Sp1-regulated expression of p11 contributes to motor neuron degeneration by membrane insertion of TASK1

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Disruption in membrane excitability contributes to malfunction and differential vulnerability of specific neuronal subpopulations in a number of neurological diseases. The adaptor protein p11, and background potassium channel TASK1, have overlapping distributions in the CNS. Here, we report that the transcription factor Sp1 controls p11 expression, which impacts on excitability by hampering functional expression of TASK1. In the SOD1-G93A mouse model of ALS, Sp1-p11-TASK1 dysregulation contributes to increased excitability and vulnerability of motor neurons. Interference with either Sp1 or p11 is neuroprotective, delaying neuron loss and prolonging lifespan in this model. Nitrosative stress, a potential factor in human neurodegeneration, stimulated Sp1 expression and human p11 promoter activity, at least in part, through a Sp1-binding site. Disruption of Sp1 or p11 also has neuroprotective effects in a traumatic model of motor neuron degeneration. Together our work suggests the Sp1-p11-TASK1 pathway is a potential target for treatment of degeneration of motor neurons.
Despite their different clinical manifestations, neurodegenerative diseases share a multitude of common underlying mechanisms. Of special relevance is intrinsic membrane excitability (IME), determinant of neuronal responsiveness to incoming stimuli, which can condition specific neuronal subpopulations to become differentially vulnerable in neurodegenerative disorders1–4.

Both passive and active membrane properties are involved in establishing neuronal IME. Passive properties, such as resting membrane potential (Vm) and membrane resistance, are determined by the density of background ion channels opened at rest, whereas active electrical signaling mainly relies on the activity of voltage-gated and ligand-gated ion channels. IME alterations interact with other pathogenic mechanisms, such as nitrosative stress (RNS) and excitotoxicity, that contribute to neuronal degeneration in many human neuropathologies1–5. For instance, RNS, a major factor mediating excitotoxic neurodegeneration6–9, enhances IME by inhibiting background potassium (K+) currents10, thus depolarizing Vm and increasing membrane resistance. On the other hand, neuronal excitotoxicity arising from disruption of Ca\(^{2+}\) homeostasis by overstimulation of glutamate receptors (GluRs)7,11 might be amplified by an increased IME. Depolarization of Vm exacerbates Ca\(^{2+}\) influx into the neuron by promoting voltage-sensitive Ca\(^{2+}\) channels (VSCCs) opening and by relieving N-methyl-d-aspartate receptors from the magnesium blockage7,12,13. Ca\(^{2+}\) overload can in turn trigger RNS, closing a vicious cycle with many still-unknown partners and leading to neuronal degeneration and cell death.

The adaptor protein p11 (S100A10) could be a promiscuous candidate to fine-tune neuronal IME by regulating plasma membrane insertion of TASK1 (TWIK-related acid-sensitive K\(^+\) subunit)1,14, a member of the KCNK family of two-pore-domain K\(^+\) channels. This family primarily determines passive membrane properties in mammalian cells, which confers it potential therapeutic impact15,16. Expression patterns of p1117 and TASK118 are sensitive to incoming stimuli, which can condition specific neuronal subpopulations to become differentially vulnerable in neurodegenerative disorders1–4.

Results

p11 determines MN IME by TASK1. Co-immunoprecipitation studies supported a physical interaction between p11 and TASK1 in the nervous system (Fig. 1a). Subsequently, we examined by whole-cell patch-clamp the impact of p11–TASK1 on MNs IME (Fig. 1b). The strong reduction in mRNA\(_{p11}\), resulting from the application of a small interfering RNA against mRNA\(_{p11}\) (siRNA\(_{p11}\)), lowered IME of hypoglossal MNs (HMNs) from brainstem slices of rat pups (postnatal day 7; P7) (Fig. 1b–e). Whether siRNA\(_{p11}\)-related IME alterations were mediated by a regulatory effect on TASK channel function was addressed by modifying extracellular pH to characterize TASK-dependent changes in Vm and input resistance (R\(_{in}\))10. Alkalination of extracellular medium (pH 8.2) from a physiological pH (7.2) opens TASK channels, enhancing K\(^+\) efflux, which leads to Vm hyperpolarization, and reduction of R\(_{in}\) (Fig. 1f). On the contrary, an acidic solution (pH 6.2) retains K\(^+\) into the cell by closing TASK channels, which causes Vm depolarization and R\(_{in}\) increase (Fig. 1f). Therefore, the impact of pH on MN IME increases in parallel to the number of functional TASK channels at the plasma membrane. Suggestive of an increase in surface expression of TASK channels, siRNA\(_{p11}\) strengthened pH impact on HMNs IME (Fig. 1g). In contrast, dexamethasone, a corticosteroid that stimulates p11 promoter activity, upregulated p11 at the hypoglossal nucleus (HN), increased IME, and reduced pH influence on HMNs IME (Supplementary Fig. 1).

To evaluate whether p11 stabilizes IME through TASK1, further experiments were performed in primary cultures of embryonic spinal cord MNs (SMNs). In our experimental conditions, IME of wild-type SMNs (SMNs\(^{wt}\)) changed over time in culture (Fig. 1h). Thus, whereas Vm and R\(_{in}\) remained steady throughout the first 3–4 days in vitro (DIV), IME of SMNs\(^{wt}\) progressively declined over the 4–6 DIV interval, and subsequently remained stable up to 8 DIV, the last time point tested (Fig. 1h). These outcomes are consistent with a progressive increase in surface expression of functional background K\(^+\) channels at the 4–6 DIV interval. Therefore, next experiments were carried out in SMNs\(^{wt}\) at 3–4 DIV (Fig. 1i), a time window over which functional expression of TASK is still low. Remarkably, p11 knockdown increased TASK1-like immunofluorescence in the neurites of SMNs\(^{wt}\) (Fig. 1i–k, Supplementary Fig. 2). Since siRNA\(_{p11}\) did not upregulate TASK1 protein (Fig. 1i), our results support that p11 regulates cell distribution rather than whole levels of TASK1 expression. Conclusively, while decline in p11 did not affect SMNs isolated from task1\(--/--\) embryos (SMNs\(_{task1--/--}\)), it lowered IME and increased TASK-dependent changes in SMNs\(^{wt}\) and SMNs\(_{task1--/--}\) (Fig. 1l, m). It was noteworthy that SMNs\(^{wt}\) either untreated or treated with a non-targeting siRNA (cRNA) were almost unresponsive to pH variations despite TASK1 expression (Fig. 1i,m, Supplementary Fig. 2), suggesting a low surface expression of pH-modulated channels at 3–4 DIV. Altogether, these findings support that physical interaction between p11 and TASK1 prevents functional expression of the channel at the plasma membrane, which in turn controls MN IME.

p11–TASK1 defines MN vulnerability to an excitotoxic insult. GluR overstimulation induces intracellular Ca\(^{2+}\) deregulation and Ca\(^{2+}\) homeostasis disruption that finally leads to cell death7,11. To evoke this harmfulness mechanism, SMNs were exposed to a toxic dose of glutamate (Fig. 2a, Supplementary Fig. 3a, b). Ca\(^{2+}\) imaging showed a glutamate-induced early rapid rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) in SMNs\(^{wt}\) which peaked and then progressively dropped to a lower plateau (Fig. 2a, Supplementary Fig. 3a, b). This plateau was transient in 90.4 ± 4.6% of cases (n = 164 SMNs\(^{wt}\)). Following this initial plateau, SMNs\(^{wt}\) generally experienced “Ca\(^{2+}\) deregulation”, a delayed and continued increase in [Ca\(^{2+}\)\(_i\)], that reached a mostly sustained and irreversible plateau (Fig. 2a, Supplementary Fig. 3a, b), indicative of impending neuronal death20,21. To characterize Ca\(^{2+}\) dynamics in SMNs, resting [Ca\(^{2+}\)\(_i\)]\(_0\), peak [Ca\(^{2+}\)\(_i\)], of the early rapid response to glutamate and time of Ca\(^{2+}\) deregulation were measured (Supplementary Fig. 3b). siRNA\(_{p11}\), strongly attenuated glutamate-evoked Ca\(^{2+}\) dynamics (Fig. 2a, Supplementary Fig. 3c–e), although did not alter the fraction of SMNs that underwent Ca\(^{2+}\) deregulation with regard to genotype (Supplementary Fig. 3f). Kaplan–Meier cumulative probability curves causally associated siRNA\(_{p11}\).
Fig. 1 p11 controls MN IME through TASK1, but not TASK3 subunits. **a** Co-immunoprecipitation (IP) of p11 and TASK1 in protein fractions of homogenized brain from an adult mouse. An anti-sheep IgG was a control. Elution 1 (E1) and 2 (E2) are displayed. **b** Left, experimental model for whole-cell patch clamp recordings from HMNs in brainstem slices. Inset, a micropipette (white arrow) close to the MN pool is showed. Right, voltage responses (top) to step depolarization and current responses (bottom) of two HMNs recorded from P7 rats receiving indicated oligonucleotides at P5. Dots, time points used to measure the recordings from HMNs in brainstem slices. Inset, a micropipette (white arrow) close to the MN pool is showed. **c** Co-immunoprecipitation (IP) of p11 and TASK1 in SMNswt by western blot. **d** mRNA levels in the brainstem at P7. *p<0.05, **p<0.01, ***p<0.001; ns, not significant; by Student t-test. **e** Changes in Vm and ε in SMNs in homogenate (E1) or recordings (E2) for 16 days in vitro (DIV) after seeding. **f** Schematic summarizing effects of extracellular pH on MN IME and TASK-mediated K⁺ currents. Int intracellular, Ext extracellular. **g** Changes in Vm and ε in SMNs in homogenate (E1) or recordings (E2) for 16 days in vitro (DIV) after seeding. **h** Time-course of Vm and Rm obtained from recorded SMNs at the indicated days-in vitro (DIV) after seeding. **i** Effect of siRNAp11 or cRNA (2 µM) on p11 and TASK1 in SMNs by western blot. **j** Confocal images showing that siRNAp11 (2 µM) leads to a drastic reduction in p11 immunolabelling (red) together with an increase in TASK1 expression (green) in neuritic processes of SMNs, as compared to cRNA (2 µM). Scale bar, 5 µm. **k** Box-plots of the mean (top) and integrated (bottom) membrane current during K⁺ channel opening in SMNs (α) in control (A) and TASK1 knock-out (B) mice, respectively. **l** Protein expression of TASK1 and α-actin in cRNA or siRNAp11-treated SMNs. **m** TASK-dependent changes in MN IME induced by variation of extracellular pH from 8.2 to 6.2.
treatment with a noticeable delay in the time of Ca$_{\text{2+}}$ deregulation (i.e., likely prolonging the cell lifespan) in SMNs$^{\text{wt}}$ and SMNs$^{\text{task3}^{-/-}}$ but, remarkably, not in SMNs$^{\text{task1}^{-/-}}$ (Fig. 2b). siRNA$_{p11}$-mediated attenuation of the intracellular Ca$_{\text{2+}}$ response to glutamate involved reduction of Ca$_{\text{2+}}$ entry through at least GluRs and VSCCs (Supplementary Note 1, Supplementary Fig. 3f–k).

Since MN IME is mainly determined by TASK channels (TASK1/3 heteromers, as well as TASK1/1 and TASK3/3 homodimers)$^{18,22}$, we first investigated their impact on SMNs...
Fig. 2 p11 controls Ca\(^{2+}\) signaling and vulnerability to an excitotoxic stimulus through TASK1, but not TASK3 subunits. a) Dimensional displays (left) and time course of [Ca\(^{2+}\)]\(\text{e}\), alterations (right) obtained from SMNs treated with cRNA or siRNA\(_{p11}\) (2 μM) from 1 DIV. Red horizontal lines indicate the glutamate (Glu, 150 μM) exposure interval. b) Log-Rank test, Kaplan–Meier analysis reported that siRNA\(_{p11}\) (dotted lines) delayed Ca\(^{2+}\) deregulation in SMNs\(_{wt}\) and SMNs\(_{task3}\) (p < 0.001), but not SMNs\(_{task1}\) (p = 0.761) relative to that in the cRNA condition. n = 36–49 SMNs. Ca\(^{2+}\) imaging studies were performed at 3–4 DIV. c) Schematic summarizing effects of listed drugs on TASK-mediated currents. d) Effects of bupivacaine (Bupiv; 40 μM, 60 min) and Glu (150 μM, 30 min), added alone or in combination, on SMNs\(_{wt}\) survival. e) Effects of isoflurane (Iso; 0.8 mM each 10 min for 70 min) and Glu, added alone or in combination, on SMNs\(_{wt}\), SMNs\(_{task1}\), and SMNs\(_{task3}\) survival. f) Influence of AEA (10 μM) on Vm of a SMNs\(_{wt}\) at 4–5 DIV. g) Changes in Vm (left) and R\(_{K}\) (right) of SMNs\(_{wt}\), SMNs\(_{task1}\), and SMNs\(_{task3}\) induced by AEA at the indicated concentrations. Number of SMNs in each group is in parentheses. h) Effects of AEA (10 μM, 60 min) added alone or in combination with Glu on the survival of the indicated groups of SMNs. i) Dose-dependent effects of siRNA\(_{p11}\) on the survival of SMNs\(_{wt}\) exposed to Glu (30 min, 150 μM); cRNA (5 μM) was taken as an additional control. Mean number of SMNs\(_{wt}\) in the untreated condition was taken as 100%. j) Mean effects of siRNA\(_{p11}\) (2 μM) on survival of SMNs\(_{task1}\), and SMNs\(_{task3}\); No NTs, culture medium not supplemented with neurotrophic factors. Schematics in d–f, h, i represent experimental protocols. Error bars, SEM. *p < 0.05, **p < 0.01, ***p < 0.001; *ns, not significant; by Student’s t-test or one-way analysis of variance (ANOVA) with post hoc Holm–Sidak method. d. e. h. j. d. e. h. j. n ≥ 3 independent experiments. k) Schematic diagram depicting a neuroprotective strategy by interfering with p11 function (with siRNA\(_{p11}\) in the example). Source data are provided as a Source Data file.

Survival. These experiments were performed in SMNs at 4–5 DIV, when an increase in surface expression of TASK channels is expected (see Fig. 1h). Drugs used for this purpose and their effects on TASK-mediated currents are summarized in Fig. 2c. A broad-spectrum TASK blocker, bupivacaine, reduced SMNs\(_{wt}\) survival like glutamate (Fig. 2d, Supplementary Fig. 4a, b); however, bupivacaine potentiated glutamate harmfullness (Fig. 2d). Interestingly, the anesthetic isoflurane, a TASK1/3 and TASK3/3 opener, and a TASK1/1 blocker, compromised survival per se and potentiated glutamate toxicity in SMNs\(_{task3}\) but blocked and reduced glutamate toxicity in SMNs\(_{wt}\) and SMNs\(_{task1}\), respectively (Fig. 2e). Furthermore, the selective TASK1 blocker anandamide (AEA, 10 μM), increased IME, reduced survival, and potentiated glutamate toxicity in SMNs\(_{wt}\) and SMNs\(_{task3}\) but, conclusively, not in SMNs\(_{task1}\) (Fig. 2f–h, Supplementary Fig. 4c). However, AEA at a lower concentration (3 μM) did not modify either IME or SMNs\(_{wt}\) survival (Fig. 2g, Supplementary Fig. 4c). The detrimental effects of AEA on SMNs\(_{wt}\) survival were insensitive to AM281, a cannabinoid receptor-1 antagonist (Supplementary Fig. 4d). Altogether, TASK channels, and among them TASK1 subunits, determine MN IME and survival, as well as MN vulnerability to an excitotoxic insult. Therefore, promoting functional expression of TASK1 by reducing p11 is a reasonable strategy to protect MNs against excitotoxicity. Accordingly, siRNA\(_{p11}\)-induced p11 downregulation protected SMNs\(_{wt}\) and SMNs\(_{task3}\) but, again, not SMNs\(_{task1}\), from glutamate (Fig. 2i, j, Supplementary Fig. 5a). Overall, p11 downregulation lowers MN IME by promoting TASK1 insertion at plasma membrane, then attenuates Ca\(^{2+}\) influx through GluRs and VSCCs, and subsequently reduces vulnerability to excitotoxic degeneration (Fig. 2k).

p11 knockdown is neuroprotective in the SOD1-G93A model. The outstanding impact of p11 on MN IME, Ca\(^{2+}\) signaling, and vulnerability to an excitotoxic insult suggests a leading role of p11 in neurodegenerative processes and highlights disruption of p11 function as a promising neuroprotective strategy. The superoxide dismutase 1 (SOD1)-G93A transgenic mouse model of familial ALS is the most widely used model in basic and preclinical studies of ALS. In this model, disease progression courses from hind to forelimbs. Hence, we focused our studies on MN pools located at the ventral horn of the lumbar spinal cord. In wild-type adult mice, MNs were the cell type in the ventral horn with highest levels of mRNA\(_{p11}\) and p11 (Fig. 3a, b). In contrast, astrocytes within and surrounding MN clusters only rarely and weakly displayed p11 immunoreactivity (Fig. 3c). At the pre-symptomatic stage (1-month-old), mRNA\(_{p11}\) and mRNA\(_{task1}\) were already upregulated in the spinal cord of transgenic mice (Fig. 3d). Although mRNA\(_{p11}\) and p11 upregulation persisted throughout the symptomatic phase (4-month-old), both mRNA\(_{task1}\) and TASK1 declined (Fig. 3d). At the early-symptomatic stage (3-month-old) that precedes MN loss, p11 was upregulated in lumbar SOD1-G93A MNs as compared to those from non-transgenic (Non-Tg) littermates (Fig. 3e). At this stage, p11-positive astrocytes were abundant within the characteristic astroglial reaction observed in the ventral horn of SOD1-G93A mice (Fig. 3f).

Experiments performed on SMNs provide evidence for a MN autonomous role of p11 upregulation in the ALS model. SMNs from SOD1-G93A embryos (SMNs\(_{G93A}\)) recapitulated p11–TASK1 dysregulation observed in the spinal cord of symptomatic mice (Supplementary Fig. 5b). In agreement with a reduced functional expression of TASK1 channels, SMNs\(_{G93A}\) exhibited a more excitable state than that of Non-Tg SMNs (SMNs\(_{Non-Tg}\)) (Supplementary Fig. 5c). Ca\(^{2+}\) imaging shed light on a gain-in-function of p11 for managing Ca\(^{2+}\) dynamics in SMNs\(_{G93A}\) (Supplementary Fig. 5d–h). Under cRNA, SMNs\(_{G93A}\) showed higher resting [Ca\(^{2+}\)]\(\text{e}\), and peak [Ca\(^{2+}\)]\(\text{e}\) of the early rapid response to glutamate, but shorter time of Ca\(^{2+}\) deregulation than SMNs\(_{Non-Tg}\) (Supplementary Fig. 5d–h). Remarkably, neuroprotective effects of siRNA\(_{p11}\) on the disturbance of Ca\(^{2+}\) dynamics were greater in SMNs\(_{G93A}\) (resting: −39.9 ± 8.8%; peak: −45.0 ± 4.1%; Δ time of Ca\(^{2+}\) deregulation: +347.8 ± 15.4 s) than in SMNs\(_{Non-Tg}\) (resting: −33.4 ± 6.4%; peak: −34.5 ± 8.6%; Δ time of Ca\(^{2+}\) deregulation: +139.7 ± 10.1 s) (Supplementary Fig. 5d–g).

Kaplan–Meier analysis of Ca\(^{2+}\) deregulation reported that, under cRNA-treatment, the probability function of SMNs\(_{G93A}\) was displaced to the left (i.e., likely shortening the cell lifespan) relative to SMNs\(_{Non-Tg}\) (p < 0.001). Noticeably, no differences (p = 0.130) were found between the two groups of siRNA\(_{p11}\)-treated SMNs (Supplementary Fig. 5h). The probability curves for both of these groups were displaced to the right (i.e., likely prolonging the cell lifespan; p < 0.001), compared to those from their respective cRNA-treated pools (Supplementary Fig. 5h). Finally, SMNs\(_{G93A}\) were more vulnerable to glutamate than SMNs\(_{Non-Tg}\) and, as expected, siRNA\(_{p11}\) robustly increased survival of SMNs\(_{G93A}\) exposed to glutamate (Supplementary Fig. 5i).

Hence, potential neuroprotective effects of siRNA\(_{p11}\) was assessed in the ALS model. We firstly tested the efficacy of a weekly injection of cRNA or siRNA\(_{p11}\) into the 4th ventricle to affect lumbar MNs, beginning at the pre-symptomatic stage (2-month-old), once the skull was developed enough to implant the injection system (Fig. 3g). By using this procedure, we observed that siRNA\(_{p11}\) consistently reduced mRNA\(_{p11}\) and p11 protein in the lumbar spinal cord after 8 weeks of treatment (Supplementary Fig. 6a, b). Quantitative analysis of p11-immunolabelling in lumbar MNs revealed that the decline in labeling intensity was already evident.
2 weeks after beginning of siRNA-p11 treatment (Supplementary Fig. 6c). Thereafter, the number of SMI32-positive MNs in the L3–L5 lumbar segment from 4-month-old mice was quantified.

Chronic administration of siRNA-p11 in SOD1-G93A mice, which reduced p11-immunoreactivity in the lumbar spinal cord (Fig. 3h), attenuated MN loss and improved motor performance (rotarod, footprint, runtime, and grip strength) as compared with cRNA-treated animals (Fig. 3i, Supplementary Fig. 6d–h). Correspondingly, siRNA-p11 delayed symptoms onset, alleviated weight loss, and prolonged survival (cRNA: 125.0 ± 1.8 days; siRNA-p11: 135.3 ± 3.0 days, p < 0.001, Student’s t-test) in SOD1-G93A mice (Fig. 3j–l). Beneficial effects did not result from different levels of transgene expression in both cohorts (Supplementary Fig. 6i).

**p11 knockdown is neuroprotective in the nerve injury model.**

In situ hybridization and immunohistochemistry studies clearly showed that HMNs, but not astrocytes, express high levels of p11 in the adult mouse (Fig. 4a, b). Bidirectional trophic...
communication between MNs and their target myocytes is necessary to keep MNs alive. Therefore, resection of a segment of the XIIth nerve, in which the possibility of nerve regeneration was minimized, led to significant loss of neurons in the HN (Fig. 4c, d). Neuronal loss was still undetectable one week post-lesion. However, a significant drop in the number of neurons was observed 2 weeks post-injury and, progressively declined up to ~40% from 4th to 8th week post-lesion (Fig. 4d). Remarkably, p11 was upregulated in the HN preceding MN loss (Fig. 4e). p11 upregulation was evident as soon as one day after injury and rose at 7th days post-lesion (Fig. 4e). p11 induction in the HN was mainly associated with upregulation in HMNs. p11-positive astrocytes were not observed in the injured side, even though the characteristic astroglial reaction occurred in the injured side (Fig. 4f, g). It was also interesting to note that, one week after nerve resection, mRNA<sub>p11</sub> declined in the injured HN (Fig. 4e), just as in the spinal cord of symptomatic SOD1-G93A mice.

To explore cell-specificity of p11 to MN loss, neuron-specific lentiviral constructions (LVVs) (Fig. 4c), which direct the expression of a small hairpin RNA against <i>lacz</i>)<sup>+</sup> and <i>lacz</i>− respectively, were used. LVV-shRNA<sub>p11</sub> reduced mRNA<sub>p11</sub><sup><i>trans</i></sup>, enhanced TASK-dependent changes, and protected SMN<sub>swt</sub> against glutamate (Supplementary Fig. 9a–c). Furthermore, DETA/NO-evoked p11 upregulation and pH-responsiveness reduction were fully blocked by retinoic acid, which post-translationally reduces p11 levels (Supplementary Fig. 9a–c), strengthening the notion that RNS inhibits TASK-like channels in a p11-dependent way. Several additional findings support a pathological NO-p11-TASK association in damaged MNs: (i) XIIth nerve crushing at P3 induces neuronal NO synthase (nNOS) expression in MNs<sup>6</sup>, and MN loss at P10, but not at P7 (Fig. 5f; Supplementary Fig. 9d, e); (ii) RhoA and p11 upregulation in the injured HN, together with hyper-excitability and reduction in TASK-mediated currents of injured HMNs, precede neuron loss (Fig. 5g–j); (iii) daily administration of the NOS inhibitor L-NAME fully prevents RhoA and p11 upregulation, and substantially preserves TASK-mediated currents in damaged HMNs (Fig. 5f–j); (iv) intracerebroventricular microinjection of siRNA<sub>p11</sub> increases TASK-dependent changes in ICE in axotomized HMNs (Fig. 5f, j); and (v) treatment of brainstem slices (P7) with retinoic acid down-regulates p11 in the injured side and partially recovers pH-responsiveness of insulted HMNs (Supplementary Fig. 9f–h).

Therefore, in axotomized MNs, RNS resulted in the recruitment of the PKG–RhoA–ROCK pathway to upregulate p11, thus reducing TASK-mediated currents underlying hyper-excitability that precedes MN loss (Fig. 5k).

RNS upregulates p11 and inhibits TASK-like currents. In order to obtain a more complete picture of this pathogenic mechanism, we looked for a widespread pathological event upstream p11 upregulation. In this context, large quantities of nitric oxide (NO) lead to RNS, a potential factor in excitotoxic neurodegeneration<sup>6–9</sup>. Interestingly, NO has been reported to upregulate p11 in human bronchial epithelial cells via protein kinase G (PKG)<sup>23</sup>. Finally, RNS inhibits TASK-mediated currents in MNs throughout PKG<sup>10</sup> (Fig. 5a). Here, RNS was simulated by long-term action (≥24 h) of DETA/NO (1 mM). At this concentration, this NO/cGMP releases physiological amounts of NO, as estimated in vivo<sup>6,7,24,25</sup>, over a 24-h period. NO inhibited TASK-like currents in HMNs independently of short-term mechanisms such as direct G<sub>Gaq</sub> co-association<sup>26</sup> and/or Rho-kinase (ROCK)-mediated phosphorylation<sup>27</sup>, since a G<sub>G</sub>q inhibitor or short-term presence (15 min) of a ROCK inhibitor did not prevent/revert long-term effects of DETA/NO (Supplementary Fig. 8a–c). However, the long-term mechanism involved RhoA–ROCK signaling, but not dysregulation of TASK1 and TASK3 expression (Supplementary Fig. 8d–g).

As reported for smooth muscle cells<sup>28</sup>, NO, via PKG, increased both, the amount of the phosphorylated form of the transcription factor ATF-1 and RhoA in the HN of neonatal rats (Fig. 5b, c). Conclusively, DETA/NO, via PKG–ROCK, upregulated mRNA<sub>p11</sub> and p11, but not mRNA<sub>TASK1</sub> or mRNA<sub>TASK3</sub> (Fig. 5c–e). Furthermore, DETA/NO-evoked p11 upregulation and pHresponsiveness reduction were fully blocked by retinoic acid, which post-translationally reduces p11 levels (Supplementary Fig. 9a–c), strengthening the notion that RNS inhibits TASK-like channels in a p11-dependent way. Several additional findings support a pathological NO-p11-TASK association in damaged MNs: (i) XIIth nerve crushing at P3 induces neuronal NO synthase (nNOS) expression in MNs<sup>6</sup>, and MN loss at P10, but not at P7 (Fig. 5f; Supplementary Fig. 9d, e); (ii) RhoA and p11 upregulation in the injured HN, together with hyper-excitability and reduction in TASK-mediated currents of injured HMNs, precede neuron loss (Fig. 5g–j); (iii) daily administration of the NOS inhibitor L-NAME fully prevents RhoA and p11 upregulation, and substantially preserves TASK-mediated currents in damaged HMNs (Fig. 5f–j); (iv) intracerebroventricular microinjection of siRNA<sub>p11</sub> increases TASK-dependent changes in ICE in axotomized HMNs (Fig. 5f, j); and (v) treatment of brainstem slices (P7) with retinoic acid down-regulates p11 in the injured side and partially recovers pH-responsiveness of insulted HMNs (Supplementary Fig. 9f–h).

Therefore, in axotomized MNs, RNS resulted in the recruitment of the PKG–RhoA–ROCK pathway to upregulate p11, thus reducing TASK-mediated currents underlying hyper-excitability that precedes MN loss (Fig. 5k).

RNS stimulates the human p11 promoter via a Sp1-binding site. To strengthen clinical and translational potential, we tested RNS effects on luciferase activity coupled to the human p11 promoter (region −1436 to +89) in the transfected and differentiated MN-like cell line NSC34 (dNSC34). In preliminary experiments, DETA/NO induced mRNA<sub>p11</sub> and p11 in dNSC34s (Supplementary Fig. 10a), indicating their capability to couple p11 expression to NO. Only DETA/NO concentrations (1–2 mM), releasing pathological amounts of NO, increased luciferase activity (Fig. 6a), in a time-dependent manner (Supplementary Fig. 10b). We chose a 48-h treatment with DETA/NO 1 mM as the optimal condition for subsequent experiments. DETA/NO stimulated luciferase activity through the NO target and PKG-activator soluble guanylyl cyclase (sGC), PKG, and ROCK
Furthermore, serial 5′ promoter deletions isolated a minimal NO-responsive element at the −96 to −70 segment (Fig. 6c). Finally, site-directed mutagenesis identified a potential binding site for Sp1 (GC-box) essential for NO-responsiveness (Fig. 6d). Since, three additional Sp1 and a NO-responsive AP-1-binding sites upstream −96 have been identified, whether p11 promoter is regulated by other NO-responsive elements and/or by still unrevealed factors remains to be investigated.

Electrophoretic mobility shift assays (EMSA) displayed two well-defined complexes when the wild-type −96 to −70 sequence was incubated with nuclear protein extracts from dNSC34s. Both complexes were absent if the sequence comprised the HN

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NO-irresponsive mutation (Fig. 6e). Competitive inhibition, by increasing the concentration of the canonical Sp1 sequence, reduced complex formation in a dose-dependent manner (Fig. 6f). In addition, incubation with antibodies against Sp1 or Sp3 either alone or in combination clearly reduced the intensity of the complex with lower electrophoretic mobility (Fig. 6g). Altogether, these outcomes support a binding of Sp1, and probably Sp3, to the NO-sensitive GC-box within the −96 to −70 segment of p11 promoter. Finally, DETA/NO at 1 mM, but not at 0.1 mM, upregulated mRNA
Sp1 and mRNA
Sp3 in SMNs
wt via the PKG–ROCK signaling (Fig. 6h, i), further supporting that DETA/NO effects on promoter activity result in an increase in transcriptional activity of the p11 gene in murine MNs.

Therefore, we hypothesize that RNS, established under neurological conditions by pathological concentrations of NO, recruits sGC–PKG–ROCK signaling and subsequently upregulates the transcription factor Sp1, which stimulates p11 promoter activity and promotes p11 expression (Fig. 6j).

Mit-A, a feasible neuroprotector that dysregulates p11–TASK1.

Based on the preceding framework, we tested the viability of Sp1 function as a neuroprotective strategy. Transient transfection of a plasmid directing the expression of a truncated form of Sp1, which acts as a dominant negative (dnSp1), strongly reduced p11 in dNSC34s (Supplementary Fig. 11a). Furthermore, a neuron-specific LVV directing the expression of dnSp1 (LVV-dnSp1) reduced mRNA
p11, strongly promoted functional expression of TASK-like channels, and almost fully blocked glutamate toxicity on SMNs
wt (Supplementary Fig. 11c–e). These data support that Sp1 controls constitutive expression of p11 in dNSC34s and SMNs
wt. Nevertheless, given the current limitations of LVVs for medical use, we tested a Sp1-interfering drug with higher clinical potential. In this context, Mit-A, a FDA-approved anticancer antibiotic that binds to GC-rich sequences, is usually used to interfere with Sp1 binding to its target sites.

Mit-A fully avoided NO-triggered stimulation of the minimal NO-responsive element of the human p11 promoter (Supplementary Fig. 12a). This antibiotic strongly reduced mRNA
p11 and, unexpectedly, increased mRNA
task3 levels in SMNs
wt, both of which would be expected to result in additive functional effects (Supplementary Fig. 12b). Thus, Mit-A drastically lowered IME and promoted TASK-mediated changes in SMNs
wt and SMNs
 taskId−/−, but, conclusively, not in SMNs
 task1−/− (Supplementary Fig. 12c–g). Mit-A also attenuated glutamate-evoked Ca2+ homeostasis disruption in a TASK1-dependent manner (Supplementary Fig. 12h–j). Kaplan–Meier analysis revealed that Mit-A caused a strong delay in the time of Ca2+ deregulation (i.e., likely prolonging the cell lifespan) in SMNs
wt and SMNs
 taskId−/−, but again not in SMNs
 task1−/− (Supplementary Fig. 12k). Finally, Mit-A protected SMNs
wt and SMNs
 taskId−/− but, once again, not SMNs
 task1−/−, against glutamate toxicity (Supplementary Fig. 12l). These results strengthen the therapeutic interest of Mit-A by reducing neuronal hyper-excitability and vulnerability against excitotoxicity.

Mit-A is neuroprotective in the SOD1-G93A model. Both Sp1 upregulation in the spinal cord from symptomatic (4-month-old) SOD1-G93A mice, and the increase of nuclear immunolabelling for Sp1 in lumbar MNs preceding cell loss (3-month-old) underpin a key role for Sp1 in the ALS model (Fig. 7a, b). Sp1 was also upregulated in SMNs
G93A (Supplementary Fig. 13a). Accordingly, Mit-A lowered IME, promoted TASK-dependent changes and strongly attenuated glutamate-triggered Ca2+ disturbance in SMNs
G93A (Supplementary Fig. 13b–g). The Kaplan–Meier analysis of Ca2+ deregulation showed that Mit-A displaced to the right the probability function of SMNs
G93A (Supplementary Fig. 13h), and lowered their vulnerability to glutamate (Supplementary Fig. 13i).

Therefore, neuroprotective action of Mit-A is underpinned by the additive action on p11 and TASK1 expression, which lowers IME and delays glutamate-evoked Ca2+ deregulation, at least in part, by promoting TASK1 expression at the plasma membrane (Fig. 7c). Then, we tested preclinical efficacy of Mit-A in SOD1-G93A mice. Initially, optimal dosage of Mit-A was determined in wild-type mice. Chronic and daily administration of growing doses of Mit-A in the drinking water for 3 weeks, reduced p11 and increased TASK1 expression in the spinal cord and attenuated the loss of lumbar MNs in 4-month-old SOD1-G93A mice (Fig. 7d, e). Mit-A delayed the frequency of skeletal muscle fibrillations (Fig. 7f–h). Antibiotic-induced neuroprotective effects were substantiated by functional benefits at the muscle. Myocytes denervation is a characteristic hallmark of ALS, identified in needle electromyography by spontaneous discharges of skeletal muscle fibers or fibrillations (Fig. 7i). Thus, the frequency of fibrillations gradually increased in transgenic mice from week 13 to week 16 of age but these were generally absent in Non-Tg animals (Fig. 7j–m). Interestingly, the rate of fibrillations...
under Mit-A treatment was drastically decreased at all ages tested (Fig. 7j–m).

Similar outcomes were observed when Mit-A treatment (30 μg kg⁻¹ day⁻¹) began at 2 months of age in ALS mice (Supplementary Fig. 15a–f). Although two additional drug regimens (30 or 150 μg kg⁻¹ day⁻¹), beginning at P90, prolonged survival and improved rotarod performance of SOD1-G93A mice (Supplementary Fig. 15c, d), the results were not conclusive on the beneficial effects of these treatments in the remaining parameters (Supplementary Fig. 15a, b, e, f). The higher dose tested even worsened body weight, footprint, and runtime outcomes over the time-course (Supplementary Fig. 15b, e, f).

Finally, Mit-A (30 μg kg⁻¹ day⁻¹) and Riluzole (25 mg kg⁻¹ day⁻¹), the most clinically used drug for ALS patients, resulted in similar beneficial outputs in SOD1-G93A mice when parallel treatments began at 2 months of age (Supplementary Fig. 15g–m).

Mit-A is neuroprotective in the motor nerve injury model. One week after resection of a 2-mm-segment of the XIIth nerve of adult mice, Sp1 was increased in the HN of the injured side (Fig. 7i).
Fig. 5 RNS upregulates p11 and inhibits TASK channels in a traumatic model of MN degeneration. a Effects of nerve injury or persistent action of the NO-PKG pathway on MN IME and TASK-mediated currents. b–e Expression of phosphorylated ATF-1 (pATF-1) and RhoA proteins, b, indicated mRNAs c, d, and p11 e in HMNs from neonatal brainstem slices incubated for 6 h in ACSF alone (control), or supplemented with either DETA/NO (1 mM), DETA/NO+PKG-i (10 μM), DETA/NO+HIP52 (20 μM), or DETA/NO+Y27632 (10 μM). In c, mRNA levels of DETA/NO versus control condition are presented. In b–e, n ≥ 3 independent experiments. f Experimental model. Crushing was inflicted to the XIIth nerve of P3 rats. Pups were untreated, L-NAMETreated, or they received a single intracerebroventricular microinjection of cRNA or siRNA-p11. g Immunobots for RhoA and p11 of HMNs from P7 rats treated as shown. gpdh and α-tub were the internal controls for qRT-PCR and western blotting, respectively. h Averaged i-V relationships of the TASK-mediated current for the indicated treatments (see experimental design in f) (see Supplementary Fig. 9b). Data were well fitted (r > 0.9) with the Goldman–Hodgkin–Katz equation (solid line). Current reversed close to −90 mV, near the reversal potential predicted for K+ (Eg). Goldman–Hodgkin–Katz fits for control and DETA/NO (dashed lines), previously reported30, are shown for comparison. i Top, current-clamp recordings of the voltage responses to a series of depolarizing and hyperpolarizing current pulses (0.5 s duration, 0.04 nA increments) from a control and a damaged HMN recorded 4 days after axonal injury. Bottom, time course illustrating alterations in current holding (Δholding) recorded in response to extracellular pH changes for two P7 HMNs held at −65 mV. j Plots represent TASK-dependent changes in Vm and Δholding for each listed treatment group. Number of independent samples is in parentheses. Error bars, SEM. *p < 0.05, **p < 0.01, ***p < 0.001; n.s., not significant; by Student’s t-test c, g or one-way ANOVA with post hoc Holm–Sidak method b, d, e, j, k Schematic diagram depicting the mechanism by which RNS impacts on MN IME. Source data are provided as a Source Data file (Fig. 8a). Sp1-immunolabelling was enhanced in the injured side at 7 days after injury (Fig. 8b, c), thus preceding HMNs loss (see Fig. 4d). Interestingly, lesion-induced suppression of Sp1 expression was avoided by 2 days treatment with the NOS inhibitor L-NAME (Fig. 8d). Furthermore, chronic administration of the NOS inhibitor or Fasudil (10 mg kg−1 day−1), an inhibitor of the downstream NO-acting ROCK (see Fig. 6j), reduced the loss of injured HMNs (Fig. 8e). All these pharmacological treatments began at 5 days post-injury, when de novo expression of nNOS in injured MNs was evidenced30. These outcomes indicate that Sp1 upregulation, downstream de novo NO synthesis, is part of a mechanism that precedes MN death after motor nerve injury.

Mit-A administration in the drinking water at two doses (30 or 300 μg kg−1 day−1) drastically reduced neuron loss observed 21 days after nerve resection (Fig. 8f). Neuroprotection was confirmed in task3−/−, but was absent in task1−/− mice (Fig. 8f). Intrapertoneal administration of the higher dose of Mit-A (300 μg kg−1 day−1) showed slight but significant neuroprotective effects (Fig. 8f). Therefore, Mit-A delays MN degeneration after nerve injury, by a mechanism involving, at least, TASK1 subunits.

Therefore, Sp1 is also a feasible partner in an overall mechanism contributing to MN degeneration and point to the therapeutic interest of Mit-A for the treatment of diverse motor pathologies regardless of causal origin.

Cell-specific Sp1–p11 knockdown protects SOD1-G93A MNs. p11 upregulation occurs in astrocytes and MNs before MN loss in SOD1-G93A mice (see Fig. 3c, f). Then, we studied a cell autonomous role of Sp1–p11 in MN degeneration. In this context, most used MN-specific conditional knockouts target the subset of MNs that is mainly spared in the disease (VAChT-Cre model)31–33, or other cells types beside MNs (Hb9-Cre model)36–38, including spinal cord interneurons involved in rhythm generation during locomotor activity. Therefore, here we performed intrasplinal microinjections of neuron-specific LVV-dnP11 or LVV-shRNA_p11 (see Fig. 4c) into the ventral horn of the lumbar segments in 2-month-old SOD1-G93A mice (Fig. 9a). A careful inspection of the L1–L3 ventral horns from 4-month-old animals showed the presence of the reporter protein GFP in p11-immunoreactive MNs, but not in glial-like structures (Fig. 9b–e). Strikingly, p11-immunolabelling was reduced in GFP-positive MNs after LVV-dnP11 or LVV-shRNA_p11 injection (Fig. 9c–f). Furthermore, the frequency of vacuolated/degenerated MNs was higher in LVV-shRNA_p11–treated SOD1-G93A mice (Fig. 9g, h). Accordingly, the number of apparently healthy MNs was higher in mice receiving LVV-dnP11 or LVV-shRNA_p11 than in those treated with LVV-shRNA_p11 (Fig. 9g–i). Therefore, these outcomes, together with those from SMNs, strongly support a cell autonomous role of Sp1/p11 in MN degeneration. However, whether p11 upregulation in glial cells also contributes in some degree to disease progression merits further investigation.

Discussion

Our study identify a mechanism that regulates neuronal IME, Ca2+ signaling, and vulnerability to degeneration. The ubiquitous transcription factor Sp1 controls the expression of p11, a retention factor at the endoplasmic reticulum for TASK114, which regulates channel expression at the cell surface, thus fine-tuning neuronal IME and Ca2+ influx via GluRs and VSCCs (Fig. 10a). Interference with Sp1 function reduces p11 expression and attenuates MN IME, Ca2+ dynamics, and vulnerability via TASK1. These effects were similar to those revealed by disrupting p11. Hence, we conclude that modulation of the Sp1–p11–TASK1 triad offers a promising strategy to minimize excitotoxic damage occurring in a multitude of neuropathologies associated with dysregulated membrane excitability and/or excitotoxic degeneration, including both acute and chronic neurodegenerative diseases3,4.

Of pathological relevance, Sp1–p11–TASK1 dysregulation occurs in two motor neurodegenerative models with very different causal origins, which shared excitotoxicity and hyper-excitability as pathophysiological hallmarks34,46. Sp1–p11–TASK1 strongly impacts on IME of MNs. Interfering with either Sp1 or p11 lowers MNs IME by promoting functional expression of TASK1, then protecting MNs against excitotoxicity. However, whether hyper-excitability is harmful or neuroprotective for MNs in ALS is a current subject of debate. Intrinsic hyper-excitability of MNs has been assumed to participates in the excitotoxic process39. In contrast, hyper-excitability is proposed as a transitory state40,41 that even might be beneficial12,43 in ALS, and which might underlie survival of S-type MNs in the ALS model42,43. Here, at early-presymptomatic (P30) stage, p11 and TASK1 upregulation could be part of a homeostatic and compensatory process to neutralize deficits during early phases of disease43. Nevertheless, persistent p11 upregulation and TASK1 down-expression occurring at the symptomatic stage might potentiate excitotoxic processes39 by strengthening, for example, the incoming synaptic excitation to MNs that might result from inhibitory bouton loss and excitatory synapse gain44. In this line, chronic interference with Sp1 and/or p11 beginning at presymptomatic (P30 or P60) or early-symptomatic (P90) stages resulted neuroprotective for SOD1-G93A MNs and/or mice.
Additional controversy comes from passive membrane properties that lie behind hyper-excitability in SOD1-G93A MNs. While embryonic (E17.5) and early-postnatal (P6–P10) spinal MNs displayed RN increase or Vm depolarization, respectively, no changes in Vm nor RN accompanied hyper-excitability in HMNs G93A (P4–P10). Here, hyper-excitable SMNs G93A (E12.5), recorded at 4–5 DIV, involved Vm depolarization and RN increase. Interestingly, hyper-excitable SMNs G93A (E12–E15) recorded at 10–30 DIV, did not display Vm and RN alterations. This agrees with an early transitory state in which these passive membrane properties of SMNs G93A are affected. All these discrepancies
might be due to different embryonic origin (spinal cord vs. brainstem), developmental stage (embryonic vs. neonatal), and/or experimental preparations.

Several findings support causality between RNS and Sp1–p11–TASK1 dysregulation. First, Sp1 expression and its DNA-binding activity increased in rat hippocampus exposed to excitotoxic insults. Second, nNOS induction preceded MN death in ALS and after nerve injury. Third, DETA/NO (0.1 mM) did not alter human p11 promoter activity or Sp1–p11 expression. However, DETA/NO (1 mM), which releases pathological concentrations of NO, increases MN IME by inhibition of TASK-like channels, upregulates Sp1–p11, and stimulates human p11 promoter via at least a Sp1-binding GC-box. Consistently, NO upregulated p11 in human epithelial cells and promoted Sp1–p11 expression. Fourth, Sp1-interfering agent Mit-A fully avoids RNS-triggered stimulation of the minimal NO-responsive element of the human p11 promoter. Fifth, inhibition of endogenous NO synthesis attenuates the Sp1–p11 upregulation and the TASK-dependent increase of MN IME in the nerve injury model. Therefore, Sp1–p11–TASK1 dysregulation downstream of RNS seems a key event in a positive feedback loop leading to excitotoxic degeneration (Fig. 10b).

Comparison of the timing of NOS-p11 upregulation after injury and in the ALS model, suggests that early p11 induction was RNS-unrelated in the two models (Fig. 10b). Thus, early slight p11 upregulation after nerve injury (1–3 days) and in the ALS model (early-presymptomatic) precede NO induction after nerve injury (3–5 days) and in SOD1-G93A mice (late-presymptomatic). Furthermore, given that NO induction occurs in MNs and glial cells in early-symptomatic SOD1-G93A mice preceding MN loss, whether p11 expression at this stage is coupled to NO from MN and/or glia origin merits further investigation (Fig. 10b).

Sp1 ubiquity and broad co-expression of p11 and TASK1 all along the CNS suggest the need for further investigation on this triad to ascertain feasible biomarkers for diverse psychiatric and neurological disorders. One of the three, p11, has recently been proposed as a biomarker for monitoring the severity of Parkinson’s disease.

The Sp1-binding inhibitor Mit-A is neuroprotective in the ALS mouse model. The oral lower dose we administered (30 μg kg⁻¹ day⁻¹) is in the lower range for its intravenous application in humans. We would expect that concentration of Mit-A that might be reached within the organism after oral administration was lower than that obtained with equivalent intravenous doses in chemotherapy. In this line, characteristic side-effects after intravenous Mit-A administration (bleeding, behavioral alterations, and weight loss) were absent in treated mice. Strikingly, beneficial outcomes of Mit-A treatment on SOD1-G93A mice were similar to those achieved under treatment with Riluzole, the most used drug for ALS patients in clinical settings. Currently, Mit-A is the subject of clinical trials for several cancers (www.ClinicalTrials.gov; Identifiers: NCT02859415, NCT01624090), thus supporting a renewed interest in the clinical use of this kind of compounds. The present results along with the emergence of Mit-A analogs with higher activity and less toxicity-61 support the use of these drugs in clinical trials for neurodegenerative disorders such as ALS.

In summary, RNS-triggered induction of Sp1–p11 decreases background K⁺ currents and switches neuron to a more vulnerable configuration in neurodegenerative disorders. In contrast, a reduction in Sp1–p11 places neurons in a more resistant state against degeneration due to an increase in the functional expression of TASK1. The broad expression of Sp1–p11–TASK1 throughout the organism suggests that interplay between these proteins might not only be circumscribed to the CNS but may also impact functioning of the immune, cardiovascular, respiratory, digestive, excretory, and reproductive systems, both in health and in sickness. Indeed, dysregulation of either Sp1, p11, or TASK1 has been independently reported in multiple pathological conditions, including inflammatory processes and cancer, affecting different organs and tissues.

Methods

Animals. Neonatal (P3–P9) wistar rats of either sex, young (1–2-month-old) and adult (>2-month-old) male mice, either CD1, SOD1-G93A (Jackson Laboratory, Bar Harbor, ME, USA) and Non-Tg littermates; and, CD1, C57BL/6J, task1⁻/⁻, task3⁻/⁻, and SOD1-G93A pregnant mice (12.5 days gestation). All these animals were provided by the local Animal Supply Services (SEPA, University of Cadiz). Animal care and handling followed the guidelines of the European Union Council (2010/63/EU, 86/609/UE) on the use of laboratory animals. Experimental procedures were approved by the local Animal Care and Ethics Committee (University of Cadiz, Cadiz, Spain) and the Ministry of Agriculture, Fisheries and Rural Development (Junta de Andalucía, Spain). task1⁻/⁻ and task3⁻/⁻ mice, a gift from Dr. Douglas A. Bayliss (University of Virginia, VA, USA), became stablished colonies and were bred in the local Animal Supply Services. Animals were individually housed in cages with water and food pellets available ad libitum, at 21 ± 1 °C, with a 12-h light/dark cycle. Neonatal animals were housed with their mother. Efforts were made to minimize the number of animals used and their suffering. All surgical procedures were carried out under aseptic conditions.

Electrophysiology. Whole-cell patch-clamp recordings were performed in HMNs from coronal brainstem slices (P6–P9 rats) or cultured SMNs (4 DIV) at 37°C. In summary, neonatal rats were decapitated under anesthesia by hypothermia (10–15 min at 4 °C). Brainstems were rapidly removed and dissected in artificial cerebrospinal fluid (aCSF) enriched with sucrose at 4 °C (in mM: 26 NaHCO₃, 10 NaCl, 1.25 NaH₂PO₄, 2 MgCl₂, and 28 sucrose), and bubbled with 95% O₂ and 5% CO₂. Transverse slices (300–400 μm thick), obtained using a vibrslicer (NVS; WPI), were transferred to normal oxygenated aCSF (in mM: 26 NaHCO₃, 10 NaCl, 1.25 NaH₂PO₄, 2 MgCl₂, and 28 sucrose)
10 glucose, 3 KCl, 1.25 NaH2PO4, 6 MgCl2, 130 NaCl, and 0.5 CaCl2) and stabilized at ~37 °C for 1 h. Slices were then transferred to a recording chamber for whole-cell patch-clamp recordings or to an incubation chamber for treatment with different drugs before HN microdissection. SMN cultures were prepared from embryonic day 12.5 (E12.5) mouse spinal cords, as described below.

Recordings of HMNs and SMNs were performed under constant perfusion (~3–4 ml min⁻¹) with normal oxygenated aCSF at 31 °C. Recordings were obtained from motoneuron somata, using a Nikon (Tokyo, Japan) Eclipse CFI60 microscope equipped with infrared differential interference contrast. Patch electrodes (1.5–3 MΩ resistance) were filled with the following internal solution (in mM): 17.5 KCl,
Fig. 7 Neuroprotective effects of the Sp1-interfering agent Mit-A in the SOD1-G93A mouse model of ALS. a mRNA (left) and protein (right) levels for Sp1 in the spinal cord of SOD1-G93A mice and their Non-Tg littermates at the indicated ages. b Confocal images showing Sp1-immunolabeled nuclei of SM132-positive lumbar MNs from 3-month-old Non-Tg and SOD1-G93A mice. Boxed areas in low magnification images for the Non-Tg condition are displayed at higher magnification together with similar areas obtained from transgenic mice. Box plot represents the mean intensity of nuclear Sp1-labeling of Non-Tg or SOD1-G93A MNs. c Mechanism of action by which Mit-A might protect neurons against excitotoxic degeneration. d Daily administration of Mit-A (30 μg kg⁻¹ day⁻¹) in the drinking water, beginning at P30, dysregulated mRNA (left) and protein (right) levels of p11 and TASK1, in the lumbar spinal cord of SOD1-G93A mice at P120. e Number of SM132-positive MNs (left), in the L3-L5 spinal cord segment of SOD1-G93A mice and their Non-Tg littermates, treated with Mit-A or vehicle. Scale bars, 50 μm. f-h Cumulative probability curves of symptoms onset f and survival h, and time course of body weight g, against the age of SOD1-G93A mice receiving the indicated treatments. Mit-A treatment as in d. i-k Representative electrophysiographic recordings from the gastronomic muscle of Non-Tg and SOD1-G93A mice at weeks 13 i, j and 15 k. Transgenic mice received treatments as in f-h. In i, f, h, i-l, and m-way ANOVA with post hoc Holm-Sidak method g. Statistical data obtained using Log-Rank test, Kaplan-Meier analysis f, h, i, j, k, l, m, Maximal (l) and mean (m) frequency of fibrillations in SOD1-G93A animals at indicated weeks treated with vehicle or Mit-A. N: number of independent samples is in parentheses. Error bars, SEM. *p < 0.05; **p < 0.01; ***p < 0.001; n.s., not significant; by Student’s t-test a, b, d, e, l, m or two-way ANOVA with post hoc Holm-Sidak method g. 122.5 K-glutamate, 9 NaCl, 1 MgCl₂, 10 HEPES, 0.2 EGTA, 3 Mg-ATP, and 0.3 GTP–Tris at pH 7.4. Recordings were obtained and low-pass Bessel filtered at 10 kHz with a MultiClamp 700B amplifier. Data were digitized at 20 kHz with a Digidata 1332A analog-to-digital converter and acquired using pCLAMP 9.2 software (Molecular Devices, Foster City, CA). Analysis of recordings was only performed for recordings with access resistance between 5 and 20 MΩ. Recordings were discarded if access resistance changed by >15% during the trial. Series resistance was routinely compensated 65–75%. The pipette offset potential was neutralized just before motoneurons were patched. Recordings were not corrected for liquid junction potential.

In the current-clamp configuration, resting Vm and Rsh were measured for the assessment of MN IME. Rsh was calculated from the current–voltage (I–V) plots obtained by injecting a series of depolarizing and hyperpolarizing current pulses (0.5 s; −0.2 to 0.2 nA). In Fig. 1b only the hyperpolarizing square pulses are exemplified. The resulting data points were then fitted with a regression line, and Rsh was estimated as the slope of the lines. Voltage-clamp recordings were all carried out in presence of tetrodotoxin (TTX, 1 μM; Tocris Cookson, Bristol, UK). Initially, the holding current (Ihold) required to keep Vm at −65 mV was measured (Supplementary Fig. 8a). I–V relationships (Supplementary Fig. 8b, right) were obtained by applying voltage steps in 5 mV increments ranging from −50 to −120 mV from a baseline holding potential of −65 mV. In this case, the instantaneous component was measured ~10 ms after the onset of the step (Supplementary Fig. 8b, e, triangles). In the experiments designed to evaluate TASK-like pH-sensitive currents, MNs were sequentially recorded at varying pH levels (pH 6.2, 7.2, and 8.2). Extracellular pH was adjusted using HCl or NaOH to aCSF. TASK-dependent currents (pH-sensitive currents) were obtained by subtraction of the I–V relationship obtained at pH 8.2 minus that measured at pH 6.2 (Fig. 5h, Supplementary Figs. 8b and 9b).

Quantitative real-time reverse transcription PCR (qRTP–PCR). Total RNA was extracted from medulla, spinal cord, microdissected HNs, dNSC34s, or SMNs (100,000 cells per well) using the TRizol Reagent (BioLine). This procedure allows the isolation of total RNA from the spinal cord, HNs, micro-dissected dNSC34s, or SMNs with specific antibodies against single sample. Subsequent treatment with the RNase-free DNase set (Invitrogen) followed by manufacturer recommendations, was performed to reduce RNA contamination. The concentration and purity of RNA samples were determined by spectrophotometry at 260 and 280 nm. Retranscription (RT) was done using random hexamers, 500 ng of total RNA as template and iScript™ cDNA Synthesis Kit (Bio-Rad). For real-time RT–PCR, each specific gene product was amplified with the MiniOpticon™ System (BIO-RAD), using iQ SYBR Green Supermix (Bio-Rad). The PCR primers sequences are stated in the Supplementary Table 1. The cDNA levels for the different samples were determined using the ΔΔct method, being gapdh the housekeeping gene. All analyses were performed in triplicate, with each experiment repeated at least twice.

Western blot analysis. Tissue samples from the medulla, spinal cord or microdissected HNs were homogenized in TNEure, as above, or in lysis buffer [50 mM Tris/HCl, pH 7.4, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate] supplemented with protease (Sigma-Aldrich) and phosphatase (Pierce) inhibitors using a 1 ml syringe. dNSC34s cells and SMNs (100,000 cells per well) were rinsed with ice-cold PBS and immediately transferred to the lysis buffer with protease and phosphatase inhibitors. Protein concentrations were determined by Bradford® protein assay (Bio-Rad) or by micro BCA protein assay kit (Thermo Scientific). Proteins were separated by SDS–PAGE and blotted to polyvinylidene difluoride membrane (Amersham). Membranes were blocked with BSA 5%, 0.1% Tween for 1 h at room temperature and subsequently incubated with specific antibodies against pATF-1 (1:200, Santa Cruz Biotechnology, Cat# sc-7978, RRID:AB_20974611), p11 (1:2000, R&D systems, Cat# AF2377, RRID:AB_2183469) developed in goat, HA (1:250, Sigma-Aldrich, Cat# 11583816001, RRID:AB_541505), RhoA (1:200, Santa Cruz Biotechnology, Cat# sc-418, RRID:AB_628218), β-actin (1:2500, Sigma-Aldrich, Cat# A5441, RRID:AB_477644) and a-tubulin (1:10000, Sigma-Aldrich, Cat# T9026, RRID:AB_477593) developed in mouse, and Sp1 (1:10000, Abcam, Cat# ab124804, RRID:AB_10974611). TASK1 (1:200, Alomone Labs, Cat# APC-024, RRID:AB_20401332, 1:200, Sigma-Aldrich, Cat# P0981, RRID:AB_260876) or TASK3 (1:200, Alomone labs, Cat# APC-044, RRID:AB_2039953) developed in rabbit. Proteins were visualized by using horseradish peroxidase-conjugated secondary antibodies (Pierce) and the enhanced chemiluminescence system (Amersham). Analysis was performed using the ImageJ 1.48v software from the National Institutes of Health. Unropped and unprocessed scans of all blots are supplied in the Source Data file.

Immunoprecipitation. Immunoprecipitation assays were performed using Pierce® co-immunoprecipitation kit (Thermo Scientific) following manufacturer’s instructions with subtle modifications. Briefly, the brain of an adult mouse (CD1) was mechanically homogenized in immunoprecipitation assay buffer (5 μl of tissue/1 ml 0.4% NP40, 1% Trit, 150 mM EDTA, 0.5 mM PMSF, 0.1% aprotinin, 0.5% sodium deoxycholate, pH 7.4 supplemented with a cocktail of protease inhibitors) and cleared by centrifugation. To adjust the protein concentration in samples prior to immunoprecipitation, it was measured using a BioRad protein assay kit according to the manufacturer’s instructions. First, Pierce spin columns containing 25 μl resin were incubated with 10 μg of anti-p11 antibody (R&D systems) for 3 h in a rotator at room temperature. As negative control, other spin columns were incubated with 10 μg of an irrelevant antibody (anti-sheep IgG, Sigma-Aldrich) in the same conditions. The homogenates were diluted with immunoprecipitation assays buffer to have a final volume of 300 μl per column, and incubated with the resins overnight in a rotator at 4 °C. Resin centrifugation steps were performed at 4°C for 45 s. Thereafter, a volume of 60 μl of the elution buffer was passed through every resin to collect p11 together with their associated-proteins in a first elution (E1) fraction. A second elution (E2) of 60 μl was collected to ensure that most p11 and p11-associated proteins were rescued from the resin. For protein denaturation and survival experiments 10 μl of the elution buffer was added to elution fraction which were subsequently heated at 95 °C for 5 min. Finally, fractions were subjected to electrophoresis and immunoblot analysis for p11 and TASK1 detection.

Primary cultures of SMNs and survival assays. Primary cultures of MNs were prepared from the spinal cord of mouse embryos at 12.5 days of gestation (E12.5), following an established protocol56. Briefly, desiccated ventral cord were first chemically (0.025% trypsin in glucose–HEPES buffer solution supplemented with 100 mM DTT, 1:m 1% streptomycin) and mechanically dissociated and subsequently plated in a 24-well Corning glass coverslips (Corning, NY) placed in 35-mm culture dishes for electrophysiology experiments (30,000 SMNs per well), Ca²⁺ imaging (30,000 SMNs per well) or...
immunocytochemistry (15,000 SMNs per well). For survival evaluation of SMNs, the cells received complete medium on the seeding day, either containing or lacking (no NTFs) the combined NTF cocktail. One and four days after seeding, culture medium with NTFs was replaced with the same medium supplemented with the experimental conditions (see treatments below). To assess neuronal survival, the number of large phase-bright cells with long neurites in the total area of triplicate wells for each condition was counted 72 h after the last treatment. Data were obtained from at least three independent experiments. Individual symbols in figures represent a single well. The mean number of surviving cells (in percent) was normalized taking the “NTF medium alone” treatment as 100%.

**Ca²⁺ imaging.** For intracellular Ca²⁺ measures, isolated SMNs, plated on laminin-coated coverslips, were washed in Krebs solution (140 mM NaCl; 5 mM KCl; 2 mM CaCl₂; 1 mM MgCl₂; 10 mM HEPES; and 11 mM glucose, pH 7.4). Afterwards, SMNs were loaded with a non-ratiometric or single wave dye that measures free cytosolic Ca²⁺. For that purpose, coverslips were incubated (30 min, 37 °C, darkness) in Krebs solution supplemented with Fluo-4 AM (1 μM, Molecular Probes, Invitrogen) and pluronic acid (0.02%). Confocal images were obtained by means of a direct Olympus FV1000 confocal laser scanning microscope using a ×40 water immersion objective. Fluo-4 AM was excited at a wavelength of 488 nm and the emission fluorescence at a wavelength of 518 nm was acquired for offline analysis.
Fig. 8 TASK1-dependent neuroprotective effects of Mit-A in the traumatic model of MN degeneration. a–e Effects of resection of a 2-mm-segment of the XIIth nerve on mRNA levels and Sp1 levels. a, number of Sp1-immunoreactive nuclei, and optical density of nuclear staining c in the HN of the injured (inj.) versus the intact (int.) side at the stated days post-injury. Coronal sections immunolabelled for Sp1 b, c, some of them counterstained with Nissl (c, bottom), obtained 7 days after nerve injury. At least 50 XIIth nerve-immunoreactive nuclei per animal and side were analyzed in c. d ImmunobLOTS for Sp1 from HN of the injured and intact sides at 7 days post-injury in mice treated with L-NAMe (180 mg kg⁻¹ day⁻¹, i.p.) or its inactive stereoisomer D-NAMe from day 5th after nerve resection. gdph and a-tub/β-act were internal controls for qRT-PCR and western blotting, respectively. e, f Plots represent the number of neurons (in percent) in the injured versus the intact side taken as 100%. In e, administration of vehicle and drugs were intraperitoneal (i.p.). Mit-A was orally administered except when indicated as i.p. applied in treatment, samples is indicated in parentheses. Error bars, SEM. *p < 0.05, **p < 0.01, ***p < 0.001; n.s., not significant; by paired (injured vs. intact side within a treatment, a–f), unpaired (comparison between two groups, f) Student t-test or one-way ANOVA with post hoc Holm–Sidak method (comparison between more than two treatments, a, f). Source data are provided as a Source Data file.

Time-lapse images were taken at 5-s intervals for at least 50 min. All experiments were performed at 31 °C. For the analysis of Ca⁺² dynamics, regions of interest including overall SMN somata were used and the background was subtracted from each selected SMN. SMN Ca⁺² concentrations were estimated using the maximal fluorescence equation:

\[
\frac{[Ca^{2+}]}{[Ca^{2+}]_0} = \frac{K_2}{R_0} \frac{F_0}{F_{max, Max}} = \frac{(1 - F/1 - F_{max})}{(1 - F/1 - F_{max})}
\]

where \(K_2\) is the Fluo-4 AM dissociation constant, that in our experimental conditions for each analyzed SMN. \(F_{max}\) was the recorded fluorescence. \(F_{max}\) and \(F_{max}\) values were determined at the end of each experiment, by exposing SMNs to the Ca⁺² ionophore ionomycin (10 μM) and subsequent addition of the Ca⁺² chelator, EGTA (15 mM), respectively. \(K_2\) and \(F_{max}\) values for the experimental conditions and cell types were estimated in vitro by using standard methods.⁶⁸

Nerve injury models. Rat pups (P3) and adult mice (>2-month-old) were subjected to XIIth nerve crushing or partial resection, respectively. Both modalities of nerve injury induce neuronal hyperexcitability preceding motoneuron loss mainly by glutamate-induced excitation⁶⁸. Anesthetized animals (1.5–3% isoflurane) were prepared for the experimental protocols. The rats in the right XIIth nerve was isolated from the surrounding tissue and the common nerve trunk was thoroughly crushed with microdissecting tweezers, applied for 30 s (Fig. 5f, Supplementary Fig. 9). In adult mice, following nerve isolation, a 2-mm segment was surgically removed (Fig. 4c). Both types of lesion were performed just proximal to the bifurcation of the main XIIth nerve trunk into lateral and medial branches. In adult mice, axonal regeneration, from the proximal stump of the injured nerves, was minimized by a tied suture thread (3/0). The incision was sutured and cleaned with saline (0.9% sodium chloride)-iodine). The animals received further injections at different time points after nerve injury, depending on the experimental paradigm. All animals received one post-operative injection of penicillin (20,000 i.u. kg⁻¹ i.m.) for post-surgery analgesia.

Histological analysis. Animals, under deep anesthesia (7% chloral hydrate), were intravenously injected with heparin and immediately perfused transcardially with phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in 100% O₂) received intramuscularly atropine (0.2 mg kg⁻¹). The medulla and/or lumbar spinal cords (L1–L3) were rapidly dissected and post-osmidized (from 0 to 250) and analyzed using the software provided by Leica. In all cases, the area delimiting p11-positive or TASK1-positive somata or Sp1-immunoreactive nerve were manually traced, and for each cell, the mean optical density (o.d.) or fluorescence intensity was measured. MNs were acquired in sections obtained from at least three animals per condition. Treatments were blinded for researcher who performed measures.

For Nissl or neutral red staining, brainstem sections containing the HN were mounted on slides, rehydrated, and immersed in Nissl (10 min, 0.5% cresyl violet) or red neutral (5 min, 1% neutral red in 0.2 M acetate buffer, pH 3.3) staining solutions. The sections were then rinsed with distilled water, dehydrated in ethanol, cleared in xylene, and covered with DePeX.

The number of neurons with a well-defined nucleus was quantified in one of each three serial sections through the whole rostrocaudal extent of the HN or in one of each three–six serial sections through the L1–L3 or L3–L5 segments of the lumbar spinal cord. SM32-identified MNs were counted in 10 horizontal or 30 coronal sections of the lumbar spinal cord from 4-month-old mice. All quantifications were carried out by researchers blinded to the lesion side, treatment and genotype groups.

SMNs, plated in four-well tissue culture dishes (15,000 cells per well), were processed by double-immunostaining for p11 and TASK1. At 3–4 DIV, cRNA-treated and siRNA₅₋₁₅-treated SMN cultures were fixed in 4% PFA (15 min, room temperature) and 100% methanol (10 min, 4 °C) and incubated overnight at 4 °C with polyclonal antibodies against p11 (1:100) and TASK1 (1:100) developed in rabbit. These were further incubated with the respective secondary antibody, followed by anti-IgG secondary antibodies conjugated with Cy3 and Cy2 (1:200; Jackson ImmunoResearch Laboratories, respectively). Dishes were analyzed using a Leica (Nussloch, Germany) confocal fluorescence microscope. Images were acquired through the z-plane containing the highest density of large ganglion cells and well defined neurites as above. The pinhole opening was 1 Airy unit. Acquisition settings were identical for cRNA and siRNA-treated cultures, which were processed and acquired in parallel. Images were captured and processed for background subtraction to obtain the maximum dynamic range of intensities (from 0 to 250) and analyzed using the software provided by Leica. In all cases, the area delimiting somata and neurites was automatically traced by applying a macro, and for each image, the integrated and mean immunofluorescence intensities of SMN cell bodies and neurites were obtained in both the cRNA-treated and siRNA₅₋₁₅-treated cultures.

Lentiviral constructions. All neuron-specific LVVs used in this work were designed, constructed, and produced under the supervision of Dr. S. Kasprow (Bristol University, Bristol, UK) during a research stay of G.R.B. in his lab. All the lentiviral constructions were made based on the vector pTFY-mCMV/SYN. This system uses a transcriptional amplification strategy (TAS) to bidirectionally enhance the transcriptional activity of human synapsin-1 (SYN) promoter, by joining upstream a minimal core promoter (from 0 to 250) and analyzed using the software provided by Leica. In all cases, the area delimiting somata and neurites was automatically traced by applying a macro, and for each image, the integrated and mean immunofluorescence intensities of SMN cell bodies and neurites were obtained in both the cRNA-treated and siRNA₅₋₁₅-treated cultures.
generate the final lentiviral plasmid. This same procedure was also followed to obtain the LVV-shRNA\textsubscript{p11} plasmid using the shRNA\textsubscript{p11} sequence (5′-TAGTCA TTGCATGCAATGGTGGTTTTGGCCACTGACTGACCACCATTGTGCAATGACTA-3′).

A different strategy was used to generate the LVV-dnSp1 vector. The full cDNA sequence for the Sp1 dominant negative\textsuperscript{71} corresponding with the C-terminal domain of the consensus sequence (622–788; a gift from Dr. Johann Rotheneder, Medical University of Vienna, Austria) was cloned into the pIRES2-eGFP. A PCR product containing the dnSp1-eGFP sequence was cloned into pTYF-mCMV/SYN plasmid to generate the final LVV-dnSp1 construction.

Transient co-transfection of HEK293F T cells with shuttle plasmids, the packaging vector pNHP, and the envelope plasmid pHEF-VSVG was performed.
to produce the LVV plasmid system derived from HIV-1 used for this study. The virus-containing medium was harvested 48 or 72 h after transfection and filtrated using a 0.45 μm filter (Millipore). Filtrated medium was carefully added on the top of a 20% sucrose solution and subsequently centrifuged at 75,000 x g for 2 h. After removal of the supernatant, the pellet was re-suspended in PBS.

Finally, the titer of the virus (10^9 to 10^11) was determined using the ELISA kit Lenti-X P24 Rapid Titer (Clontech) which measures the amount of lentiviral capsid protein p24. Cells were transfected with the different LVVs at a five times multiplicity of infection.

**Plasmid constructions, deletions, and site-directed mutagenesis**

The promoter region of the p11 human gene (about 1.4 kb of the gene 5’ flanking region) was amplified using primers designed to add complementarities overhangs in BglII-HindIII and the bacterial artificial chromosome (BAC) RP11-139D23 as genomic DNA template (developed by Dr. Kazutoyo Osoegawa, and acquired through the BACPAC Resource Center, Children’s Hospital Oakland Research Institute, California, USA). PCR conditions used were 95 °C 5 min, (95 °C 30 s, 52 °C 30 s, 72 °C 1 min) x 35 cycles, and 72 °C 5 min for final extension. The PCR amplicon was purified using a PCR Purification Kit (Qiagen), digested with BglII-HindIII and cloned into the pG4.10 luciferase Firefly reporter plasmid (Promega) in BglII-HindIII to generate − 4836 + 6911-F1 (pG4.10-p11-F1) construct. The human promoter was sequenced by performing the PCR.

To narrow down NO-responsive regions in the promoter sequence, we performed serial 5’ deletions of the promoter by PCR, changing the 5’ sense primer. The common −89 HindIII 3’ primer was 5’-AAGCTTACCTTGCGGGCCCAGCGGGGCGG-3’. Primers used for 5’ deletions of the p11 promoter are indicated in Supplementary Table 1. The amplicons were cloned into pG4.10 as above. When these regions were identified, we looked for transcription factors with the ability to bind to the deleted promoter segments using a virtual transcription element search system. Site-directed mutagenesis of the transcription factors’ binding sequences were then performed by PCR.

For site-directed mutagenesis, modified versions of the −96 fragment 5’ primer were used to develop PCR. The related primers were used as indicated in Supplementary Table 1.

**Luciferase reporter assay**

dNSC34 cells were transiently co-transfected with 5 μg of the different constructions cloned in pG4.10-F1 and 0.5 μg of the wild-type Renilla luciferase control reporter vector pGL4.74-REN (Promega) to determine the transfection efficiency, using the calcium phosphate precipitation method. 40,000 dNSC34s per well were transfected. After 24 h in the transfection medium, this medium was washed out and replaced by culture medium supplemented with diverse drugs. Finally, luciferase activity was measured using the Dual-luciferase Reporter Assay System (Promega). Promoter activity was measured in relative units of luciferase (RLU) of Firefly versus Renilla activities and normalized to the control.

Undifferentiated NSC34s (CELLutions Biosystems Inc., Toronto, Canada) were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM l-glutamine and 1% (v/v) penicillin/streptomycin. To induce maturation, NSC34s were plated at low density (4,000 cells cm^-2) in 1:1 DMEM plus Ham’s F12 medium supplemented with 1% (v/v) FCS, 1% penicillin/streptomycin, and 1% modified Eagle’s medium non-essential amino acids (Sigma-Aldrich).\(^\text{65}\) Differential phenotype of these cells was visually verified after 7–9 days in differentiating medium and transfections were carried out as above.

**Electrophoretic mobility shift assays**

Nuclear extracts were isolated from −4 x 10^6 dNSC34 cells. dNSC34s were gently washed in PBS and collected by centrifugation in a microcentrifuge at 8,600 x g. Cells were re-suspended in isotonic buffer (10 mM Tris–HCl pH 7.5, 2.5 mM MgCl2, 3 mM CaCl2, 0.3 M sucrose, 1 mM DTT) plus a protease inhibitors cocktail (Sigma). Next, plasma membrane was disaggregated in a 10% NP40 solution and the total mix was centrifuged at 8,600 x g (30 min, 4 °C). Subsequently, the pellet was re-suspended in extraction buffer (20 mM Hepes pH 7.9, 1.5 mM MgCl2, 0.2 mM EDTA, 25% Glycerol (v/v)). After further centrifugation (14,000 x g, 10 min, 4 °C), the resultant supernatant, enriched in nuclear proteins, was aliquoted.

Double stranded probes were obtained by mixing equal volumes of both complementary primers (wild-type and Mut 5; Supplementary Table 1) at equimolar concentration (25 pM). We next placed the probes in a standard heat-block at 90–95 °C for 3–5 min followed by cooling at room temperature overnight. Then, the probes were labelled with a T4 Polynucleotide Kinase (Promega Gel Shift Assay System) using [γ^32P]-ATP (3,000 Ci mmol^-1) at 10 mCi ml^-1. Labeled probes were purified by spin-column chromatography (Microspin G-50 Columns, GE Healthcare).

Binding reactions between the nuclear extracts and radiolabelled probes were performed by incubating 1 μl of nuclear extract, 4 μl of binding buffer (50 mM Tris–HCl pH 7.5, 250 mM NaCl, 2.5 mM EDTA, 2 mM DTT, 20% Glycerol), and 1 μl of poly (dI-dC)/(dI-dC) (1 mM μl^-1 in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA) in the presence or absence of competitive antibodies [anti-Sp1 (2 μg ml^-1), Abcam, Cat# ab34804, RRID:AB_10794111, or anti- Sp3 (2 μg ml^-1), Santa Cruz Biotechnology, Cat# sc-13018, RRID: AB_2194471]. Nuclear extracts were pre-incubated for 1 h at 4 °C with antibodies or with oligonucleotides comprising the canonical sequence for Sp1 (5’-ATTCGATCAGGGGGCGGCGGCGG-3’; 1.75, 3.5 and 5.25 pmol μl^-1) or AP-2 (5’-GATCGAATGCCCCGGCCGCGCCGC-3’; 5.25 pmol μl^-1) before adding the radiolabeled probe for 25 min at RT. Products were electrophoresed at 30 mA for 3 h on 4.8% polyacrylamide gels in high ionic strength buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5). Dried gels were analyzed by autoradiography (Personal Molecular Imager™ (PMI™) System, BioRad).

**Evaluation of disease progression in SOD1-G93A mice**

Transgenic males were pre-treated at pre-symptomatic stage (P30). Then, habituation sessions for rotated and grip strength began at P45. One or two valid measures were obtained before cannula implantation for chronic oligonucleotide administration at P60 to discard surgical effects on motor performance. Only animals with no evident motor deficits were included in the study. Researchers blinded for treatments did all behavioral analyses.

Surgery to implant the cannula infusion system (Plastics One Inc., Roanoke, VA, USA) for intracerebroventricular chronic administration of oligonucleotides was performed in 2-month-old animals. Briefly, under general anesthesia (as above), mice were fixed with ear bars in a stereotaxic frame and the skin was incised and subsequent disinfecting. The guide cannula and its respective dummy cannula were carefully advanced through a drilled round window (0.5 mm in diameter) in the midline of the interparietal–occipital bone junction. Cannula was placed in parallel and close to the inner portion of the occipital bone (Fig. 3g). The infusion system was finally attached to the skull by means of acrylic resin once the end of guide cannula was positioned near to the bregma. To gain in stability and endurance, the system was cemented with the same resin to two screws fixed in the left and right parietal bones. Post-operative cares were as indicated in Supplementary Table 1.

The body weight for each mouse was weekly recorded, beginning one week after surgery. Individual weights were normalized taking the mean value of initial measures as 100%. At the beginning of the study (66 days), mean body weight was similar for the two cohorts (cRNA: 23.4 ± 0.5 g; siRNA: 24.2 ± 0.4 g; p = 0.124, Student t-test). Survival times were analyzed by means of Kaplan–Meier survival analysis with Log-Rank tests for statistical significance. The end stage was defined as the day when mice were unable to right themselves 30 s after being placed on their side. At this time point, animals were euthanized using a carbon dioxide-enriched atmosphere.
Evaluation of motor function was assessed by rotarod, footprint, runtime, and grip strength tests. Rotarod test was carried out to judge motor coordination, strength, and balance. Animals were daily trained for one week before data acquisition. Mice were placed individually on a cylinder (3.4 cm in diameter), rotating at a constant speed of 15 rpm. The longest latency without falling for each animal was recorded once per week, after three attempts to keep onto the rotarod (Rotarod LE8500, Panlab SA, Barcelona, Spain). Rotarod recordings began one week before surgery and an additional test was performed one day before surgery. The cut-off time was taken at 180 s. As starting point to construct the cumulative probability curve of symptoms onset, we used the first day in which a mouse

Fig. 10 Schematic diagram modeling the role of the Sp1–p11–TASK1 triad in neurodegeneration. a Physiological expression of the transcription factor Sp1 maintains baseline levels of p11, which regulates IME by controlling TASK1 trafficking from the endoplasmic reticulum (ER) to the plasma membrane. p11–TASK1 complex dissociation is required to overcome ER retention and normal recycling of TASK1 to the surface. Under healthy conditions, when neuronal IME is fine-tuned, moderated Ca^{2+} influx, through GluRs and/or VSCCs, leads to physiological responses, which are controlled by mechanisms of intracellular Ca^{2+} buffering and extrusion. In neurological conditions, over-expression and/or de novo expression of nNOS could be the switch on (star) to initiate a positive feedback loop that contributes to the degeneration and death of sick neurons. Usually, NO production in the nervous system is directed by Ca^{2+} influx driven by NMDA receptors. The calmodulin (CaM)–Ca^{2+} interaction, which is now enabled to activate nNOS, allows for the coupling of pathological NO synthesis to Ca^{2+} entry. NO reacts rapidly with the superoxide anion (O_{2}^{-}) to form the highly toxic product peroxynitrite (ONOO^{-}) and/or promotes S-nitrosylation, triggering protein misfolding and aggregation, thus leading to neurotoxicity (omitted for simplicity). In addition, a persistent pathological concentration of NO, involving the sGC/PKG-RhoA/ROCK pathway (omitted for simplicity), induces Sp1 and stimulates the human p11 promoter depending on, at least, a Sp1-binding site (GC-box). Subsequent p11 upregulation sequesters TASK1 to the ER, thus disrupting the normal recycling of this subunit to the surface. This TASK1 uptake/replenishment unbalance enhances IME, which, in turn, increases the opening probability of VSCCs and, GluRs in the presence of glutamate, thereby exacerbating Ca^{2+} entry into the cell. Ca^{2+} overload might contribute to neurodegeneration and, finally, to neuron death. Whether NO of glial origin and/or other unidentified factors promote Sp1 expression/binding and/or p11 expression are not discarded and merit for further investigation.
showed motor function deficits on the rotarod test (+10% reduction in the time to fall) followed by progressive deterioration in performance in the next sessions.

The rat hind limb and hind forelimb were performed weekly starting one week after surgery. The hind feet were immersed into innocuous black ink and animals were placed on a conventional calibrated paper along a gangway of 50 cm length. Footprints were analyzed with respect to the step length as illustrated in Supplementary Fig. 6e. Additionally, the time duration required by the mice to run along the gangway was measured.

Muscle strength of forelimbs and hind limbs was monitored on a grip strength meter (Grip Strength Meter, BIOSEB, Chaville, France) twice a week for each mouse. The animal held by the tail, once ensured that its fore-limbs or hind-limbs were gripping the bars correctly, was released as quickly as possible, and the peak force at which time the hand was dropped was recorded and expressed in grams. The highest outcome of three trials per mouse was recorded for each session. Mean value per week for each mouse was taken to construct time course of grip strength for the two cohorts, cRNA-treated or siRNA-treated SOD1-G93A mice.

Electromyography was performed by inserting a concentric needle electrode into the gastrocnemius muscle of anaesthetized mice (1.5% isoflurane mixed with 100% O2). To ground the system, a monopolar needle electrode was inserted into the tail of the mouse. The electrical signals were amplified and filtered at a bandwidth of 10 Hz–10 kHz for display and digitization purposes. Spontaneous activity of the muscle was monitored and recorded for 30 min, after a period of 5 min of stabilisation. Only spontaneous activity with an amplitude of at least 50 μV was considered as a significant signal. Mean and maximum frequency of fibrillation potentials were calculated from automatically selected single intervals all along the recording session (30 min). Thus, 1,800 data per animal and session were used to construct box-plots (Fig. 7, m).

Drugs and treatments. SMN cultures were treated at different DIV depending on the experimental series. Oligonucleotides (cRNA, siRNA, 2 µm each) and LJV’s (LVV-shRNAnc, LVV-shRNA, LVV-dnShp1; 2 µl in 350 µl of culture medium) were added to the culture medium at 1 DIV, and were washed out at 4 DIV. For patch-clamp recordings, SMNs (4 DIV) were initially perfused with normal aCSF to obtain baseline control data. Next, SMNs were superfused for 10 min with aCSF containing AEA (3 or 10 µM) before voltage responses were acquired again. Finally, a last round of acquisition was taken after a 10 min washout with AEA-free aCSF (Fig. 2c). For survival experiments, bupivacaine (40 µm, 60 min), silonurate (0.8 mM, each 10 min for 70 min), AEA (0.03, 0.05, 0.1, 0.3, 5, 10, or 30 µM, 50 or 60 min), ethanol (0.07%, v/v, 60 min), AM281 (0.5 µM, 65 min), AEA+AM281, alone or in combination with glutamate (150 µM, 30 min) were added to the culture medium at 4–5 DIV (Fig. 2d, e, Supplemental Fig. 4c,d). For Ca+ imaging experiments, Flu-o-charged SMNs were initially perfused (~5 min) with Krebs buffer containing [Ca++] gene expression (20 µM), a-conotoxin GVIA (0.5 µM), α-agatoxina (0.1 µM), NBQX (20 µM), or (DL)-APV (50 µM) were added, for at least 5 min or until a new plateau was reached. Then, glutamate (150 µM) was co-added to the solution for 30 min. The Sp1-interfering agent Mit-A and Riluzole did not lead to additive or synergistic effects (not shown). Combined treatment with the LVVs (Fig. 4c). p11 upregulation in the HN began as soon as 1 day after XHII nerve transection. In order to minimize feasible harmful effects of p11 upregulation, LJV’s were microinjected in the midline and 0.7 mm below the dorsal surface of the medulla at the time of injection. The intramedullar injection rate was 0.1 µl min-1 and the microprobe remained in situ for 10 min before being slowly withdrawn at the end of injection. The incision was sutured and cleaned with an aseptic solution (povidone-iodine). 24 days after lentiviral infusion, animals were processed for immunohistochemistry as above.

In another experimental series, adult mice under deep anesthesia (as above) were placed in a stereotoxic device and surgically prepared to receive a single injection of LVVs or vehicle (sterile PBS). Briefly, a midline dorsal incision was performed to expose the spinal cord, and after hemostasis was achieved (~25 µm tip diameter), connected to an oil-filled tubing system connected to a Hamilton syringe. After the injection, the cannula was left in place for 5 min and then slowly retracted. Sham mice were similarly implanted with the chronic injection system and, weekly manipulated as the experimental group, but volume delivery was omitted.

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Statistical Analysis. Data are all presented as mean ± s.e.m. The number of animals per group was determined based on preliminary experiments showing the expected level of variability. For comparisons involving more than two groups, a one-way or two-way ANOVA followed by Bonferroni's multiple comparisons test was used. For pairs of means, an unpaired Student’s t-test was used. A probability level of P < 0.05 was considered statistically significant. The results are expressed as mean ± s.e.m. for each experiment. Data points were excluded from the statistical analysis unless otherwise noted.
Studies using SOD1-G93A mice were all blinded to researcher until data were grouped. The remaining experiments were not blinded unless otherwise noted.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The source data underlying Fig. 1d, e, g-i, k, 1b, d, e, g-j, 3d, e-i, l, d, h, j, 6a-i, 7a, b, d-h, l, m, 8, 9f, and Supplementary Figs. 1, 2, c-f, i-k, 4b-d, 5a-c, e-i, 6a-f, i-l, 7b-g, 9a-c, e-g, b, 10-12, 13a-c, e-i, 14 and 15 are provided as a Source Data file. In situ hybridization images were obtained from © 2004 Allen Institute for Brain Science. Allen Mouse Brain Atlas. Available from: http://mousebrain-map.org/ (Fig. 4a) and from © 2008 Allen Institute for Brain Science. Allen Mouse Spinal Cord Atlas. Available from: http://mousespinalbrain-map.org/ (Figs. 3a, 9a, b). All other data, raw images and/or supplemental materials are available from the corresponding author upon reasonable request.

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Author contributions

B.M.-L. conceptualized, designed the study, organized data, prepared figures and, wrote and reviewed the paper. I.G.-P., G.R.-B., V.G.-M., G.D.-V., F.P., A.G.-C., N.I. and D.G.-F. performed experiments and data analysis. B.M.-L., A.C.-G., and R.M.S. obtained the funding. D.G.-F. supervised initial electrophysiological experiments. R.M.S. and A.G. supervised initial experiments using primary cultures of embryonic spinal motoneurons and calcium-imaging. A.C.-G. supervised experiments using the human p11 promoter. All authors participated in critical review of the manuscript.

Additional information

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