Early and Persistent Abnormal Decoding by Glial Cells at the Neuromuscular Junction in an ALS Model

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Amyotrophic lateral sclerosis (ALS) is a late-onset neuromuscular disease characterized by progressive loss of motor neurons (MNs) preceded by neuromuscular junction (NMJ) denervation. Despite the importance of NMJ denervation in ALS, the mechanisms involved remain unexplored and ill defined. The contribution of glial cells in the disease has been highlighted, including axonal Schwann cell activation that precedes the decline of motor function and the onset of hindlimb paralysis. Because NMJ denervation occurs early in the process and that perisynaptic Schwann cells (PSCs), glial cells at the NMJ, regulate morphological stability, integrity, and repair of the NMJ, one could predict that PSC functions would be altered even before denervation, contributing to NMJ malfunctions. We tested this possibility using a slowly progressive model of ALS (*SOD1*^{G37R} mice). We observed a normal NMJ organization at a presymptomatic stage of ALS (120 d), but PSC detection of endogenous synaptic activity revealed by intracellular Ca^{2+} changes was enhanced compared with their wild-type littermates. This inappropriate PSC decoding ability was associated with an increased level of neurotransmitter release and dependent on intrinsic glial properties related to enhanced muscarinic receptor activation. The alteration of PSC muscarinic receptor functions also persists during the preonset stage of the disease and became dependent on MN vulnerability with age. Together, these results suggest that PSC properties are altered in the disease process in a manner that would be detrimental for NMJ repair. The impairments of PSC functions may contribute to NMJ dysfunction and ALS pathogenesis.

Key words: motor unit properties; muscarinic receptors; perisynaptic Schwann cells; *SOD1*; synaptic transmission

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the progressive loss of upper and lower motor neurons (MNs). One of the primary pathogenic events that occur in ALS is the denervation and destruction of neuromuscular junctions (NMJs) (Frey et al., 2000; Fischer et al., 2004; Pun et al., 2006; Dupuis and Loeffler, 2009). An important distinction has been shown whereby fast-fatigable MNs are known to be affected first, whereas the slow-type MNs are partially resistant, purportedly attempting to reinnervate previously denervated muscle fibers (Frey et al., 2000; Atkin et al., 2005; Schaefer et al., 2005; Pun et al., 2006; Hegedus et al., 2007, 2008).

ALS is a non-cell-autonomous disease, involving glial cells (astrocytes, microglia, and oligodendrocytes) in pathogenesis and progression (Clement et al., 2003; Boillée et al., 2006; Yamanaka et al., 2008; Wang et al., 2011; Kang et al., 2013). Also, it was shown that activation of axonal Schwann cells (myelinating and nonmyelinating) precedes the decline of motor function and the onset of hindlimb paralysis (Keller et al., 2009). However, while the impact of the mutation in axonal Schwann cells was tested (Lobsiger et al., 2009; Turner et al., 2010; Wang et al., 2012), the contribution of perisynaptic Schwann cells (PSCs), glial cells at the NMJ, remains unknown.

Interestingly, PSCs may have an important contribution in NMJ dysfunction in ALS because they are essential for the maintenance of synaptic structure and function and govern the induction and guidance of nerve terminal sprouting during reinnervation (Reynolds and Woolf, 1992; Son et al., 1996; O'Malley et al., 1999; Reddy et al., 2003; Feng and Ko, 2008). They decode synaptic transmission and regulate it in a Ca^{2+} -dependent manner (Reist and Smith, 1992; Robitaille, 1998; Rochon et al., 2001; Colomar and Robitaille, 2004; Rousse et al., 2010; Todd et al., 2010; Darabid et al., 2013). Importantly, this decoding ability determines the fate of the NMJ because it controls PSC response to injury and regulates gene expression in PSCs via muscarinic receptors (mAChRs) (Reynolds and Woolf, 1992; Georgiou et al., 1994, 1999; Son et al., 1996). These data indicate that mAChR activation maintains PSCs in a state of maintenance, preventing

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gene expression required for NMJ repair. Hence, an altered PSC decoding ability with abnormal mAChRs activation would deter on PSC functions of maintenance and repair.

Because of their role as synaptic modulators and their involvement in NMJ maintenance and repair, we postulate that the decoding ability of PSCs is altered at a very early stage of disease and persist in time. To evaluate this, we used the *SOD1*^{G37R} mouse model (line 29), which features a late-onset and slowly progressing phenotype (Wong et al., 1995). We report that the ability of PSCs to decode synaptic transmission is altered in *SOD1*^{G37R} mice, elicited by higher muscarinic activation. Because enhanced muscarinic activation would not favor NMJ repair, this suggests that PSCs could play an important role to the onset and/or progression of the disease.

Materials and Methods

Animals

Soleus (SOL) muscles and their innervation were dissected from three animal groups: (1) transgenic mice expressing the human *SOD1* gene carrying the G37R mutation (Gly → Arg) (line 29) (*SOD1*^{G37R}) where a moderate (4-fold to 5-fold) increase in mutated *SOD1* level in brain and spinal cord was reported (Wong et al., 1995); (2) their age-matched wild-type (WT) littermates; and (3) h*SOD1* transgenic mice (*SOD1*^{WT}), which overexpress the WT human *SOD1* gene, were used as age-matched controls. The *SOD1*^{G37R} were purchased from The Jackson Laboratory and maintained in a C57BL/6 genetic background. The transgenic mice were genotyped by PCR amplification for the human *SOD1* gene performed on tail biopsy samples taken at the time of the weaning (~21 d of age). The *SOD1*^{G37R} transgenic mice develop symptoms and pathology resembling human ALS, with paralysis in one or more limbs attributable to the eventual loss of MN from the spinal cord. *SOD1*^{WT} have not been reported to develop ALS-like disease. *SOD1*^{WT} express three times the normal level of *SOD1* in the blood, brain, and fibroblasts (Audet et al., 2010).

To avoid ambiguity associated with gender-related differences, only male mice were used in the present study. Presymptomatic animals were used at postnatal day 107–130 (P120), and preonset animals were used at P357–P404 (P380). A smaller cohort of animals was used at P60. An autopsy was performed on every preonset animal. They were discarded if any sign of abnormal anatomical features of abdominal organs were observed. As proposed earlier, we refer to the onset of the disease by the peak of the body weight curve (Boillée et al., 2006). This age also coincides with initial axonal retraction from the neuromuscular synapses in the SOL (data not shown). Therefore, the disease onset was carefully monitored and assessed every week with animal weight and behavioral performances, including the presence of tremors and lack of hindlimb extension reflex (median age of onset = 426 d). No animal that passed the onset point was used in this study. The fragility and the state of the nerve-muscle preparations after the onset of the disease prevented us from performing electrophysiological recordings and calcium imaging experiments with a sufficient success rate.

All experiments were performed in accordance with the guidelines for maintenance and care of animals of the Canadian Council of Animal Care and Université de Montréal.

Nerve-muscle preparations

SOL nerve-muscle preparations were pinned in a Sylgard-coated experimental chamber filled with normal Rees' Ringer's saline solution containing the following (in mM): 110 NaCl, 5 KCl, 1 MgCl₂, 25 NaHCO₃, 2 CaCl₂, 11 glucose, 0.3 glutamate, 0.4 glutamine, 5 BES (C₆H₁₅NO₅S), 4.34 × 10^{−7} cocarboxylase, and 0.036 choline chloride. Experiments were performed at 28 ± 2°C under continuous perfusion of oxygenated (95% O₂, 5% CO₂) saline solution. The pH of oxygenated solution was at 7.3.

Electrophysiological recordings of synaptic transmission

Stimulation of the tibial nerve was performed using a suction electrode filled with extracellular saline. Endplate potentials (EPPs) were recorded

using glass microelectrodes (1.0 mm OD; WPI) pulled to 40–70 MΩ (filled with 3 mM KCl) with a Brown–Flaming micropipette puller (Sutter Instruments). Synaptic responses were amplified by an AM Systems 1600 amplifier and further amplified (100×) and filtered (2 kHz) by a Warner Instruments DC amplifier. The recordings were digitized using a national Instruments BNC 2110 board and subsequently acquired with WinWCP software (John Dempster, Strathclyde University, Strathclyde, UK).

Synaptic strength of recorded NMJs was determined by measuring the paired-pulse facilitation (PPF) and the quantal content (m). These were obtained using a low Ca²⁺ (1 mM) and high Mg²⁺ (6.4 mM) Ringer's solution. PPF was obtained using two stimuli of 0.1 ms duration at 10 ms interval, elicited at 0.2 Hz. PPF was calculated as the mean amplitude of the second EPPs divided by the mean amplitude of the first EPPs, including failures. Quantal content (m) was determined using the amplitude of the miniature endplate potentials (MEPPs), as described previously (Del Castillo and Katz, 1954): m = (mean amplitude of EPPs/mean amplitude of the MEPPs). MEPP amplitude and frequency were determined using 5–10 min of recordings without motor nerve stimulation. A minimum of 100 MEPPs were used to calculate m for each NMJ. Recordings with an initial membrane potential depolarized >−65 mV or with >5 mV variation from holding potential were not included for analysis. Muscle fibers were impaled ~50–100 μm from the NMJ to be studied, avoiding mechanical distortion of the NMJ, yet providing a morphological landmark for finding the NMJ for *post hoc* morphological analysis.

Calcium imaging of PSCs

Nerve-muscle preparations were incubated for 90 min (2 × 45 min) in a preoxygenated Ringer's saline solution containing 10 μM fluo-4 AM (Invitrogen) and 0.02% pluronic acid (Invitrogen) at 26 ± 1°C. This method is known to preferentially load PSCs at NMJs (Georgiou et al., 1999; Rousse et al., 2010; Todd et al., 2010). PSCs were easily identified on surface NMJs with transmitted light microscopy. Excitation of the Ca²⁺ indicator (fluo-4) was achieved using the 488 nm line of the argon ion laser of an FV1000 Olympus confocal microscope and a 20× or a 60× water-immersion objective (respectively, 0.95 NA, XLUMPlanFI; and 0.90 NA, Olympus). Emitted fluorescence was detected using a bandpass filter (500–545 nm). Basal level of fluorescence was always set within the same range of arbitrary units, using the same software and hardware settings. Changes in fluorescence were measured by subtracting the background fluorescence from the neighboring muscle fiber, and changes in fluorescence were measured over PSC soma and expressed as follows: % ΔF/F = (F − F rest)/F rest × 100. Experiments were discarded when focus drift occurred.

Calcium responses evoked in PSCs by endogenous release of neurotransmitter were obtained by stimulating the tibial nerve at 50 Hz during 5 s using a suction electrode. To prevent muscle contractions, postsynaptic cholinergic receptors were blocked with D-tubocurarine chloride (2.2–2.6 μg/ml, Sigma), which does not affect PSC excitability (Reist and Smith, 1992; Todd et al., 2010). Preparations were allowed to stabilize with constant bath perfusion for at least 20 min before attempting motor nerve stimulation and Ca²⁺ imaging. In certain experiments, PSC Ca²⁺ responses were elicited by local application of agonists using a brief, small pulse of positive pressure (20–40 PSI, 150–200 ms) generated by a Picospritzer II (Parker Instruments) applied on a glass pipette (5 MΩ, ~2-mm-tip diameter) positioned in proximity of the cells. Adenosine 5'-triphosphate (ATP), muscarine, or acetylcholine (10 μM, Sigma) was dissolved in the same Ringer's solution used for the experiment. One NMJ per muscle was imaged when motor nerve stimulation was applied to the preparation, but PSCs at several NMJs were imaged per muscle with agonist applications. A recovery of at least 20 min was allowed between each application when several applications were performed on the same cells.

Antagonist applications

In some experiments, antagonists were bath applied for at least 45 min before the start of experiments. P2_Y antagonists (Reactive Blue 2 or RB2, 20 μM, Alexis), large-spectrum P2 antagonists (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid, 20, 60, and 100 μM, and suramin, 100 μM, Sigma) and mAChRs antagonist (atropine, 5–20 μM, Sigma) were diluted in the same extracellular saline solution.

TEA application

Neurotransmitter released by either WT or *SOD1*^{G37R} nerve terminals was increased using a K⁺ channel blocker, tetraethyl ammonium (TEA, 0.2 mM, Sigma). TEA was bath applied for 30 min, and synaptic activity was monitored (frequency of stimulation = 0.2 Hz). Then, nerve-muscle preparations (now potentiated by TEA) were stimulated at high frequency (50 Hz for 5 s), and the corresponding PSC Ca²⁺ responses were recorded. In some control experiments, agonists (ATP, 10 μ M) were locally applied in the presence of TEA by micro-pressure as indicated above.

Immunohistochemistry and confocal imaging

Labeling of synaptic compartments. Immunohistochemical labeling of the three synaptic components at the NMJ was done according to the method previously described (Todd et al., 2010; Darabid et al., 2013). SOL muscles were dissected in oxygenated Rees' Ringer's solution and pinned in a Sylgard-coated 10 mm Petri dish. Muscles were then fixed for 10 min in 4% formaldehyde diluted in PBS buffer (in mM as follows: 137 NaCl, 2.7 KCl, 10 Na₂HPO₄, 2 KH₂PO₄) at room temperature and permeabilized in 100% cold-methanol at –20°C for 6 min. Nonspecific labeling was blocked by incubating muscles with 10% normal donkey serum in PBS containing 0.01% Triton X-100 for 20 min at room temperature. First, Schwann cells were labeled with a rabbit anti-S100 β antibody (1:250, Dako) for 2 h, and then axons (chicken anti-neurofilament M [NF-M], 1:2000, Rockland Immunochemicals) and nerve terminals (mouse IgG1 anti-synaptic vesicular protein 2, 1:2000; Developmental Studies Hybridoma Bank) were labeled for 90–120 min. After washing, muscles were incubated with secondary antibodies, goat anti-mouse IgG1 Alexa-488, donkey anti-chicken Alexa-448, and donkey anti-rabbit Alexa-647 (all 1:500, Jackson ImmunoResearch Laboratories) for 1 h. Postsynaptic nicotinic acetylcholine receptors (nAChRs) were labeled with Alexa-594-conjugated- α -bungarotoxin (1.33–2.0 μ g/ml, Invitrogen) for 30–45 min. All antibody incubations were performed in PBS containing 0.01% Triton X-100 and 2% normal donkey serum at room temperature. After each step (except blocking), muscles were rinsed three times in PBS containing 0.01% Triton X-100 for 5 min. Muscles were then mounted in Prolong Gold antifade reagent (Invitrogen). Observations were done on an Olympus FV1000 (WT, *SOD1*^{WT}, and *SOD1*^{G37R} groups) or a Zeiss LSM 510 (C57BL/6 controls) confocal microscope. No image manipulations were performed after acquisition.

Labeling of muscle fiber type. Muscle fiber types were determined at the end of all physiological experiments. Muscles were fixed in 4% formaldehyde at room temperature for 10 min and rinsed with PBS (3 $\times$ 5 min). Muscles were then permeabilized in 100% cold methanol for 6 min at –20°C and incubated in a solution of 10% normal donkey serum and 0.01% Triton X-100 for 20 min to minimize nonspecific labeling. Muscles were washed in PBS containing 0.01% Triton X-100 (3 times, 5 min each) after each of the following steps. Muscles were incubated with a mouse anti-Type I myosin heavy chain IgGIIb and anti-Type IIa myosin heavy chain IgG1 (1:100–200, SC-71-c and 1:75–100, BA-D5-c, Developmental Studies Hybridoma Bank, respectively) for 120 min at room temperature. After rinsing in PBS (3 $\times$ 5 min), preparations were incubated with CY5 or Alexa 647 anti-mouse IgGIIb (1:500) secondary antibodies for 60 min and then incubated with Alexa-488 anti-mouse IgG1 (1:1000) for 60 min at room temperature. Finally, muscles were incubated with α -bungarotoxin (Alexa-594, 1.33–2.0 μ g/ml) for 45 min. Preparations were then mounted in Prolong Gold antifade reagent and all labels observed simultaneously using a Zeiss LSM 510 confocal microscope or the spectral detection feature of an Olympus FV1000. No further manipulations of the images were performed after acquisition.

Histological analysis

Seven criteria similar to those described in previous studies (Wright et al., 2009; Valdez et al., 2012) were used to analyze NMJ morphology and are detailed in Table 1. We did not evaluate receptor fragmentation as this was observed on NMJs from P120 C57BL/6 controls animals (data not shown) and reported in detail previously (Wright et al., 2009; Valdez et al., 2012). Axonal diameter was not evaluated as changes in the labeling could be due to changes in NF-M expression in the disease or switch to

Table 1. Definition and criteria used in the morphological analysis of NMJs

	Criteria	Definition
Presynaptic	(1) Denervation	<i>Partial</i> : when part of the endplate is not recovered by the presynaptic nerve terminal. <i>Complete</i> : when the presynaptic element is absent from the endplate.
	(2) Terminal sprouting	When the presynaptic element, originating from the endplate, extends at least 20 μ m away in any direction or contacts another NMJ. Only sprouts on NMJs fully innervated by a myelinated axon were counted as originating from that NMJ.
	(3) Polyinnervation	When at least two distinct axons or sprouts were seen entering a single postsynaptic site. Sprouts from unknown origin on partially denervated NMJs or NMJs without a visible large myelinated axon were automatically counted as a source of innervation rather than a sprout originating from that endplate.
Postsynaptic	(4) Ectopic AChR	When two nAChR clusters of at least 3 μ m of diameter belonging to the same endplate were at least separated by 5 μ m. nAChRs are considered as belonging to the same endplate if they are on the same muscle fiber as evaluated by background fluorescence or transmitted light images.
	(5) Faint clustered nAChR	When the postsynaptic site was noticeably faint, clustered, and had a lack of organization reminiscent of an immature NMJ (Darabid et al., 2013).
Glial	(6) Incomplete glial coverage	When glial cells were not fully covering the presynaptic element. Only <i>en face</i> NMJs were analyzed because of the limited penetration of the S100 β antibody in the tissue.
	(7) Glial sprouting	<i>Sprouting</i> : when PSCs extended a process associated with a presynaptic sprout. <i>Bridge</i> : when at least one PSC extended a process at least 20 μ m away or contacting another NMJ without being associated with a presynaptic element.

another isoform (neurofilament heavy). Also, the NF-M antibody did not penetrate the tissue very well such that labeling axons located under the first two layers of muscle fibers was unreliable.

The following procedure was used to ensure that the muscle fiber typing was performed on the same NMJs on which physiological measurements were performed. First, we captured an image at low magnification (20 $\times$) to determine its position within the field of view in the microscope relative to the surrounding NMJs and the position of the main nerve entry by counting the number of fibers between them. Sometimes, the position of the nerve entry into the junction could be used as an additional criterion. Second, after electrophysiological recordings, we performed deliberate perforation of the surrounding muscle fibers with a glass pipette. The map was then used in the subsequent imaging sessions to locate the junction of interest. Third, we labeled the nAChRs with α -bungarotoxin (Alexa-594, 10 μ g/ml, 20 min) for each Ca²⁺ imaging experiment. This toxin does not affect PSC excitability (Rochon et al., 2001). The unique “pretzel-like” pattern of the bungarotoxin-labeled nAChRs of an NMJ and of the surrounding ones served as selective markers. Also, this staining allowed us to generate a 3D reconstruction of the endplate to further detail its organization.

Statistical analysis

Results are presented as mean $\pm$ SEM. *N* represents the number of animals, and *n* refers to the number of NMJs or PSCs. Paired *t* tests were performed when comparing synaptic responses induced by the motor nerve stimulation during the same experiment. Unpaired *t* tests were performed to compare two different conditions from different experiments. One-way Kruskal–Wallis ANOVA test with Dunn's Multiple-

Comparison post-test was used to compare three groups or more. Two-way ANOVA test with Bonferroni's Multiple Comparison post-test were used when there were more than one independent variable and multiple observations for each independent variable. All tests were used at a confidence level of 95% ($\alpha = 0.05$).

Results

The decoding ability of PSCs with mAChR activation represents a central element for appropriate PSC responses in the regulation of NMJ morphology and stability. Thus, we hypothesized that PSC properties could be altered at an early presymptomatic period in an ALS mouse model in which predictable NMJ destruction will occur. In this work, we investigated two fundamental PSC properties that are essential for their normal function: the regulation of morphological organization and the decoding of synaptic activity.

NMJ morphology is unchanged at a presymptomatic stage

PSCs actively participate in the maintenance and repair of neuromuscular synapses (Reynolds and Woolf, 1992; Son et al., 1996; Georgiou et al., 1999; O'Malley et al., 1999; Reddy et al., 2003; Feng and Ko, 2008). Hence, any sign of PSC morphological disorganization coupled to NMJ morphological alteration would be a clear and sensitive sign of PSCs' inability to maintain NMJ integrity.

To distinguish between the impacts of the mutated SOD1 protein and the overexpression of the human WT SOD1 protein, we analyzed the NMJ morphology at an asymptomatic stage of the disease (P120) using immunohistological labeling of PSCs, presynaptic and postsynaptic elements from WT, *SOD1^{WT}*, and *SOD1^{G37R}* animals (Fig. 1A). Seven previously documented NMJ-related alterations (Table 1) were quantified from images, such as those shown in Figure 1A (described in Experimental procedures). Only 12% of NMJs of WT, 19% of NMJs of *SOD1^{WT}*, and 11% of NMJs of *SOD1^{G37R}* exhibited one or two of the seven criteria (Table 2; no statistical difference between any of these criteria in the different groups). For instance, the presynaptic terminal branch morphology or postsynaptic receptor appearance was unaltered, and all NMJs of each group were innervated (Fig. 1A; Table 2). Consistent with O'Malley et al. (1999), we found that PSC somata and processes were precisely aligned with nerve terminal branches and postsynaptic ACh receptors (O'Malley et al., 1999) (Fig. 1A; Table 2). Hence, these results reveal that NMJs from *SOD1^{G37R}* were indistinguishable from WT and *SOD1^{WT}* at P120.

We next examined whether the proportion of Type I and Type IIa fibers was different at this age. This was tested using specific antibodies against MHC isoform Types I and IIa, corresponding to the slow and fast fatigue-resistant muscle fibers, respectively, present in the SOL muscle (Fig. 1B). All unlabeled fibers were identified as fiber IIX, the remaining fiber subtype in the SOL (Valdez et al., 2012). However, as shown in Figure 1C and as previously reported in a young adult C57BL/6 mouse (Valdez et al., 2012) and *SOD1^{WT}* mice (Hegedus et al., 2007), we found no difference in the proportion of fiber types (WT: $N = 5$ muscles, $n = 295$ fibers; *SOD1^{WT}*: $N = 4$ muscles, $n = 220$ fibers; *SOD1^{G37R}*: $N = 5$ muscles, $n = 295$ fibers; Fig. 1C, two-way ANOVA, $F = 0.83$, $p = 0.5158$). Finally, there was no significant difference in the percentage of NMJs per muscle associated with each fiber type (WT: $N = 5$ muscles, $n = 176$ NMJs; *SOD1^{WT}*: $N = 4$ muscles, $n = 147$ NMJs; *SOD1^{G37R}*: $N = 5$ muscles, $n = 154$ NMJs; Fig. 1D, two-way ANOVA, $F = 1.61$, $p = 0.1951$).

Thus, neither the overall number of NMJs according to the fiber type nor the morphology was altered at P120 of WT,

SOD1^{WT}, and *SOD1^{G37R}* mice. This suggests that PSC properties that ensure the stability of the neuromuscular synapse were normal at this early age and stage of the disease.

Excitability of PSCs is altered early at P120

PSCs regulate morphological stability and integrity and actively participate in the reinnervation of the NMJ, events that are under the influence of synaptic communication. Indeed, in healthy conditions, interruption of synaptic communication between PSCs and presynaptic nerve terminals leads to changes in PSCs phenotype that facilitate NMJ plasticity and reinnervation (Reynolds and Woolf, 1992; Son et al., 1996; O'Malley et al., 1999). Importantly, the PSC switch from maintenance mode to repair mode is regulated by their ability to detect synaptic transmission (Georgiou et al., 1994, 1999), suggesting that their role as synaptic modulators is tightly linked to synaptic maintenance and repair.

Thus, we examined the ability of PSCs to respond to synaptic activity elicited by motor nerve stimulation. Stimulation was performed at 50 Hz for 5 s, a frequency reported for firing of motor neurons innervating adult SOL muscle (Chipman et al., 2010) and for PSC responsiveness (Todd et al., 2007; Rousse et al., 2010). PSC activity was assessed by monitoring changes in intracellular Ca^{2+} levels, which is a well-known and reliable reporter of their level of excitation and responsiveness (Robitaille, 1998; Rochon et al., 2001; Rousse et al., 2010; Darabid et al., 2013).

As shown in Figure 2A, synaptic activity evoked by stimulation of the motor nerve induced Ca^{2+} responses in PSCs. As indicated by the number of cells activated, the propensity of PSCs to respond to synaptic activity was similar for NMJs from both control groups (WT: 58%, 22 PSCs activated out of 38; *SOD1^{WT}*: 47%, 8 PSCs activated out of 17) and the *SOD1^{G37R}* mice (58%, 22 activated PSCs out of 38). However, and somewhat surprisingly, the amplitude of PSC Ca^{2+} responses was larger in *SOD1^{G37R}* mice. Indeed, the average amplitude of PSC Ca^{2+} responses from WT mice and *SOD1^{WT}* was $34.3 \pm 4.3\%$ $\Delta F/F$ and $24.1 \pm 2.8\%$ $\Delta F/F$, respectively, whereas it was $108.9 \pm 18.1\%$ $\Delta F/F$ from *SOD1^{G37R}* mice (Fig. 2B; $N = 10$, $n = 22$ for WT, $N = 5$, $n = 8$ for *SOD1^{WT}* and $N = 9$, $n = 22$ for *SOD1^{G37R}*, one-way ANOVA, $p = 0.0004$). However, this difference was not observed at an earlier age (P60) (data not shown; $N = 5$, $n = 9$ for WT and $N = 4$, $n = 10$ for *SOD1^{G37R}*, unpaired t test, $p = 0.3065$). These results indicate that the PSCs at NMJs of an ALS mouse model at P120 detected transmitter release but with an enhanced excitability.

We next tested whether PSC excitability reflected MN properties because MNs differ not only in their physiological properties but also in their anatomical plasticity and susceptibility to loss of neuromuscular connectivity. This was tested by performing immunohistological staining of MHC isoforms after each Ca^{2+} imaging experiment (Fig. 2C). Because PSC Ca^{2+} responses from WT and *SOD1^{WT}* mice were indistinguishable, we compared only the WT and the *SOD1^{G37R}* groups for these experiments. As shown in Figure 2D, Ca^{2+} response amplitude from *SOD1^{G37R}* was 3.0- to 3.5-fold larger than WT for each fiber type (MHC-I: $N = 4$, $n = 8$ for WT and $N = 4$, $n = 9$ for *SOD1^{G37R}*, unpaired t test, $p = 0.0279$) (MHC-IIa: $N = 7$, $n = 12$ and for WT and $N = 4$, $n = 8$ for *SOD1^{G37R}*, unpaired t test, $p = 0.0021$). Together, these results suggest that alterations in PSC Ca^{2+} responses do not correlate with the predicted and specific vulnerability of MNs at this presymptomatic stage of the disease. These alterations are expressed at least 4 months before the earliest signs of sensorimo-

Figure 1. Morphology is unaltered at presymptomatic stage of the disease. **A**, Confocal images of NMJs from controls (WT and SOD1^{WT}) and an ALS mouse model (SOD1^{G37R}). P120 soleus NMJs were labeled for the nerve terminals (NT) (synaptic vesicular protein 2 and NF-M, green), PSC (S100 β , cyan), and postsynaptic nAChRs (α -bungarotoxin, red). Note the similar staining patterns between each animal group. **B**, Confocal images of soleus muscle from an SOD1^{G37R} mouse. Muscle fibers were stained using anti-myosin heavy chain (MHC) monoclonal antibodies: Type I fibers (MHC-I; blue), Type IIa fibers (MHC-IIa; green), and α -bungarotoxin (red) to identify the localization of the endplates. Note the alternation of slow- and fast-twitch fibers in the soleus muscle. **C**, Histogram of the percentage of surface fibers in the soleus of each animal group. **D**, Histogram of the percentage of surface NMJs per fiber type in the soleus of each animal groups. Histograms in **C** and **D** represent mean $\pm$ SEM. Scale bars: **A**, 10 μ m; **B**, 50 μ m.

Table 2. Percentage of NMJs that meet criteria used in the morphological analysis for each animal group

	Criteria	WT	SOD1 ^{WT}	SOD1 ^{G37R}	<i>p</i>
Presynaptic	(1) Denervation	0	1.3 ± 1.3	0	0.4053
	(2) Terminal sprouting	1.0 ± 1.0	2.3 ± 2.3	0	0.5589
	(3) Polyinnervation	0	3.0 ± 1.8	0	0.1007
Postsynaptic	(4) Ectopic AChR	0	11.4 ± 3.7	2.5 ± 1.4	0.3939
	(5) Faint clustered nAChR	9.2 ± 3.4	11.4 ± 3.7	6.4 ± 2.6	0.5648
Glial	(6) Incomplete glial coverage	0	0	0	NA
	(7) Glial sprouting	2.8 ± 1.7	2.8 ± 2.8	3.2 ± 1.9	0.9871

NA, Not applicable.

Figure 2. Altered PSC excitability at presymptomatic stage of the disease. **A**, False color images of PSCs (*) loaded with fluorescent Ca^{2+} indicator Fluo-4 AM, before (baseline), during (stimulation), and after (recovery) motor nerve stimulation at NMJs of WT, SOD1^{WT}, and SOD1^{G37R}. Representative changes in fluorescence are illustrated on the right for the PSCs indicated by the arrowhead. **B**, Histogram depicting the mean $\pm$ SEM of the amplitude of the Ca^{2+} responses elicited in PSCs by transmitter release evoked by motor nerve stimulation (50 Hz, 5 s). PSCs at NMJs from SOD1^{G37R} mice had larger Ca^{2+} responses (one-way ANOVA, $p = 0.0004$). **C**, Examples of an NMJ imaged during Ca^{2+} imaging experiment (top) and the same NMJ imaged after MHC and BTX staining (bottom). This NMJ was associated with a fiber Type I (MHC-I, blue) and not a fiber Type IIa (MHC-IIa, green). **D**, Histogram illustrating the mean $\pm$ SEM of the amplitude of the Ca^{2+} responses elicited in PSCs by motor nerve stimulation as a function of each fiber type. The altered PSC excitability did not correlate with motor neuron vulnerability at a presymptomatic stage of the disease. Scale bars: **A**, 10 μ m; **C**, 20 μ m. * $p < 0.05$, ** $p < 0.01$.

tor and cognitive dysfunctions in this SOD1^{G37R} transgenic mouse model of ALS (Filali et al., 2011).

Neurotransmitter release is increased in the soleus muscle of an ALS mouse model at P120

Next, we tested the hypothesis that the synaptic strength from SOD1^{G37R} mice might be altered. This would be consistent with

the early alterations in MN excitability (Pieri et al., 2003; Kuo et al., 2004; Amendola et al., 2007; Bories et al., 2007) that seems to diminish toward the symptomatic stage of the disease (Delestrée et al., 2014) and with the properties of PSCs at immature and mature NMJs, which decode different levels of synaptic activity by producing different Ca^{2+} responses (Rousse et al., 2010; Todd et al., 2010; Darabid et al., 2013). To this end, we performed

Figure 3. Enhanced synaptic transmission at NMJs from *SOD1^{G37R}* mice at presymptomatic stage of the disease. **A**, Examples of spontaneous MEPP recordings from WT, *SOD1^{WT}*, and *SOD1^{G37R}* NMJs. Histogram showing the mean $\pm$ SEM of the amplitude (**B**) and the frequency (**C**) of the MEPP. **D**, Examples of EPPs evoked by paired-pulse stimulation of the motor nerve (10 ms interval) from WT, *SOD1^{WT}*, and *SOD1^{G37R}* NMJs. **E**, Histogram showing the mean $\pm$ SEM of the amplitude of the first EPP. **F**, Calculated quantal content obtained from electrophysiological recordings of synaptic transmission and determined as EPP amplitude/MEPP amplitude. NMJs from *SOD1^{G37R}* mice had higher quantal content. **G**, Calculated PPF obtained from electrophysiological recordings of synaptic transmission and determined as the mean amplitude of the second EPPs divided by the mean amplitude of the first EPPs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

intracellular recordings from WT, *SOD1^{WT}*, and *SOD1^{G37R}* NMJs. Synaptic strength was determined using the quantal content (m) and PPF.

First, we evaluated the spontaneous activity of the NMJs. No difference in MEPP amplitude was observed between WT and *SOD1^{G37R}* mice (WT, 0.36 ± 0.01 mV, $N = 9$, $n = 15$; *SOD1^{WT}*, 0.30 ± 0.02 mV, $N = 6$, $n = 8$; *SOD1^{G37R}*, 0.35 ± 0.01 mV, $N = 9$, $n = 14$; one-way ANOVA, $p = 0.0145$; Fig. 3A,B). Surprisingly, MEPP amplitude from *SOD1^{WT}* mice was slightly but significantly smaller than the WT and the *SOD1^{G37R}* mice (Fig. 3A,B). Importantly, however, MEPP frequency was significantly in-

creased in the SOL muscle of the *SOD1^{G37R}* mice (Fig. 3A) where it was 0.60 ± 0.05 Hz for the WT mice, 0.48 ± 0.05 Hz for the *SOD1^{WT}* mice, and 0.99 ± 0.12 Hz for the *SOD1^{G37R}* mice (Fig. 3C; $N = 8$, $n = 16$ for WT, $N = 6$, $n = 8$ for *SOD1^{WT}* and $N = 8$, $n = 14$ for *SOD1^{G37R}*, one-way ANOVA, $p = 0.0008$). Importantly, a difference in MEPP frequency was observed between WT and *SOD1^{G37R}* mice in Type I fibers (data not shown; $N = 4$, $n = 8$, 0.50 ± 0.04 Hz for WT and $N = 5$, $n = 6$, 0.66 ± 0.06 Hz for *SOD1^{G37R}*, unpaired t test, $p = 0.0475$) and Type IIa fibers ($N = 5$, $n = 7$, 0.72 ± 0.09 Hz for WT and $N = 5$, $n = 7$, 1.27 ± 0.13 Hz for *SOD1^{G37R}*, unpaired t test, $p = 0.0045$). These results are

Figure 4. PSC decoding ability is unaffected by an increase in transmitter release. **A**, Changes of EPP amplitude before and during (red bar) bath application of TEA (0.2 mM). Insets, Examples of EPPs (black) before and 30 min after TEA application (red). TEA increased synaptic transmission of both WT and *SOD1*^{G37R} nerve terminals. Effect of TEA (0.2 mM) bath application on corresponding PSC Ca²⁺ responses induced by synaptic activity (50 Hz, 5 s) from WT (**B**) and *SOD1*^{G37R} (**C**) animals. Gray zones represent the mean $\pm$ SEM of the amplitude of the Ca²⁺ responses elicited by motor nerve stimulation in control (without TEA). PSC Ca²⁺ responses elicited by potentiated WT nerve terminals remain smaller than those triggered by the *SOD1*^{G37R} and were not significantly different from the ones without TEA (unpaired *t* test, *p* > 0.05). Ca²⁺ responses elicited in PSCs by local applications of ATP in WT (**D**) and *SOD1*^{G37R} NMJs (**E**). The ability of PSCs to produce larger Ca²⁺ responses was not affected by TEA because local application of ATP (red arrow) elicited larger Ca²⁺ responses in PSC from WT and *SOD1*^{G37R} mice. In **B–D**, black and gray traces represent the mean and SEM, respectively.

consistent with changes of the presynaptic mechanisms of transmitter release.

Second, we evaluated the nerve-evoked activity of the NMJs. Amplitude of nerve-evoked EPPs (Fig. 3D; stimulation at 0.2 Hz) in the *SOD1*^{G37R} (1.18 ± 0.05 mV, *N* = 9, *n* = 14) was significantly larger than WT (0.89 ± 0.09 mV, *N* = 9, *n* = 15) and *SOD1*^{WT} (0.55 ± 0.05 mV, *N* = 5, *n* = 8) (Fig. 3D,E; one-way ANOVA, *p* < 0.0001). Again, data from *SOD1*^{WT} mice was significantly smaller than the WT and the *SOD1*^{G37R} mice.

Third, and consistent with these observations, quantal content of NMJs from *SOD1*^{G37R} mice (3.40 ± 0.18 , and *N* = 9, *n* = 14) was significantly higher compared with WT (2.44 ± 0.20 , *N* = 9, *n* = 15) and *SOD1*^{WT} mice (1.93 ± 0.14 , *N* = 5, *n* = 8) (Fig. 3F; one-way ANOVA, *p* < 0.0001). Moreover, the quantal content in Type I fibers for the *SOD1*^{G37R} was significantly higher

than the WT mice (data not shown; *N* = 5, *n* = 9, 2.41 ± 0.28 for WT and *N* = 5, *n* = 6, 3.35 ± 0.19 for *SOD1*^{G37R}, unpaired *t* test, *p* = 0.0269) but not in Type IIa fibers (*N* = 5, *n* = 5, 2.71 ± 0.27 for WT and *N* = 5, *n* = 7, 3.43 ± 0.32 for *SOD1*^{G37R}, unpaired *t* test, *p* = 0.1363). Interestingly, these alterations in synaptic properties did not follow the typical prediction that stronger synapses should present smaller PPF (Mallart and Martin, 1968). Indeed, the *SOD1*^{G37R} NMJs had a PPF value similar to the two control groups (Fig. 3G; WT, 1.33 ± 0.03 , *N* = 9, *n* = 15; *SOD1*^{WT}, 1.33 ± 0.03 , *N* = 5, *n* = 8; *SOD1*^{G37R}, 1.36 ± 0.02 , *N* = 9, *n* = 14; one-way ANOVA, *p* = 0.6966).

As a whole, this detailed synaptic analysis suggests that complex presynaptic alterations result in an enhancement of neurotransmitter release in *SOD1*^{G37R} mutants, primarily in Type I fibers.

Decoding ability also relies on PSC intrinsic properties at P120

Because PSCs are sensitive to transmitter release, one could argue that large Ca²⁺ response amplitude in *SOD1*^{G37R} may be due to a larger level of transmitter release at these NMJs. However, it has been demonstrated, at immature and adult NMJs, that the decoding ability of PSCs also relies on their intrinsic properties (Rousse et al., 2010; Darabid et al., 2013). Hence, we examined whether the increase in transmitter release could be sufficient to explain the level of PSCs excitability or if their intrinsic properties are at play.

To discriminate between these two possibilities, we argued that, if the difference in PSCs activity between WT and *SOD1*^{G37R} was solely dependent on the level of transmitter release, increasing transmitter release should result in a direct change in the amplitude of PSC Ca²⁺ responses. Because quantal content from WT and *SOD1*^{WT} mice was not significantly different (Fig. 3F), we compared *SOD1*^{G37R} mice with WT littermates for these experiments. We monitored nerve-evoked Ca²⁺ responses in PSCs while potentiating transmitter release using a K⁺ channel blocker (TEA, 0.2 mM), which does not directly affect PSCs (Rousse et al., 2010; Darabid et al., 2013). As shown in Figure 4A, TEA significantly increased EPP amplitude approximately two-fold, as expected, in WT (Ctrl, 0.79 ± 0.01 , TEA, 1.51 ± 0.02 , *N* = 4; paired *t* test *p* < 0.0001) and in *SOD1*^{G37R} (Ctrl, 1.08 ± 0.02 , TEA, 1.95 ± 0.02 , *N* = 4; paired *t* test *p* < 0.0001). PSC excitability appears unaltered by TEA because Ca²⁺ responses elicited by local application of agonists were unaffected (data not shown).

Interestingly, even though transmitter release was doubled, bath application of TEA had no effect on the average amplitude of PSC Ca²⁺ responses on WT synapses induced by nerve stimulation (50 Hz for 5 s) (Fig. 4B; $26.2 \pm 5.2\%$ $\Delta F/F$, *N* = 4, *n* = 6,

unpaired *t* test, $p = 0.3650$). Similar results were obtained when potentiating the *SOD1*^{G37R} synapses (Fig. 4C; $77.6 \pm 29.9\%$ $\Delta F/F$, $N = 4$, $n = 9$, unpaired *t* test, $p = 0.3666$). In addition, the percentage of responsive cells remained unchanged in WT and *SOD1*^{G37R}. Importantly, this cannot be explained by a saturation of the Ca^{2+} signal or the inability of PSCs to produce larger Ca^{2+} responses because larger Ca^{2+} responses were elicited when ATP was applied locally on the cells monitored following nerve-evoked stimulation (Fig. 4D; WT: $521.4 \pm 56.3\%$ $\Delta F/F$, $N = 4$, $n = 10$; Fig. 4E; *SOD1*^{G37R}: $680.2 \pm 51.8\%$ $\Delta F/F$, $N = 4$, $n = 6$). These results are consistent with previous observations (Rousse et al., 2010; Darabid et al., 2013) and indicate that an acute increase of neurotransmitter release cannot explain PSC properties in *SOD1*^{G37R} mice. This strongly suggests that PSC decoding of synaptic activity in an ALS mouse model relies on their intrinsic properties.

PSC ability to detect purinergic and muscarinic signals is unaltered at P120

At adult mammalian NMJ, ACh and ATP released during synaptic activity activate PSC purinergic and muscarinic receptors to elicit Ca^{2+} responses (Rochon et al., 2001). Hence, one possible mechanism that might explain the difference in the decoding ability of PSCs of WT and *SOD1*^{G37R} to nerve terminal stimulation is that PSC detection of these neurotransmitters may be altered. In this scenario, one would predict that Ca^{2+} responses elicited by local application of agonists for these receptors should also be altered in *SOD1*^{G37R}.

The ability of PSCs to detect ATP applied locally was examined first. Local application of ATP (10 μM) induced Ca^{2+} responses in 100% of PSCs with a mean of $564.0 \pm 25.8\%$ $\Delta F/F$ ($N = 6$, $n = 49$) for the WT, $463.7 \pm 27.8\%$ $\Delta F/F$ ($N = 4$, $n = 33$) for *SOD1*^{WT}, and $525.4 \pm 22.3\%$ $\Delta F/F$ ($N = 6$, $n = 55$) for *SOD1*^{G37R} (Fig. 5A). There was no significant differences between these amplitudes (Fig. 5B; one-way ANOVA, $p = 0.0917$). Also, we found no significant difference in Ca^{2+} responses associated with Type I fibers (Fig. 5C; $N = 4$, $n = 27$ for WT and $N = 3$, $n = 13$ for *SOD1*^{G37R}, unpaired *t* test, $p = 0.5544$) or Type IIa fibers ($N = 3$, $n = 12$ for WT and $N = 4$, $n = 21$ for *SOD1*^{G37R}, unpaired *t* test, $p = 0.6259$).

We next investigated the ability of a muscarinic agonist to evoke Ca^{2+} responses (Fig. 5D–F). Muscarine (10 μM) induced Ca^{2+} responses in 67.9% of PSCs of WT (36 of 53 PSCs), 48.9% of PSCs for *SOD1*^{WT} (23 of 47 PSCs), and 50.8% of PSCs for *SOD1*^{G37R} (31 of 61 PSCs). Local application of muscarine induced Ca^{2+} responses with a mean of $173.5 \pm 29.5\%$ $\Delta F/F$ ($N = 9$, $n = 36$) for the WT, $208.1 \pm 36.8\%$ $\Delta F/F$ ($N = 4$, $n = 23$) for *SOD1*^{WT}, and $200.8 \pm 28.0\%$ $\Delta F/F$ ($N = 8$, $n = 31$) for *SOD1*^{G37R} (Fig. 5D). These responses were not statistically different (Fig. 5E; one-way ANOVA, $p = 0.6294$). Similar results were obtained when ACh was locally applied (data not shown). We also tested whether PSC detection reflected MN properties. Similar to our observation using ATP local applications, we found no significant difference between Ca^{2+} response amplitude from PSCs associated with Type I fibers for WT and *SOD1*^{G37R} mice (Fig. 5F; $N = 5$, $n = 19$ for WT and $N = 5$, $n = 11$ for *SOD1*^{G37R}, unpaired *t* test, $p = 0.2081$) and Type IIa fibers (Fig. 5F; $N = 5$, $n = 13$ for WT and $N = 4$, $n = 14$ for *SOD1*^{G37R}, unpaired *t* test, $p = 0.9285$).

Together, these results indicate that PSC receptors are present and functional in this ALS mouse model at P120. Moreover, the PSC detection of local application of agonist appears normal, and this cannot explain the difference in PSC Ca^{2+} response evoked by synaptic activity.

PSC muscarinic detection of synaptic transmission is enhanced in mutant *SOD1* mice at P120

The lack of difference in Ca^{2+} responses of WT and *SOD1*^{G37R} mice could be explained by the fact that local applications of agonist activated PSC synaptic and extrasynaptic receptors, whereas nerve-evoked Ca^{2+} responses were induced by a subset of receptors during synaptic communication. This mechanism is at play to allow PSCs to distinguish two competing terminals at poly-innervated NMJs (Darabid et al., 2013). If this was the case, selective antagonism of each type of receptor should differentially alter PSC nerve-evoked Ca^{2+} responses of *SOD1*^{G37R} compared with WT. Because of the rundown of Ca^{2+} responses (Jahromi et al., 1992; Rochon et al., 2001), each preparation was stimulated once and amplitude of Ca^{2+} responses elicited by nerve stimulation presented in Figure 2B was used as control.

First, we examined the impact of purinergic receptor blockade using broad-spectrum P2 receptor antagonists (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid and suramin) and RB2 as a general P2_Y receptor antagonist. Consistent with our previous observations (Rochon et al., 2001), there were no clear purinergic mechanisms that were identified in PSCs of either WT or *SOD1*^{G37R} NMJs (data not shown). This suggests that, in the experimental conditions tested, activation of PSC purinergic contribution would be produced by other ATP-dependent mechanisms that still remain unidentified (Rochon et al., 2001).

Second, we examined the impact of mAChR blockade on nerve-evoked Ca^{2+} responses because they are the main receptor system through which PSCs are activated in adult NMJs (Rochon et al., 2001). Before testing the contribution of the PSC mAChR during synaptic transmission, we performed local application of agonists on PSC soma in the presence of the general mAChR antagonist atropine (5–20 μM) applied in the bath. As shown in Figure 6A, B, the presence of atropine completely abolished Ca^{2+} responses elicited by muscarine in all cells tested ($N = 4$, $n = 10$ for WT mice and $N = 4$, $n = 8$ for *SOD1*^{G37R} mice) but did not prevent PSC Ca^{2+} responses elicited by local application of ATP on the same cells. This suggests that atropine was specific for PSC mAChRs and did not interfere with the ability of PSCs to produce large Ca^{2+} responses. This blockage was reversible because Ca^{2+} responses were elicited by muscarine application following 45 min of washout (data not shown; $N = 3$, $n = 3$).

Activation of PSCs during synaptic transmission is known to be blocked by atropine (Rochon et al., 2001). As shown in Figure 6A, C, PSC Ca^{2+} responses of WT mice were significantly smaller in the presence of atropine ($22.2 \pm 2.8\%$ $\Delta F/F$ in atropine, $N = 10$, $n = 15$ vs $34.3 \pm 4.3\%$ $\Delta F/F$ in control, $N = 10$, $n = 22$; unpaired *t* test, $p = 0.0433$). This represented a 35% contribution of the muscarinic signaling pathway during synaptic transmission. From all tested NMJs, 50% of PSCs responded to motor nerve-evoked release of neurotransmitters in the presence of atropine (15 of 30 PSCs). No significant difference was found in PSC Ca^{2+} responses in WT mice in association with Type I and Type IIa fiber types (Fig. 6E; MHC-I: $N = 4$, $n = 8$ for WT without atropine and $N = 6$, $n = 9$ for WT with atropine, unpaired *t* test, $p = 0.3661$; MHC-IIa: $N = 7$, $n = 12$ for WT without atropine and $N = 4$, $n = 6$ for WT with atropine, unpaired *t* test, $p = 0.1075$).

Similar to WT NMJs, blockage of mAChR with atropine on *SOD1*^{G37R} NMJs also reduced the amplitude of Ca^{2+} responses induced by nerve-evoked transmitter release (Fig. 6B). However, this reduction was much more pronounced than in the WT (atropine, $24.9 \pm 3.0\%$ $\Delta F/F$, $N = 11$, $n = 25$; control, $108.9 \pm 18.1\%$ $\Delta F/F$, $N = 9$, $n = 22$; unpaired *t* test, $p < 0.0001$). This represented a 77% contribution of muscarinic signaling during

synaptic transmission. From all tested NMJs, 78% of PSCs responded to neurotransmitter release in the presence of atropine (25 of 32 PSCs). Interestingly, the remaining amplitude of PSCs Ca^{2+} responses in the presence of atropine was comparable for WT and $\text{SOD1}^{\text{G37R}}$ (Fig. 6C,D). Furthermore, because NMJ associated with fast fatigue-resistant MN and Type IIa fiber will degenerate first in the SOL during disease progression and PSC mAChR inactivation is required to repair the synapse, one would expect that PSC muscarinic contribution in these fibers to be lower than the one from fiber Type I. However, we found a significant difference between the average PSCs Ca^{2+} responses from $\text{SOD1}^{\text{G37R}}$ mice with and without atropine associated with fiber Type I (3.7-fold) and fiber Type IIa (5.5-fold) (Fig. 6F; MHC-I: $N = 4$, $n = 9$ for $\text{SOD1}^{\text{G37R}}$ without atropine and $N = 7$, $n = 17$ for $\text{SOD1}^{\text{G37R}}$ with atropine, unpaired t test, $p = 0.0013$; MHC-IIa: $N = 4$, $n = 8$ and for $\text{SOD1}^{\text{G37R}}$ with atropine and $N = 5$, $n = 8$ for $\text{SOD1}^{\text{G37R}}$ with atropine, unpaired t test, $p = 0.0022$).

As a whole, our data show that major synaptic alterations are already observed at an early stage of the disease (P120), particularly in Type I fibers, whereas PSCs show a muscarinic hyperactivation in both Type I and Type IIa fibers. Hence, these results reveal a larger contribution of PSC mAChR during synaptic communication in $\text{SOD1}^{\text{G37R}}$ mice that is independent of MN vulnerability at a pre-symptomatic stage of the disease. All these functional changes took place without any NMJ morphological alterations. These data suggest that ALS-associated events start long before symptom onset and involve several elements at the NMJ.

Enhanced neurotransmitter release in $\text{SOD1}^{\text{G37R}}$ mutants in the soleus muscle at P380

To determine whether the observed alterations progress in the course of the disease or are simply transient, we further examined the properties of NMJ at an age close to the disease onset.

First, we evaluated the synaptic properties of $\text{SOD1}^{\text{G37R}}$ mice at P380. We observed no difference in MEPP amplitude between WT and $\text{SOD1}^{\text{G37R}}$ mice (WT, 0.27 ± 0.01 mV, $N = 7$, $n = 21$; $\text{SOD1}^{\text{G37R}}$, 0.26 ± 0.01 mV, $N = 8$, $n = 22$; unpaired t test, $p = 0.2672$; Fig. 7A,B). Surprisingly, unlike P120, no difference in MEPP frequency was observed in the SOL muscle of the $\text{SOD1}^{\text{G37R}}$ mice at P380 where it was 0.88 ± 0.12 Hz for the WT mice and 0.74 ± 0.13 Hz for the $\text{SOD1}^{\text{G37R}}$ mice (Fig. 7A,C; $N = 6$, $n = 17$ for WT and $N = 7$, $n = 15$ for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p = 0.4601$). Importantly, no difference in MEPP frequency and amplitude was observed between WT and $\text{SOD1}^{\text{G37R}}$ mice in Type I fibers and Type IIa fibers (data not shown).

The properties of nerve-evoked synaptic activity of NMJs from mice at P380 were similar to the ones at P120 (stimulation at 0.2 Hz) where amplitude of nerve-evoked EPPs in the $\text{SOD1}^{\text{G37R}}$ (0.89 ± 0.09 mV, $N = 8$, $n = 23$) was significantly larger than WT

(0.66 ± 0.04 mV, $N = 9$, $n = 28$) (Fig. 7D,E; unpaired t test, $p = 0.0184$). Importantly, a difference in EPP amplitude was observed between WT and $\text{SOD1}^{\text{G37R}}$ mice in Type I fibers (data not shown; $N = 5$, $n = 11$, 0.68 ± 0.07 Hz for WT and $N = 5$, $n = 9$, 1.05 ± 0.08 Hz for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p = 0.0017$) but not in Type IIa fibers ($N = 5$, $n = 10$, 0.63 ± 0.05 Hz for WT and $N = 5$, $n = 6$, 0.62 ± 0.03 Hz for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p = 0.8234$).

Third, and consistent with these observations, quantal content of NMJs from $\text{SOD1}^{\text{G37R}}$ mice (3.45 ± 0.29 , $N = 8$, $n = 22$) was significantly higher compared with WT (2.57 ± 0.15 , $N = 7$, $n = 21$) (Fig. 7F; unpaired t test, $p = 0.0092$). Although the quantal content in Type I fibers was significantly higher in the $\text{SOD1}^{\text{G37R}}$ than in the WT mice (data not shown; $N = 5$, $n = 9$, 2.85 ± 0.20 for WT and $N = 5$, $n = 9$, 3.97 ± 0.31 for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p = 0.0084$), no difference was observed in Type IIa fibers (data not shown; $N = 5$, $n = 9$, 2.40 ± 0.25 for WT and $N = 5$, $n = 6$, 2.51 ± 0.12 for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p = 0.7260$). Again, similar to the P120 data, the $\text{SOD1}^{\text{G37R}}$ NMJs had a PPF value similar to the control group (Fig. 7G; WT, 1.31 ± 0.02 , $N = 9$, $n = 28$; $\text{SOD1}^{\text{G37R}}$, 1.35 ± 0.02 , $N = 8$, $n = 23$; unpaired t test, $p = 0.073$).

As a whole, electrophysiological data at a preonset age (P380) indicate that most synaptic properties observed very early on in the disease process were maintained throughout the nonsymptomatic phase of the disease.

Excitability of PSCs remains altered just before the onset of the disease

The ability of PSCs to detect and decode synaptic transmission is critical for their proper role in modulating synaptic function and maintenance (Ko and Robitaille, 2014). Hence, we evaluated whether the ability of PSCs to decode synaptic activity were altered at the preonset stage of the disease.

Using the same motor nerve stimulation (50 Hz for 5 s), we observed that endogenous release of neurotransmitter induced Ca^{2+} responses in PSCs at NMJs of $\text{SOD1}^{\text{G37R}}$ mice ($145.4 \pm 19.3\%$ $\Delta\text{F}/\text{F}$) that were significantly larger than in WT ($31.3 \pm 4.1\%$ $\Delta\text{F}/\text{F}$) (Fig. 8A; $N = 10$, $n = 15$ for WT and $N = 17$, $n = 39$ for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p = 0.0006$). These results indicate that PSCs at NMJs of an ALS mouse model still detected transmitter release at a preonset stage of the disease but with a persistent enhanced excitability and propensity to respond to synaptic activity (WT: 31%, 15 of 48 PSCs activated; $\text{SOD1}^{\text{G37R}}$: 51%, 39 of 76 activated PSCs). PSC Ca^{2+} responses of WT and SOD1 mice were not different from the ones observed at P120 (unpaired t test: for WT, $p = 0.6320$ and for $\text{SOD1}^{\text{G37R}}$, $p = 0.2155$).

We next tested whether PSC excitability reflected MN properties. As shown in Figure 8B, C, Ca^{2+} response amplitude from $\text{SOD1}^{\text{G37R}}$ was 4.4- to 4.8-fold larger than WT for each fiber type (MHC-I: $N = 6$, $n = 9$ for WT and $N = 5$, $n = 17$ for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p = 0.0098$) (MHC-IIa: $N = 4$, $n = 5$ for WT and $N = 12$, $n = 20$ for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p = 0.0413$). Together, again, these results suggest that alterations in PSC Ca^{2+} responses do not correlate with the predicted and specific vulnerability of MNs before the onset of the disease.

Persistent alteration of the PSC muscarinic detection in mutant SOD1 mice

Results described above showed that the muscarinic activation of PSCs was higher at NMJs of SOD1 mutant mice at an early stage of the disease. We wondered whether PSCs retained that property

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Figure 5. PSC Ca^{2+} responses to local application of ATP and muscarine. **A**, False color confocal images of PSCs (*) loaded with fluorescent Ca^{2+} indicator Fluo-4 AM, before (baseline), during (ATP application), and after (recovery) local ATP applications at NMJs of WT, SOD1^{WT} , and $\text{SOD1}^{\text{G37R}}$. Representative changes in fluorescence are illustrated on the right for the PSCs indicated by the arrowhead. Higher magnification of PSCs marked with the arrowhead in left are illustrated on the three other images for each group. **B**, Histogram depicting the mean $\pm$ SEM of the amplitude of the Ca^{2+} responses elicited in PSC by ATP application. **C**, Histograms depicting the mean $\pm$ SEM of the amplitude of the Ca^{2+} responses elicited in PSC by ATP application as a function of fiber type. **D–F**, Similar representation as in **A–C**, but illustrating the muscarine application. There was no statistical difference between the different animal groups either for the ATP or the muscarine application (one-way ANOVA, $p > 0.05$). Scale bar, 10 μm .

at preonset, a time when synaptic denervation becomes more prominent.

As shown in Figure 8A, PSC Ca^{2+} responses of WT mice were significantly smaller in the presence of atropine ($18.3 \pm 2.6\%$ $\Delta\text{F}/\text{F}$ in atropine, $N = 11$, $n = 15$ vs $31.3 \pm 4.1\%$ $\Delta\text{F}/\text{F}$ in control, $N = 10$, $n = 15$; unpaired t test, $p = 0.0129$). This represents a 42% contribution of the muscarinic signaling pathway during synaptic transmission. From all tested NMJs, 37% of PSCs responded to motor nerve-evoked release of neurotransmitters in the presence of atropine (15 of 41 PSCs). No significant difference was found in PSC Ca^{2+} responses in WT mice in association with Type I and Type IIa fiber types (Fig. 8B; MHC-I: $N = 6$, $n = 9$ for WT without atropine and $N = 7$, $n = 8$ for WT with atropine, unpaired t test, $p = 0.0998$; MHC-IIa: $N = 4$, $n = 5$ for WT without atropine and $N = 5$, $n = 7$ for WT with atropine, unpaired t test, $p = 0.0561$).

Similar to WT NMJs, blockage of mAChR with atropine on $\text{SOD1}^{\text{G37R}}$ NMJs also reduced the amplitude of Ca^{2+} responses induced by nerve-evoked transmitter release (Fig. 8A). However, this reduction was much more pronounced than in the WT (atropine, $21.9 \pm 2.2\%$ $\Delta\text{F}/\text{F}$, $N = 10$, $n = 15$; control, $145.4 \pm 19.3\%$ $\Delta\text{F}/\text{F}$, $N = 17$, $n = 39$; unpaired t test, $p = 0.0016$). This represents a 85% contribution of muscarinic signaling during synaptic transmission. From all tested NMJs, 36% of PSCs responded to neurotransmitter release in the presence of atropine (15 of 42 PSCs). Again, we found a significant difference between the average PSC Ca^{2+} responses from $\text{SOD1}^{\text{G37R}}$ mice with and without atropine associated with fiber Type I (6.4-fold) and fiber Type IIa (7.4-fold) (Fig. 8C; MHC-I: $N = 5$, $n = 17$ for $\text{SOD1}^{\text{G37R}}$ without atropine and $N = 6$, $n = 9$ for $\text{SOD1}^{\text{G37R}}$ with atropine, unpaired t test, $p = 0.0061$; MHC-IIa: $N = 12$, $n = 20$ for $\text{SOD1}^{\text{G37R}}$ without atropine and $N = 5$, $n = 6$ for $\text{SOD1}^{\text{G37R}}$ with atropine, unpaired t test, $p = 0.0139$).

As a whole, these results reveal a larger contribution of PSC mAChR during synaptic communication in $\text{SOD1}^{\text{G37R}}$ mice at P380, which remains independent of the known MN vulnerability.

PSC ability to detect muscarinic signals is altered at P380

PSC sensitivity to direct application of muscarinic and purinergic agonists was unaltered at P120. However, a fundamental prerequisite for PSCs to adapt their phenotype to the state of the NMJ (i.e., innervated vs denervated or mature vs immature) is to increase the sensitivity of the purinergic receptor signaling and lower the ones of the mAChRs (Darabid et al., 2013). We performed local applications of ATP and muscarine to test whether PSC sensitivity may be altered at a preonset period of the disease.

Local application of ATP ($10 \mu\text{M}$) induced Ca^{2+} responses in 100% of PSCs with a mean of $576.1 \pm 38.7\%$ $\Delta\text{F}/\text{F}$ ($N = 8$, $n =$

22) for the WT and $625.2 \pm 24.1\%$ $\Delta\text{F}/\text{F}$ ($N = 6$, $n = 29$) for $\text{SOD1}^{\text{G37R}}$ (Fig. 9A,B; no significant difference, unpaired t test, $p = 0.2649$). Also, we found no significant difference in Ca^{2+} responses associated with Type I fibers (Fig. 9C; $N = 4$, $n = 13$ for WT and $N = 4$, $n = 11$ for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p = 0.3097$). Interestingly, we did find an increased Ca^{2+} responses in association with Type IIa fibers ($N = 5$, $n = 9$ for WT and $N = 5$, $n = 18$ for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p = 0.0012$).

We next investigated the ability of a muscarinic agonist to evoke Ca^{2+} responses (Fig. 9D–F). Local application of muscarine ($10 \mu\text{M}$) induced Ca^{2+} responses in 69.6% of PSCs of WT (32 of 46 PSCs) and 56.5% of PSCs for $\text{SOD1}^{\text{G37R}}$ (52 of 92 PSCs). Average of Ca^{2+} responses was $139.5 \pm 20.0\%$ $\Delta\text{F}/\text{F}$ ($N = 9$, $n = 32$) for the WT and $248.6 \pm 21.5\%$ $\Delta\text{F}/\text{F}$ ($N = 10$, $n = 52$) for $\text{SOD1}^{\text{G37R}}$ (Fig. 9D). These responses were statistically different (Fig. 9E; unpaired t test, $p = 0.0009$). Finally, we tested whether PSC detection reflected MN vulnerability. Similar to our observation with ATP activation, we found no significant difference between Ca^{2+} response amplitude from PSCs associated with Type I fibers for WT and $\text{SOD1}^{\text{G37R}}$ mice (Fig. 9F; $N = 7$, $n = 15$ for WT and $N = 7$, $n = 36$ for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p = 0.2339$). However, we observed a significant increase in the amplitude of Ca^{2+} responses from PSCs associated with Type IIa fibers (Fig. 9F; $N = 6$, $n = 14$ for WT and $N = 5$, $n = 12$ for $\text{SOD1}^{\text{G37R}}$, unpaired t test, $p < 0.0001$).

Together, our results show that, close to symptom onset, NMJ synapses undergo persistent alterations of the synaptic functions whereby synaptic strength remains elevated only in Type I fibers, although spontaneous events are no longer enhanced. However, not only the glial alterations are maintained until the preonset in both fiber types, but an additional enhanced muscarinic sensitivity appeared in PSCs of Type IIa fibers.

As summarized in Figure 10, our results show that altered synaptic properties are maintained from an early, nonsymptomatic period, up to a preonset period. However, the glial alterations seem to correlate more with the disease progression. Indeed, PSC decoding abilities were unaltered at P60, whereas PSC excitability was enhanced at P120 through enhanced muscarinic activation. These features were maintained at P380, but an additional enhanced muscarinic contribution was unraveled only in Type IIa fibers, the ones that are more vulnerable to denervation.

As a whole, these results indicate that PSC phenotype is abnormal, with an imbalanced muscarinic and purinergic activation. As a consequence, the ability of PSCs to efficiently contribute to NMJ reinnervation could be impaired once the disease ultimately manifests itself, particularly in the more vulnerable fiber Type IIa.

Discussion

Using $\text{SOD1}^{\text{G37R}}$ mice, we report an inappropriate PSC decoding ability based on enhanced activation of mAChRs present early in the disease process (P120) and progressed until the preonset period of the disease (P380). Alteration of PSC mAChR functions is of particular importance because of their regulation of NMJ stability and repair. Our data suggest that the phenotype of PSCs is inconsistent with NMJ plasticity and repair, a condition that NMJs will necessarily undergo in ALS. To our knowledge, this is the earliest, persistent change reported in this mouse model (Ezzi et al., 2010; Filali et al., 2011).

Figure 6. Larger contribution of mAChR activation of PSCs during synaptic transmission in $\text{SOD1}^{\text{G37R}}$ mice. PSC Ca^{2+} responses evoked by motor nerve stimulation in the presence of atropine ($5\text{--}20 \mu\text{M}$) for WT (A) and $\text{SOD1}^{\text{G37R}}$ (B) mice. No Ca^{2+} responses were elicited in PSCs by local application of muscarine in the presence of atropine while responses were still elicited by local application of ATP. Dark trace represents the average of PSC Ca^{2+} responses. Dotted line indicates the SEM. Gray boxes represent the mean $\pm$ SEM of the amplitude of the Ca^{2+} responses elicited by nerve stimulation or agonist application without atropine. Histogram depicting the mean $\pm$ SEM of the amplitude of the Ca^{2+} responses elicited in PSCs by transmitter release evoked by motor nerve stimulation (50 Hz , 5 s) during bath application of mAChR antagonist (atropine, $5\text{--}20 \mu\text{M}$) for WT (C) and $\text{SOD1}^{\text{G37R}}$ (D) mice. Histogram depicting the mean $\pm$ SEM of the amplitude of the Ca^{2+} responses elicited in PSC by motor nerve stimulation in presence of atropine as a function of each fiber type for WT (E) and $\text{SOD1}^{\text{G37R}}$ (F) mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 7. Enhanced synaptic transmission at NMJs from *SOD1*^{G37R} mice at preonset stage of the disease. **A**, Traces of spontaneous activity (MEPPs) recorded from WT and *SOD1*^{G37R} NMJs. Histogram showing the mean $\pm$ SEM of the amplitude (**B**) and the frequency (**C**) of MEPP events. **D**, Examples of EPPs evoked by paired-pulse stimulation (10 ms interval) from WT and *SOD1*^{G37R} NMJs. **E**, Histogram showing the mean $\pm$ SEM of the amplitude of the first EPP. **F**, Histogram showing the quantal content determined as the ratio of EPP amplitude/mEPP amplitude. NMJs from *SOD1*^{G37R} mice had a larger quantal content. **G**, Histogram showing the PPF determined as the mean amplitude of the second EPPs divided by the mean amplitude of the first EPPs. * $p < 0.05$.

Differential alteration of presynaptic and glial properties in ALS

The abnormal nerve-evoked synaptic properties remained throughout the adulthood life of the animal, which is particularly striking considering that SOL has a delayed degeneration in ALS (Frey et al., 2000; Atkin et al., 2005; Pun et al., 2006; Hegedus et al., 2007; Valdez et al., 2012). The abnormalities in the presynaptic functions in *SOD1*^{G37R} mice described here are consistent with the ones of the diaphragm preparation from *SOD1*^{G93A} mice at a presymptomatic stage of the disease (Rocha et al., 2013), a muscle with the same fiber composition as the SOL (Zardini and Parry, 1994; Gregorevic et al.,

2008). Interestingly, others found that synaptic transmission was reduced at NMJs of fast twitch muscles of larval zebrafish carrying *TDP-43* or *FUS* mutations (Armstrong and Drapeau, 2013a, b). Hence, it would be important to determine the properties of PSCs in a fast-twitch muscle knowing that PSCs at NMJs of different muscles have different properties (Rousse et al., 2010).

Similar to the hyperexcitability of mutant *SOD1*^{G93A} spinal MN in culture and from organotypic slices (Pieri et al., 2003; Kuo et al., 2004), we observed an enhanced excitability and synaptic strength of the nerve terminal of *SOD1*^{G37R}. Interestingly, recent data suggest that the higher resilience to the disease of fast fatigue-

Figure 8. Altered PSC excitability at preonset stage of the disease. **A**, Dark traces represent the mean $\pm$ SEM of the Ca²⁺ responses elicited in PSCs by transmitter release evoked by motor nerve stimulation (50 Hz, 5 s) at NMJs of WT and SOD1^{G37R}. Larger Ca²⁺ responses were elicited in PSCs of NMJs from SOD1^{G37R} mice (unpaired *t* test, $p = 0.0006$). Blue traces represent the mean $\pm$ SEM of the PSC nerve-evoked (50 Hz, 5 s) Ca²⁺ responses in presence of atropine (20 μ M) for WT and SOD1^{G37R} mice. PSC Ca²⁺ responses of WT (unpaired *t* test, $p = 0.0129$) and SOD1 mice (unpaired *t* test, $p = 0.0016$) were significantly smaller in the presence of atropine; this represents a 42% and 85% contribution of muscarinic signaling during synaptic transmission, respectively. **B**, Histograms depicting the mean $\pm$ SEM of the amplitude of the Ca²⁺ responses elicited in PSCs by motor nerve stimulation in the presence of atropine depending of each type of fiber for WT and SOD1^{G37R} (**C**) mice. The altered PSC-detecting ability did not correlate with MN vulnerability at a preonset stage of the disease. * $p < 0.05$, ** $p < 0.01$.

resistant and slow MNs is related to higher MN excitability (Saxena et al., 2013). Our results support this possibility because the quantal content of the slow MN alone (the more disease-resistant ones) was significantly higher in the SOD1^{G37R} mice. Furthermore, increased MEPP frequency at P120 has been observed in motor endplate disease, a hereditary disorder of NMJs caused by a progressive failure of neurotransmission (without denervation), muscle inactivity, and atrophy (Duchen and Stefani, 1971). Our data are also consistent with increased MEPP frequency (Uchitel et al., 1988; Appel et al., 1991) and quantal content at the mouse NMJ (O'Shaughnessy et al., 1998) induced by immunoglobulins from ALS patients. A common mechanism may be related to presynaptic Ca²⁺-dependent regulation (Uchitel et al., 1988; Appel et al., 1991; O'Shaughnessy et al., 1998; Armstrong and Drapeau, 2013a; Rocha et al., 2013) because of the larger Ca²⁺ accumulation in motor nerve terminals from ALS patients (Siklós et al., 1996) and the greater Ca²⁺ influx in nerve terminals that release more neurotransmitters (Pawson and Grinnell, 1990). However, the normal MEPP frequency observed at P380 is not consistent with this possibility, pointing to another molecular mechanism of exocytosis.

Unlike the abnormal synaptic properties, changes in PSC properties were first observed at P120 and evolved during the course of the disease. Indeed, PSC muscarinic and purinergic

sensitivity increased with age in an MN vulnerability-dependent manner. Interestingly, similar to neurons, PSCs undergo plastic changes following an alteration of synaptic activity (Bélair et al., 2010), resulting in changes of receptor contribution and sensitivity. For instance, PSC purinergic receptor contribution is increased and the muscarinic one decreased in conditions when morphological plasticity is required, such as NMJ maturation (Darabid et al., 2013) and after nerve injury (Georgiou et al., 1999, 1994; Perez and Robitaille, personal communication).

Interestingly, there is an important mismatch between the synaptic and the PSC properties of NMJs on Type I and Type IIa MNs. Indeed, although synaptic output was only higher for Type I MNs, PSCs muscarinic excitability was high in both types. Although this glial feature would help maintain NMJs during ALS pathogenesis and be consistent with the stability of the Type I NMJs, it appears detrimental for the Type IIa. Indeed, permissiveness of PSCs for NMJ repair requires a reduced muscarinic receptor activity (Darabid et al., 2014). However, although Type IIa MNs are targeted before Type I, PSCs at these NMJs still present a high muscarinic sensitivity close to the disease onset. Hence, this imbalance of PSC receptors appears as a limiting factor for NMJ repair in ALS.

Overall, our data indicate that the synaptic properties are present early and appear maintained until the disease onset,

Figure 9. PSC Ca^{2+} responses to local application of ATP and muscarine at preonset stage of the disease. **A**, False color confocal images of PSCs (*) loaded with fluorescent Ca^{2+} indicator Fluo-4 AM, before (baseline), during (ATP application), and after (recovery) local ATP applications at NMJs of WT and SOD1^{G37R}. Representative changes in fluorescence are illustrated on the right for the PSCs indicated by the arrowhead. Higher magnification of PSCs marked with the arrowhead in left are illustrated on the three other images for each group. **B**, Histogram depicting the mean $\pm$ SEM of the amplitude of the Ca^{2+} responses elicited in PSCs by ATP application. **C**, Histograms depicting the mean $\pm$ SEM of the amplitude of the Ca^{2+} responses elicited in PSC by ATP as a function of each fiber type. **D–F**, Similar representation as in **A–C**, but for muscarine application. Note the statistical difference between the different animal groups for the muscarine application and for the PSC Ca^{2+} responses associated with fiber Type IIa for ATP and muscarine application. Scale bar, 10 μ m. *** $p < 0.001$.

Figure 10. Timeline of changes in NMJ structure and function at presymptomatic stages of the disease in SOL muscle. Summary of the changes in the presynaptic properties (top), PSC properties (middle), and the morphological features (bottom) is presented as a function of the age of the animals. It shows that synaptic properties are observed early and only in Type I fibers and maintained throughout. However, PSC properties are altered in both fiber types and further evolve at a presymptomatic stage. Morphological features are normal at P120 but altered at the presymptomatic stage. ↑ indicates a significant increase in SOD1 compared with WT, and "No $\neq$ " indicates that there was no statistical difference.

whereas PSC properties gradually evolve and worsen toward disease onset. In addition, our data indicate that functional changes in synaptic and glial activities are more sensitive indicators of the state of NMJ functions in ALS than are the morphological characteristics, the former appearing several months before the latter. Hence, it would be important to develop tools to measure these properties, providing a more sensitive readout of the NMJ functions and an earlier detection of NMJ dysfunctions.

Impact of PSC abnormalities in ALS

The roles of axonal Schwann cells in ALS were studied using selective expression or knock-out of mutant SOD1 in these cells (Lobsiger et al., 2009; Turner et al., 2010; Wang et al., 2012). However, these studies were not designed to specifically assess PSC contributions in ALS pathogenesis. This is important considering that PSCs are quite different from axonal Schwann cells based on their properties and functions at the NMJ (Auld and Robitaille, 2003).

Implication of glial cells at the NMJ in ALS remains ill-defined, although the involvement of glial cells in MN death has received strong support and selective rescue of MN death only modestly delayed denervation and improved lifespan (Gould et al., 2006). An interesting possibility is that the adverse relationship between glial cells and MNs in the spinal cord in ALS may be replicated at the NMJ (Carrasco et al., 2010). Indeed, using whole-muscle transplants in *SOD1^{G93A}* mice, Carrasco et al. (2010) hypothesized that the properties of nerve terminal and PSCs, and not the muscle transplant, were determinant for the degenerative changes observed at the NMJ. This suggests that interactions between PSCs and motor nerve ter-

minal are crucial to understand how the degenerative changes begin and progress at NMJs in ALS.

Detrimental consequences for the stability and repair of the NMJ in ALS may emerge from the altered PSC decoding ability and mAChR sensitivity. For instance, the larger activation of PSC mAChR during synaptic transmission at P120 and P380 in SOD1 mutants implies that their detection threshold is decreased, which could maintain their muscarinic activation even in conditions of reduced synaptic activity. This would maintain gene expression compatible with NMJ maintenance, preventing PSCs to switch to the repair phenotype needed when denervation occurs. One possibility is that the formation and/or extension of PSC processes may be altered. However, our observations and those of others (Frey et al., 2000; Gould et al., 2006) argue against this suggestion because PSC processes have been observed in ALS mouse models. Another interesting possibility is that the guidance of the motor nerve terminal and the remodeling/stabilization of the new synapse may be altered (Kang et al., 2014). An altered propensity to repair and reform NMJs following nerve injury in ALS models supports this possibility (Gordon et al., 2004). Furthermore, the clearance of debris (Kang and Lichtman, 2013) and lack of metabolic support can also be involved (Moloney et al., 2014). Hence, we postulate that the inadequate muscarinic activation of PSCs leads to an improper repair of NMJ structure and function during the course of the disease.

Potential mechanisms underlying an increased muscarinic excitability

The inadequate mAChR activation of PSC may have several origins. The distribution and density of the mAChRs on PSC pro-

cesses close to active zones and/or changes in the functionality of the receptor per se can be altered. Other mechanisms may include changes in the receptor subtypes on PSC (M1, M3 and M5) (Wright et al., 2009; Darabid et al., 2013), affinity, insertion, and recycling or their association with internal molecular machinery activating different downstream signaling pathways.

Another interesting mechanism is that the soluble *SOD1*^{G37R} protein impacts on mAChR functionality. Indeed, it was suggested that soluble SOD1 protein interacts with cellular membrane and alter muscarinic receptor activation of phospholipase C pathway (Damiano et al., 2013).

In conclusion, because of their roles in regulating the balance between synaptic efficacy, maintenance, and repair following injury, the enhanced mAChR activation of PSCs would impinge the quality and reliability of NMJ repair during ALS progression. Future demonstration of this PSC malfunction as a potential contributor to ALS and also in other ALS models (e.g., *TDP-43* and/or *FUS*) will highlight the importance and broad implication of such mechanisms. Our study is the first direct evidence of glial alteration at the NMJ despite the importance of NMJ malfunction and the reported involvement of other glial cells in ALS. The intrinsic PSC properties could represent a very important and novel therapeutic target in ALS.

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