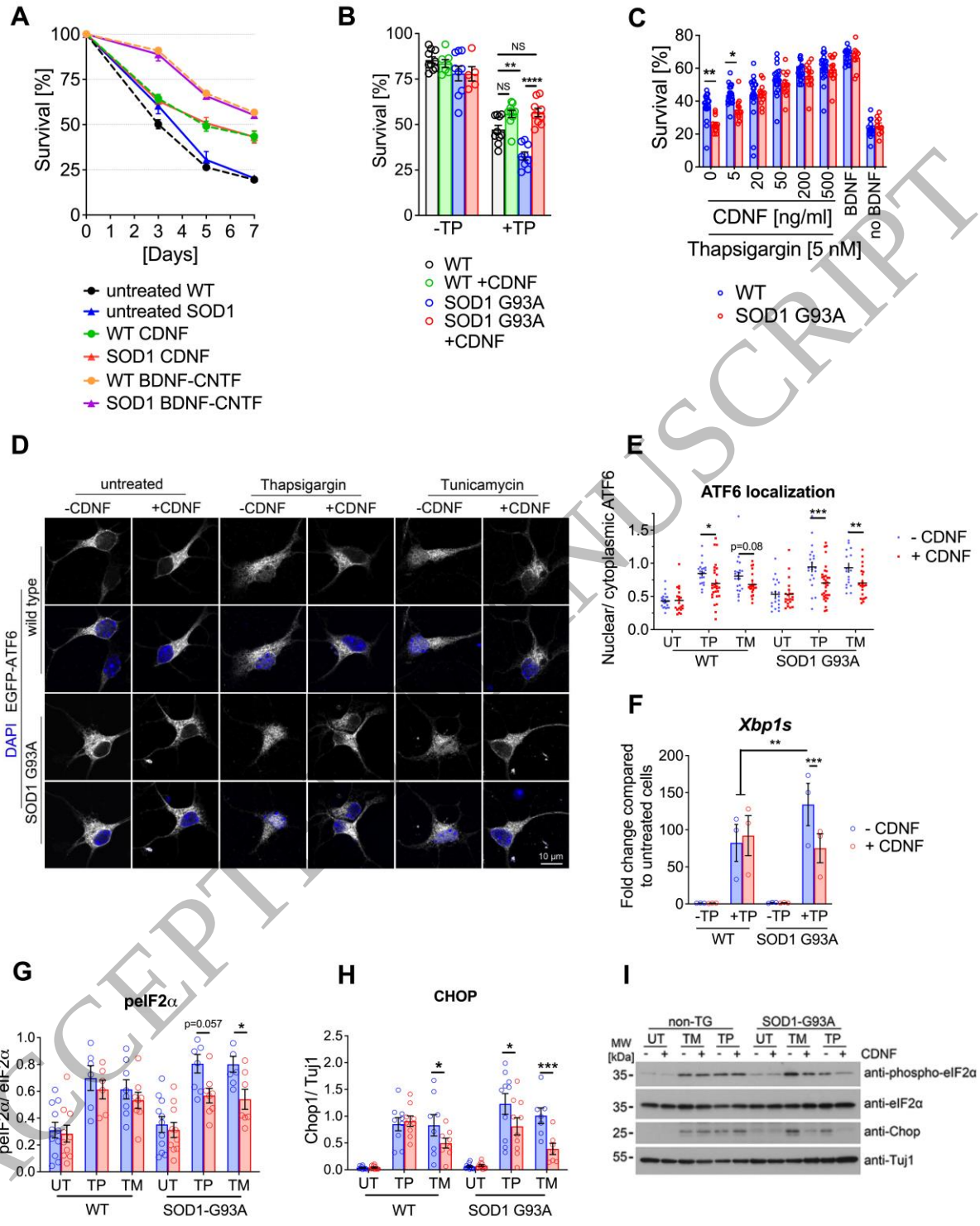

Figure 5
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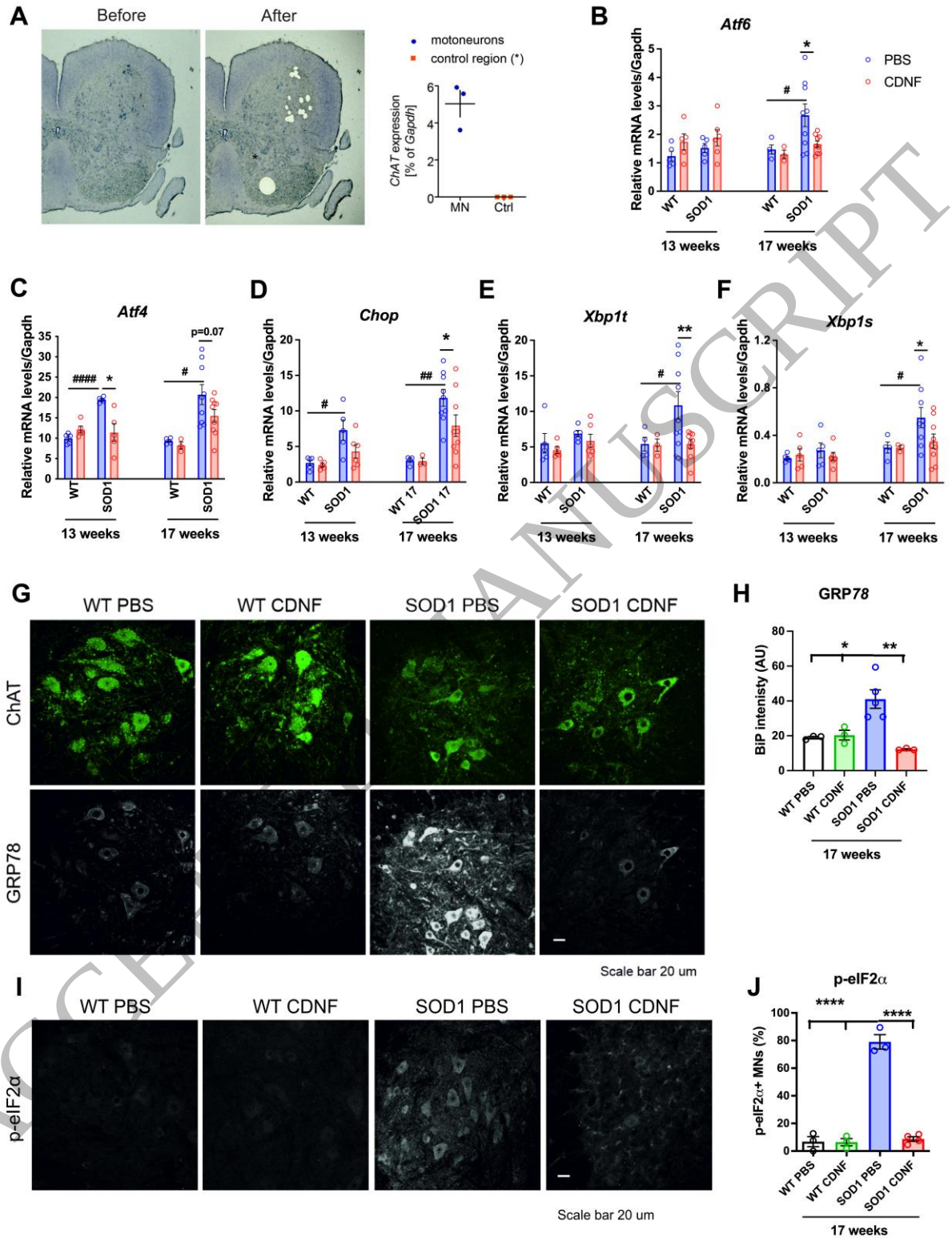

Figure 6
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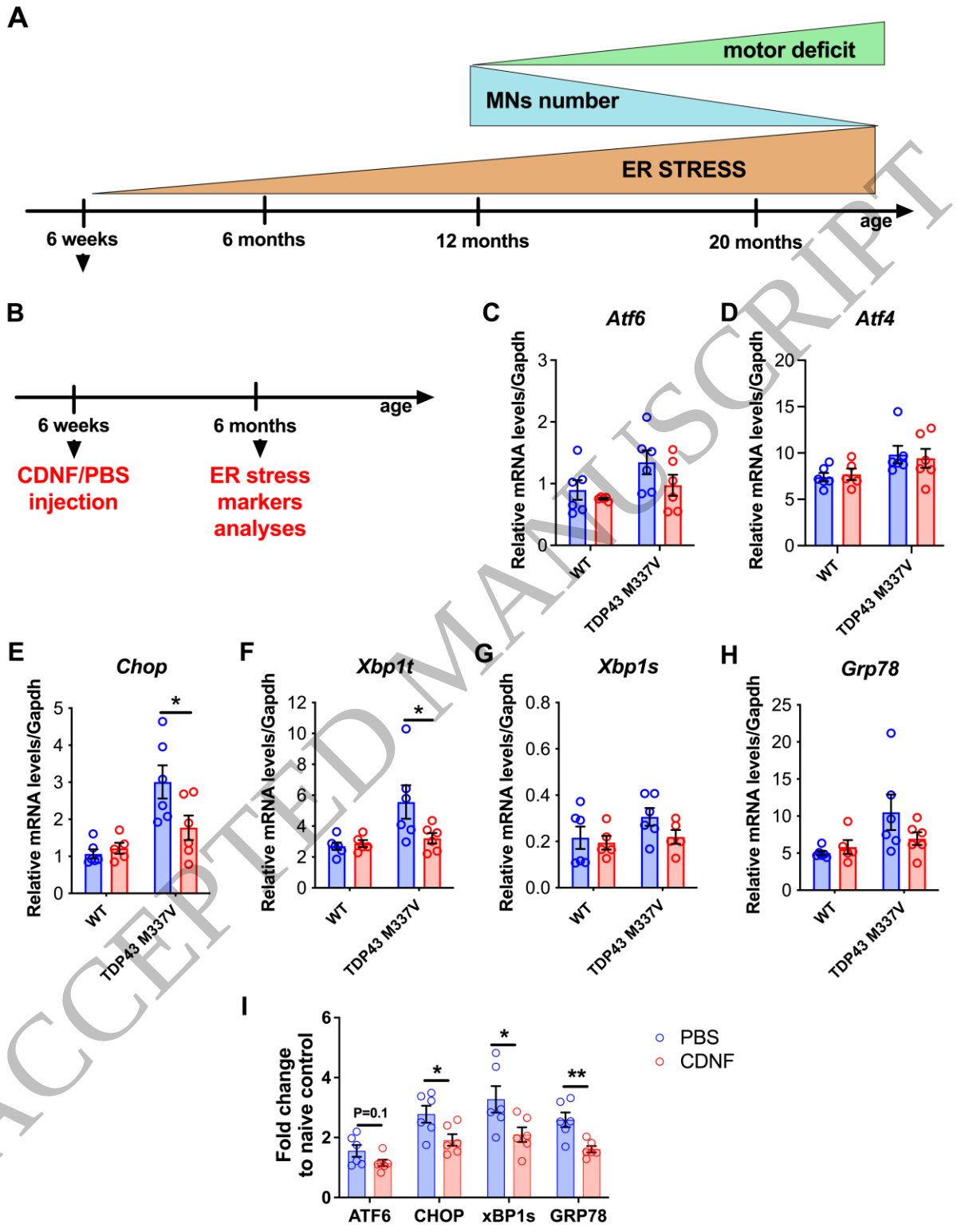

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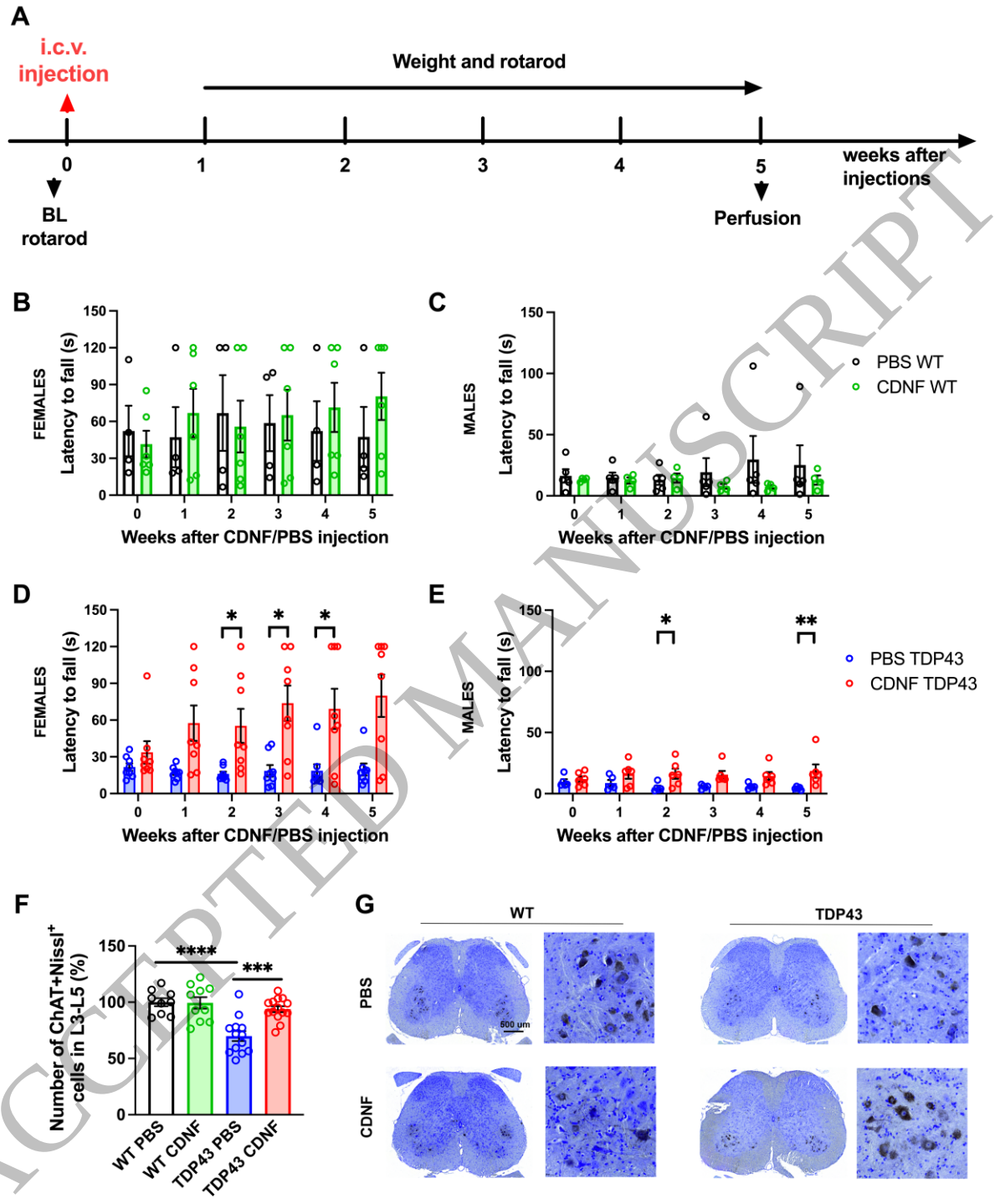

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