Enhanced neuronal Met signalling levels in ALS mice delay disease onset

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Signalling by receptor tyrosine kinases (RTKs) coordinates basic cellular processes during development and in adulthood. Whereas aberrant RTK signalling can lead to cancer, reactivation of RTKs is often found following stress or cell damage. This has led to the common belief that RTKs can counteract degenerative processes and so strategies to exploit them for therapy have been extensively explored. An understanding of how RTK stimuli act at cellular levels is needed, however, to evaluate their mechanism of therapeutic action. In this study, we genetically explored the biological and functional significance of enhanced signalling by the Met RTK in neurons, in the context of a neurodegenerative disease. Conditional met-transgenic mice, namely Rosa26LacZ-stop-Met have been engineered to trigger increased Met signalling in a temporal and tissue-specific regulated manner. Enhancing Met levels in neurons does not affect either motor neuron (MN) development or maintenance. In contrast, increased neuronal Met in amyotrophic lateral sclerosis (ALS) mice prolongs life span, retards MN loss, and ameliorates motor performance, by selectively delaying disease onset. Thus, our studies highlight the properties of RTKs to counteract toxic signals in a disease characterized by dysfunction of multiple cell types by acting in MNs. Moreover, they emphasize the relevance of genetically assessing the effectiveness of agents targeting neurons during ALS evolution.

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Signalling by receptor tyrosine kinases (RTKs) is involved in cell communication events regulating tissue morphogenesis during development and tissue homeostasis during adulthood.1 In vivo, RTK signalling levels vary according to a number of parameters, such as RTK expression levels, ligand availability, action of positive/negative signalling regulators, and components of the signalling cascade. The levels of RTK signalling determine qualitatively different biological outcomes.2 Given the multiple roles of RTKs in coordinating basic biological processes, modulating their activation levels is a means of achieving different cellular responses in normal processes and in pathological conditions. Notably, RTK activation is tightly regulated in healthy adult tissues as aberrant signalling in susceptible cells can cause pathologies, such as cancer.1 Conversely, studies on degenerative diseases have shown that following stress or cell damage there is nearly always a reactivation of RTK signalling, coinciding with periods of active fight for survival/repair.4,5 Therefore, for repair of damaged tissues.4 In contrast, studies on cultured cells and on animal models have shown that symptoms linked to degenerative diseases can be ameliorated when activation of appropriate RTKs is achieved through exogenous ligand administration.4,6 However, it is still not clear to which extent levels of remobilized endogenous RTKs are limiting for effective neuronal repair in disease CNS.

Over the last years, a regional and temporal map of RTK-dependency has emerged, suggesting that an appropriate enhancement of RTK signalling might be beneficial to efficiently counteract disease onset and progression. This issue is particularly relevant for neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), an adult onset motor neuron (MN) disease caused by pathological processes occurring in both neuronal and non-neuronal cells.7 ALS involves progressive degeneration of upper and lower MNs, culminating in muscle wasting, and death mostly due to respiratory failure. Although the aetiology of most cases remains unknown, 10–20% of familial ALS is caused by mutations in the superoxide dismutase1 (SOD1) gene.
Consistently, transgenic mice expressing the mutant forms of human SOD1 recapitulate a number of ALS symptoms and have been instrumental in evaluating the molecular and cellular events underlying ALS pathology. Moreover, genetic and cell biological studies based on the differential expression of mutant SOD1 in distinct cell types have demonstrated that although death of MNs causes ALS symptoms, the disease also renders other cells, such as astrocytes and microglia, dysfunctional. Thus, MN loss, in addition to a cell-autonomous origin, is also triggered by non-cell-autonomous defects involving toxicity of other unhealthy cells. Notably, these different cell types play distinct roles in ALS pathogenesis. Damage within MNs is primarily associated with disease onset and its early progression phase, whereas damage within microglia and astrocytes accelerates disease. Among several strategies to alleviate ALS symptoms, a major hope has been placed on the ability of trophic factors, both extrinsic and intrinsic, to function extrinsically and/or intrinsically to modify disease. The need to understand the relative contribution of providing beneficial signals to different cell types involved in the disease.

In this study, we assessed the biological and functional significance of enhanced signalling, above endogenous levels, downstream of the Met RTK specifically in neurons during neuro-degenerative diseases, such as ALS. A number of features makes the Met receptor of special interest to address RTK signalling levels during neuro-degeneration. During development, activation of the Met receptor by its ligand HGF regulates MN fate at multiple levels, including identity acquisition, axonal growth, and survival. Consequently expression of the Met chimeric protein (Mettg) in neural tissues (Figures 1c and d). Western blot analysis of E15.5 embryos and P7 mice revealed the presence of Mettg in dissected brains and spinal cords only after recombination (Figure 1d). To estimate Mettg levels versus endogenous Met, we performed western blot analysis of brain and spinal cord protein extracts at different developmental stages using antibodies recognizing the kinase domain of both endogenous and Mettg. Quantification analyses indicated that Mettg levels were at least 5/7-fold increased in brains and spinal cords of heterozygous Nes-R26Mettg, when compared with endogenous mouse Met (Figures 1e and f). Consistently, Mettg levels were twofold higher in homozygous mice compared to heterozygous littermates (Figures 1d–f).

We next characterized the R26lacZ-stop-Met b mice before and after recombination by following the expression of the lacZ-stop cassette and met19 transcript in adult brains and spinal cords. We found a decrease in β-galactosidase activity and lacZ transcripts in Nes-R26Mettg compared with R26lacZ-stop-Met transgenics, indicating that recombination in several brain regions occurred as expected (Figures 2a–d, Supplementary Figures 1 and 2). Conversely, met19 was expressed in brains only after recombination in a pattern complementary to lacZ distribution (Figures 2e and f). High levels were found in the hippocampus, cerebellum, cerebral cortex, and cervical spinal cord (Figures 2g–i). Expression studies performed on adult lumbar spinal cord sections revealed that the expression of the lacZ-stop cassette and met19 transcript occurred in approximately 56% of these cells (56.3 ± 1.1; P < 0.0001; Figure 3g).

Molecular and cellular characterization of Nes-R26Mettg mice. Although the Rosa26 locus drives gene expression ubiquitously, we observed that the lacZ distribution in brains and spinal cords of adult R26lacZ-stop-Met b mice appeared restricted to distinct cell types (Figures 2a–d and 3a–f, Supplementary Figures 1 and 2). Colocalization studies revealed β-galactosidase activity predominantly in...
NeuN-positive neurons, but not in glial fibrillary acidic protein (GFAP)-positive astrocytes (Figures 4a–h). The restricted neuronal lacZ expression was also observed in cultured cells (Figures 4i–n). Thus, the genetic setting we adopted (CMV-enhancer/β-actin-promoter in Rosa26) results in an animal model with a restricted expression of the transgene, indicating that mettg should be predominantly confined to neurons after nestin-cre-mediated recombination. Consistently, mettg transcripts colocalized with Smi32-positive neurons, but not with GFAP-positive astrocytes (Supplementary Figure 3). The restricted expression of mettg in neurons was also observed in Nes-R26Met adult spinal cords.
where it was found in dorsal horn neurons, intermediate lateral neurons, and MNs, but not in GFAP-positive astrocytes (Figure 5). Thus, mettg is predominantly restricted to neurons in Nes-R26Met mice.

We next examined whether Mettg protein was active by following its phosphorylation state using anti-phospho-Met antibodies. High levels of phosphorylated Mettg were found in the pons, medulla, lateral ventricles, rostral-migratory stream, olfactory bulbs, cerebral and lumbar spinal cords (Figure 6 and data not shown). These results show that Mettg is predominantly functional in spatially restricted domains, which possibly correlate to a map of cellular competence for Mettg activation influenced by a combination of parameters, such as environmental contexts (e.g., endogenous HGF levels) or permissive intracellular mechanisms (e.g., signalling modulators). Altogether these data show that Nes-R26Met mice can be a useful animal model to investigate the functional consequences of enhancing Met levels in distinct cell types, using available tissue-specific cre-lines.

**Phenotypical characterization of Nes-R26Met mice.** As previously discussed, Met regulates specification, axonal growth, and survival of MN subtypes during development.17–20 We therefore evaluated whether enhanced Met levels influence MN numbers in Nes-R26Met mice. By staining spinal cord sections either with cresyl violet or with vesicular-acetylcholines-transporter (VACht) antibodies, similar MN numbers were found at thoracic (data not shown) or lumbar spinal cord levels in Nes-R26Met and control animals (P > 0.05; Figures 7a–d and g). As expected by the

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**Figure 2** Molecular characterization of Nes-R26Met and R26LacZ-stop-Met mice in adult brains. (**a–d**) Expression of the lacZ reporter gene was followed by measuring β-galactosidase (X-Gal) activity (a and b) or its transcript levels (c and d) on sagittal sections. Decrease in X-Gal and lacZ transcripts were found after nestin-cre-mediated recombination. (e and f) Expression of the met chimeric transgene in sagittal section of adult Nes-R26Met and R26LacZ-stop-Met brains. (g–i) High levels of transgenic met transcripts were detected in the hippocampus (g), cerebellum (h), cervical spinal cord (i). Panels (g–i) show enlarged views of brain areas delineated by the rectangles in (e). cb, cerebellum; cc, cerebral cortex; h, hippocampus; sc, spinal cord
colocalization studies, no differences in GFAP-fluorescence intensity were observed (P > 0.05; Figures 7e, f and h).

As body weight is a generic indicator of animal physiology influenced by body metabolism, activity, and feeding behaviour, the weight of Nes-R26Met mice was followed over-time and no significant differences were found versus controls (P > 0.05; Figure 7j). We next performed the rotarod test to evaluate the overall motor function and no significant differences were found in Nes-R26Met versus controls (P > 0.05; Figure 7i). Altogether, these studies show that enhancing Met levels in neurons does not cause gross physiological abnormalities.

Neuronal-enhanced Met levels counteract ALS symptoms in SOD transgenic mice. We next investigated the functional relevance of genetically enhancing Met signalling levels in the context of a neurodegenerative disorder, such as the ALS. The Nes-R26Met mice were therefore crossed with a strain carrying high copy numbers of the SOD1G93A transgene to generate an ALS animal model with increased Met levels in neurons. Five groups of mice were generated: (a) wild-type; (b) Rosa26 LacZ/C0 stop/C0 Met, (c) Nes-R26Met, (d) SOD1G93A (referred to as SOD), and (e) Nes-R26Met;SOD1G93A (referred to as Nes-R26Met-SOD). As no significant changes were observed between wild-type, Rosa26 LacZ/C0 stop/C0 Met and Nes-R26Met for all parameters examined, results of control animals included these three groups.

By evaluating the life span of SOD and Nes-R26Met-SOD mice, we found that neuronal-enhanced Met levels prolonged the survival by 13 days (mean age, SOD: 136 ± 2 days; Nes-R26Met-SOD: 149 ± 3 days; P = 0.0021; Figure 8a). As reduction of body weight is an objective measure of ALS disease, we examined its evolution in SOD and Nes-R26Met- SOD mice, and found that neuronal-enhanced Met levels delayed body weight loss. In particular, loss of 10% body weight was retarded for 13 days (SOD: 123 ± 3 days; Nes-R26Met-SOD: 136 ± 5 days; P = 0.0094; Figure 8b), whereas no significant differences were observed during disease progression (SOD: 12.9 ± 2.5 days; Nes-R26Met-SOD: 13 ± 2.4 days; P > 0.05; Figure 8c). Together, these results indicate that enhanced Met levels in neurons counteract ALS symptoms in SOD transgenic mice.

Improved motor performance and delayed onset of paralysis in SOD mice with neuronal-enhanced Met levels. The neuro-degeneration defects causing ALS disease lead to progressive muscle weakness, atrophy, and paralysis. Screwed hindlimbs and locomotor defects are among the first symptoms affecting transgenic ALS mice. We
monitored the appearance and progression of motor defects in SOD versus Nes-R26Met-SOD compared with controls by employing swimming tank and footprint assays. Onset of swimming defects was delayed by 14 days, as estimated by the increased time that mice needed to execute this motor task (SOD: 113 ± 3 days; Nes-R26Met-SOD: 127 ± 3 days; P = 0.0015; Figure 8d), whereas disease progression was unchanged (SOD: 30 ± 3.9 days; Nes-R26Met-SOD: 22 ± 2.7 days; P > 0.05; Figure 8e). Therefore, neuronal-enhanced Met improved motor strength and swimming performance of SOD mice by acting specifically on disease onset.

The motor capability of Nes-R26Met-SOD versus SOD mice was further evaluated by performing footprint studies (Supplementary Figure 4). The forepaw/hindpaw overlap analysis revealed a delay of 19 days in the locomotor gait dysfunction (SOD: 108 ± 3 days; Nes-R26Met-SOD: 127 ± 3 days; P = 0.0015; Figure 8f), whereas disease progression was unchanged (SOD: 30 ± 3.9 days; Nes-R26Met-SOD: 22 ± 2.7 days; P > 0.05; Figure 8e). Therefore, neuronal-enhanced Met improved motor strength and swimming performance of SOD mice by acting specifically on disease onset.

**Figure 4** Subcellular localization of the lacZ reporter gene in Nes-R26Met and R26lacZ-stop-Met mice. (a and b) Colocalization studies of X-Gal activity with NeuN protein showing the reporter activity in neuronal cell types. (c and d) Enlarged view of the hippocampus area. (e–h) Colocalization studies of X-Gal activity with GFAP protein showing that the lacZ reporter gene is not predominantly expressed in astrocytes. Panels (g and h) correspond to an enlarged view of the hippocampus. (i–k) Mixed cell cultures derived from E12.5 R26lacZ-stop-Met spinal cords were immunostained for neurofilament-160 (red) and β-galactosidase (green) proteins. (l–n) Astrocyte cultures from P2 R26lacZ-stop-Met spinal cords immunostained for GFAP (red) and β-galactosidase (green). DAPI was used to counterstain nuclei (blue), scale bar: 20 μm.

**Figure 5** Subcellular localization of chimeric met transcripts in lumbar spinal cords of adult Nes-R26Met mice. (a–c) Colocalization studies of exogenous met transcript with NeuN protein showing met expression in neuronal cell types. (d–f) Colocalization studies of exogenous met transcript with GFAP protein showing that transgenic met is not predominantly expressed in astrocytes. Panels (b and d) correspond to an enlarged view of spinal cord areas indicated by red and black rectangles in (a and b), respectively. Arrowheads in (c) point to MNs co-expressing chimeric met transcript and NeuN protein. Arrows and arrowheads in (f) indicate GFAP-positive astrocytes and MN expressing chimeric met transcripts, respectively.
Neuronal-enhanced Met levels attenuate MN loss in spinal cords of SOD mice. As motor performances were transiently improved in Nes-R26Met-SOD mice by delaying disease onset, we evaluated the neuroprotective effects of increased Met levels by quantifying lumbar spinal cord MNs. For these studies, we selected three animals among the Nes-R26Met-SOD, SOD, and controls at the symptomatic disease phase (120 days). This stage was chosen because all behavioural studies showed significant differences between groups. Lumbar spinal cord sections were stained with VAChT antibodies and MN numbers were determined (Figures 9a–c and m). As expected, we observed a significant 60% MN loss in SOD mice compared with controls ($P=0.0065$). By contrast, neuronal-enhanced Met in Nes-R26Met-SOD mice led to an improvement of MN maintenance as the surviving MN numbers increased by 32% compared with SOD ($P=0.0002$; Figure 9m).

We next assessed to what extent enhancing Met function in neurons influenced astrogliosis and microglia activation, which appear at disease onset and become more prominent during progression. In contrast to controls, activated GFAP-positive astrocytes (changes in fluorescence intensity) were detected in lumbar spinal cords, although reduced in Nes-R26Met-SOD compared with SOD (SOD: $15.5 \pm 1.9 \times 10^8$; Nes-R26Met-SOD: $9.9 \pm 1.4 \times 10^8$; $P=0.0164$; Figures 9d–f and n). Similarly, the number of microglial cells was reduced in Nes-R26Met-SOD mice compared with SOD (SOD: $281.7 \pm 14.6$; Nes-R26Met-SOD: $222.1 \pm 15.5$; $P=0.0016$; Figures 9g–i and o). Analysis of muscle innervation revealed that the increased MN numbers in Nes-R26Met-SOD was accompanied by an enhanced integrity of neuromuscular junctions (NMJs; SOD: $16 \pm 1.8\%$; Nes-R26Met-SOD: $41.7 \pm 11.4\%$; $P=0.041$; controls: $84.4 \pm 7.9\%$; Figure 9p). Thus, increased neuronal Met levels elicit a combination of protective effects in different cell types: (1) cell-autonomous protective effects on spinal cord MNs and for NMJ maintenance; (2) non-cell-autonomous delay of astrocyte activation and increased microglia cell numbers.

Discussion

Most of neurodegenerative diseases result from a combinatorial action of pathological signals produced by neurons themselves and by neighbouring cells acting in a non-cell-autonomous manner. A number of molecules including trophic factors and their receptors can elicit beneficial effects on disease-related cells when applied in vitro and/or when delivered in disease animal models. Understanding how these
molecules act on dysfunctional cells remains a key topic to clarify disease mechanisms and to evaluate their use for therapies. We show here that enhanced signalling by Met has an impact on a specific stage of ALS pathology when it is selectively upregulated in neurons. Indeed, transgene-mediated neuronal expression of Met elicits a beneficial effect in SOD1G93A by delaying disease onset, but not progression.

Our results are based on a genetic approach involving the generation of conditional met transgenic mice, in which Met signalling levels is modulated in a temporally and spatially regulated manner. Such an approach offers the possibility of exploring how enhanced Met signalling above endogenous levels influences cell fate in developmental events, in adult physiology, and in pathological conditions. Loss of Met function during development interferes with identity acquisition, axonal growth, and survival of MN subsets.2,17–20 We show here that neuronal-enhanced Met signalling levels do not affect either MN development or function in adulthood, whereas it influences MN maintenance in ALS pathological conditions. Thus, it is likely that excessive Met functions are restrained by mechanisms such as tissue homeostasis or by limiting amounts of ligand. Importantly, the dispensable function of Met in several adult tissues is in contrast to its requirement in counteracting degenerative processes following injuries, such as axotomy,21,22 hepatectomy,28 and skin-wound.29 These regenerative studies together with our findings indicate that in a pathological context, cell types like neurons become sensitive to the beneficial effects provided by Met. The generation of compound transgenics by crossing R26LacZ/C0 stop transgenic mice with available cre-lines will offer a unique genetic setting for determining tissue-specific sensitivity to enhanced Met signalling either during development, in adulthood, or in pathologies.

The pleiotropic functions elicited by the HGF/Met system in neurons have boosted the interest in exploring its potential for...
Genetically enhancing Met levels in ALS neurons

M Genestine et al

Cell Death and Disease
Figure 9  Effects of enhanced Met signalling in MN maintenance and astrogliosis at ALS symptomatic phase (120 days). (a–c) VAChT-stained sections through the ventral horn of the lumbar spinal cord showed an increase in MN maintenance in Nes-R26Met-SOD compared with SOD transgenics. (d–f) GFAP-stained sections through the ventral horn of the lumbar spinal cords showing astrogliosis in Nes-R26Met-SOD and SOD mice. (g–i) Iba-1-stained sections through the ventral horn of the lumbar spinal cords showing an increase in the number of microglial cells in Nes-R26Met-SOD and SOD mice. (j–l) Immunofluorescence staining of gastrocnemius muscle with α-bungarotoxin-tetramethylrhodamine (red), anti-neurofilament (green). Scale bar 50 μm. (m) Quantification of MN numbers (VAChT-positive) among the three groups of mice, showing a 32% increase in Nes-R26Met-SOD compared with SOD mice (Nes-R26Met-SOD versus SOD: \( P = 0.0002 \); SOD versus control: \( P < 0.0001 \); Nes-R26Met-SOD versus control: \( P = 0.006 \); \( n = 3 \)). (n) Quantification of fluorescence intensity of GFAP-immunopositive cells. Significant differences were observed between the three groups (Nes-R26Met-SOD versus SOD: \( P = 0.0164 \); SOD versus control: \( P = 0.002 \); Nes-R26Met-SOD versus control: \( P = 0.0084 \); \( n = 3 \)). (o) Quantification of Iba-1-positive cells. Significant differences in microglia numbers were observed between the three groups (Nes-R26Met-SOD versus SOD: \( P = 0.0016 \); SOD versus control and Nes-R26Met-SOD versus control: \( P < 0.0001 \); \( n = 3 \)). (p) The percentage of α-Bungarotoxin-stained end-plates showing complete or partial colocalization with neurofilament staining was evaluated in the three groups (Nes-R26Met-SOD versus SOD: \( P = 0.0416 \); controls versus SOD: \( P = 0.0143 \); controls versus Nes-R26Met-SOD: \( P = 0.0256 \)). Values are expressed as means ± S.E.M. ***\( P < 0.001 \), **\( P < 0.01 \), *\( P < 0.05 \).
ALS therapy through several strategies. HGF intrathecal administration at disease onset provided evidence that, when present at high doses and accessible to different disease cells, HGF/Met attenuates MN degeneration and retards disease progression by 11 days.\(^3\)\(^6\) However, these studies did not clarify whether, and to what extent, the HGF/Met system exerts support on MNs in a cell-autonomous manner. Insights to this issue come from studies based on genetic neuronal delivery of HGF in the low copy number SOD1\(^{G93A}\) mice. In particular, hgf expression driven by the neuron-specific enolase-promoter delayed disease onset by approximately 28 days, rather than influencing its progression.\(^3\)\(^9\) However, as exogenous HGF is expressed and secreted by neurons, these hgf transgenic mice did not allow discriminating between the HGF effects on MNs versus those elicited on astrocytes, which in turn influence MNs and microglia function. Consistently, a decrease in the number of microglia, reactive astrocytes, and MN loss was observed in hgf transgenics. Our mouse model allowed discrimination between the cell-autonomous effects elicited by enhanced Met signalling in MNs and those influenced by HGF on other dysfunctional cells. Moreover, the Nes-R26\(^{Met}\) mouse established that Met signalling in MNs selectively counteracts ALS disease onset. It remains to be investigated what contribution enhanced Met signalling, above endogenous levels in astrocytes and microglia, could have with respect to disease evolution.

The severity of ALS and the lack of effective therapeutic strategies are driving efforts to explore agents, applied either separately or in combination, to counteract disease symptoms. Concerning trophic factors, HGF could offer therapeutic advantages at multiple levels. It is noteworthy that endogenous Met is upregulated starting from ALS onset in ventral spinal cords of SOD1\(^{G93A}\) mice (data not shown and see Sun et al.\(^2\)\(^3\)). These observations indicate that endogenous Met is turned on to counteract ALS symptoms, but endogenous HGF levels may not be sufficient to reverse the damage, as was found studying regeneration after optic-nerve axotomy (unpublished results). Moreover, as HGF/Met elicits functions in muscle cells, it is possible that ectopic HGF in muscles would also counteract neuro-muscular-junction denervation and muscle atrophy. Indeed, HGF favours the formation of NMJs during development.\(^3\)\(^1\)\(^3\)\(^2\) Although enhanced Met activation is often associated with tumour formation and metastasis, we did not observe side effects such as neoplasia in Nes-R26\(^{Met}\) mice despite Met\(^9\) expression levels in regions of the nervous system. Thus, the beneficial effects of HGF/Met on ALS degenerative processes appear to be well tolerated in healthy nervous system tissues.

The use of the conditional ALS mouse model, in which the mutant SOD1 gene can be deleted according to the cre-transgenic line used, has been instrumental in clarifying the influence of distinct cell types during ALS evolution. In particular, selective reduction of mutant SOD1 in MNs predominantly impacts on disease onset and its early phase. In contrast, reduced mutant SOD1 either in astrocytes or in microglia mainly influences the late stage of disease progression.\(^8\)\(^9\) Thus, although recombination in Nes-R26\(^{Met}\) mice did not occur in 44% of lumbar spinal cord MNs, rebalancing the levels of stress and survival signals and/or protecting presynaptic terminals in ALS MNs, either by providing RTK support (our studies), or by removing signals such as BAX, influencing NMJ integrity,\(^1\)\(^2\) or by depleting mutant SOD,\(^8\)\(^9\) ameliorates ALS by selectively delaying disease onset. It is known that the initial MN damage is followed by a progressive increase in cytotoxic and inflammatory mediator levels, which further affect MN themselves and neighbouring cells. Astrocytes and microglia are the predominant cell types driving disease progression towards death. Although disease progression is unchanged in Nes-R26\(^{Met}\) mice, our genetic analysis show that enhanced-neuronal Met in SOD mice impacts on distinct cell types involved in the ALS disease: MNs, in a cell-autonomous manner, possibly by providing trophic support and/or maintenance of NMJ integrity and astrocytes and microglia, in a non-cell-autonomous manner, by delaying their activation. Future studies will establish the relative contribution of each individual event in delaying the onset of the disease by neuronal Met signalling and uncover the underlying mechanisms.

Understanding how therapeutic reagents act at cellular levels is needed to accelerate the progress of promising treatments towards the clinic. Our findings emphasize the relevance of genetically assessing the effects of agents on distinct cell types implicated in a disease and during its evolution. Although successful therapies for ALS will possibly require concomitant actions on distinct dysfunctional cells, our results highlight the considerable therapeutic potential of modulating RTK signalling in MNs to combat degenerative signals.

**Materials and Methods**

**Generation of R26\(^{LacZ-stop-Met}\) mice.** The R26\(^{LacZ-stop-Met}\) mice were generated by taking advantage of the loxp-flanked (‘floxed’)-stop cassette system. To generate the chimeric mouse–human gene, the human Met cDNA encoding the transmembrane and cytoplasmic portion was fused in-frame with the mouse extraacellular cDNA sequence using the PvuII site present in both sequences. We chose such a strategy to ensure that the Met chimeric protein interacts efficiently with the endogenous mouse HGF, but can still be identified from the endogenous Met using the CMV enhancer-chicken \(/\)-actin promoter followed by the loxp-flanked \(/\)-geo/3xpA cassette. This vector is referred to as pCALL2. The targeting vector was electroporated into R1 ES cell lines. Cell culture, electroporation, selection, and Southern blot analyses were performed as previously described.\(^3\)\(^2\) To identify recombined clones, genomic DNA was digested with EcoRI or EcoRV and probed with an external or internal probe, respectively. Two selected ES cell lines carrying the homologous recombination were used to generate the R26\(^{LacZ-stop-Met}\) mice through blastocyst injection.

**Transgenic mice.** The mouse line expressing cre recombinase under the nestin promoter was previously described.\(^3\)\(^5\) The B6SJL-Tg(SOD1\(^{G93A}\)) Tg1Gur(SOD1\(^{G93A}\)) mouse line was used as ALS disease model.\(^3\)\(^4\) Both R26\(^{LacZ-stop-Met}\) and nestin-cre mice were backcrossed into the B6SJL genetic background before breeding with the SOD1\(^{G93A}\) transgenics. The number of mice used for behavioural and immuno-histochemical studies are indicated in figure legends. Each genetic group consisted of a mixed population of equal numbers of males and females. The presence of a vaginal plug in the morning was considered as 0.5 embryonic day (E0.5). All procedures involving the use of animals were performed in accordance with the European Community Council Directive of 24 November 1986 on the protection of animals used for experimental purposes (86/609/EEC). The experimental protocols were carried out in compliance with institutional ethical committee guidelines for animal research. All efforts were made...
to minimize the number of animals used and their suffering. When paralysis started, food and water were placed directly into the cage. To reduce animal pain, mice were killed when they were unable to right themselves within 30 s when placed on their back.

**Antibodies.** Antibodies used were anti-tubulin and anti-GFAP and anti-VACH (1:1000; Sigma-Aldrich, St. Louis, MO, USA), anti-Met/FGF (1:1000; R&D systems, Minneapolis, MN, USA), and anti-phospho Y1234-1235-Met (1:500; Cell Signaling, Danvers, MA, USA), anti-human Met (1:500; Santa-Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-Sm22 (1:500; Stemgerber monoclonals, Covance, Dallas, TX, USA), anti-NeuN (1:200; Chemicon, Millipore, Billerica, MA, USA), and anti-neurofilament-145 (1:1000; AB1897; Millipore), anti-mouse or rabbit fluorescent-coupled secondary antibodies (1:400; Jackson, West Grove, PA, USA), anti-mouse or rabbit biotin-coupled secondary antibodies (1:500; Jackson). For western blot analyses, the following secondary antibodies were used: anti-rabbit IgG-peroxidase or anti-mouse IgG-peroxidase (1:4000, Jackson).

**Histological analysis.** Anesthetized mice were intra-cardiacally perfused first with PBS then with 4% para-formaldehyde (PFA, Sigma, Germany). In situ hybridization, immune-histochemistry, and X-Gal staining were performed as previously described.37,38,39 MN numbers were determined on 16-10 ng/ml CNTF) and maintained at 37°C on poly-ornithine/laminin-treated coverslips in supplemented Neurobasal medium (1:500; Jackson, West Grove, PA, USA), anti-mouse or rabbit biont-coupled secondary antibodies (1:500; Jackson). For western blot analyses, the following secondary antibodies were used: anti-rabbit IgG-peroxidase or anti-mouse IgG-peroxidase (1:4000, Jackson).

**Biochemical studies.** Protein extracts were prepared from freshly dissected brains and spinal cords at the appropriate stages and western blot analyses were performed as previously described.37,38 Quantifications were done by measuring band intensities with the Image J software.50,51,52

**Statistical analysis.** Results were expressed as the mean ± S.E.M. Statistical significance was assessed by Student’s t-test or ANOVA, followed by post-hoc test with appropriate P value. Data analysis was performed with Graph Pad (Graph Pad Software, San Diego, CA, USA).

**Conflict of interest**

The authors declare no conflict of interest.

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281
Genetics enhancing Met levels in ALS neurons
M Genestine et al
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