Postactivation depression of the Ia EPSP in motoneurons is reduced in both the G127X SOD1 model of amyotrophic lateral sclerosis and in aged mice

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Hedegaard A, Lehnhoff J, Moldovan M, Grøndahl L, Petersen NC, Meehan CF. Postactivation depression of the Ia EPSP in motoneurons is reduced in both the G127X SOD1 model of amyotrophic lateral sclerosis and in aged mice. J Neurophysiol 114: 1196–1210, 2015. First published June 18, 2015; doi:10.1152/jn.00745.2014.—Postactivation depression (PActD) of Ia afferent excitatory postsynaptic potentials (EPSPs) in spinal motoneurons results in a long-lasting depression of the stretch reflex. This phenomenon (PActD) is of clinical interest as it has been shown to be reduced in a number of spastic disorders. Using in vivo intracellular recordings of Ia EPSPs in adult mice, we demonstrate that PActD in adult (100–220 days old) C57BL/6J mice is both qualitatively and quantitatively similar to that which has been observed in larger animals with respect to both the magnitude (with ~20% depression of EPSPs at 0.5 ms after a train of stimuli) and the time course (returning to almost normal amplitudes by 5 ms after the train). This validates the use of mouse models to study PActD. Changes in such excitatory inputs to spinal motoneurons may have important implications for hyperreflexia and/or glutamate-induced excitotoxicity in the neurodegenerative disease amyotrophic lateral sclerosis (ALS). With the use of the G127X SOD1 mutant mouse, an ALS model with a prolonged asymptomatic phase and fulminant symptom onset, we observed that PActD is significantly reduced at both presymptomatic (16% depression) and symptomatic (17.3% depression) time points compared with aged-matched controls (22.4% depression). The PActD reduction was markedly altered by symptom onset. Comparing these PActD changes at the EPSP with the known effect of the depression on the monosynaptic reflex, we conclude that this is likely to have a much larger effect on the reflex itself (a 20–40% difference). Nevertheless, it should also be accounted that in aged (580 day old) C57BL/6J mice there was also a reduction in PActD although, aging is not usually associated with spasticity.

postactivation depression; aging; ALS

The simplest and earliest characterized reflex, the stretch reflex, is mediated by Ia afferents from muscles forming monosynaptic connections with spinal motoneurons innervating the same (or synergistic) muscles. Although much research has investigated Ia excitatory postsynaptic potentials (EPSPs) in motoneurons evoked by single shocks, physiological activation of the Ia afferents usually occurs in trains. In such cases, mechanisms related to the presynapse operate to impose a frequency related depression of the EPSPs evoked at the Ia afferent-motoneuron synapse. This is of two distinct forms: the first of these, presynaptic inhibition, was first described by Eccles (1964) and has been considered to be mediated by GABAergic interneurones, which synapse directly onto the terminals of Ia afferents, activating GABA_A receptors (Rudomin and Schmidt 1999). Importantly, this effect is relatively short lasting with a duration not longer than a few hundred milliseconds (Eccles 1964). A second, longer lasting type of depression is also seen following repetitive activation of Ia afferents and has been termed postactivation depression (PActD) (Crone and Nielsen 1989) or homosynaptic depression (Curtis and Eccles 1960).

PActD differs from classical presynaptic inhibition not only with respect to time scale (lasting up to 10 s) (Hultborn et al. 1996) but also in terms of mechanisms. Unlike presynaptic inhibition, it is not affected by blocking GABA_A receptors (Lev-Tov and Pinco 1992). PActD is not accompanied by a dorsal root potential (Hultborn et al. 1996) and is not observed in terminal potentials of Ia afferents recorded extracellularly (Lev-Tov and Pinco 1992), suggesting that it is not due to an impairment of action potential invasion to the afferent terminals. In contrast to presynaptic inhibition, PActD is restricted to homonymous Ia afferents (Hultborn et al. 1996) and is observed with no change in membrane potential, conductance (Hultborn et al. 1996), input resistance, or membrane time constant (Lev-Tov and Pinco 1992). Taken together these findings suggest a presynaptic mechanism for PActD, which has been hypothesized to be a depletion in the levels of readily releasable neurotransmitters in the presynaptic terminal (Lev-Tov and Pinco 1992).

PActD of Ia EPSPs in motoneurons can be measured both directly using intracellular recordings of EPSPs in animal models or indirectly in humans by examining frequency depression of the stretch reflex or its experimental electrical analog, the Hoffman reflex (H reflex) (Hultborn et al. 1996).

PActD is of clinical interest given that it has consistently been shown to be reduced across a range of disorders in which spasticity is present including spinal cord injury (Aymard et al. 2000; Field-Fote et al. 2006; Ishikawa et al. 1966), stroke (Lamy et al. 2009), cerebral palsy (Achache et al. 2010), and multiple sclerosis (Grey et al. 2008; Nielsen et al. 2005). Furthermore, of all of the factors known to affect the efficacy of the stretch reflex, a reduction in PActD is the only one that has consistently been shown to correlate with the degree of spasticity (Achache et al. 2010; Aymard et al. 2000; Field-Fote et al. 2006; Ishikawa et al. 1966; Lamy et al. 2009; Masakado et al. 2005).

Spasticity is also a feature of the motoneuron disease amyotrophic lateral sclerosis (ALS). Little research, however, has focused on factors influencing the stretch reflex in ALS although abnormalities in H reflexes do suggest that presynaptic
inhibitory mechanisms may be impaired (Schieppati et al. 1985). Changes in excitatory inputs to motoneurons are also of interest in ALS due to their potential contribution to an increased excitation of spinal motoneurons resulting in excitotoxic cell death. The hypothesis of glutamate-induced excitotoxicity of motoneurons in ALS has received considerable support from investigations in both humans with ALS (Lin et al. 1998; Plaitakis and Carosol 1987; Plaitakis et al. 1988; Plaitakis and Constantakakis 1993; Rothstein et al. 1990, 1992, 1995; Spauws-Varoquaux et al. 2002) and animal models of the disease (Guo et al. 2003; Howland et al. 2002; Trott et al. 1999).

The discovery of mutations in the gene coding for the superoxide dismutase-1 (SOD1) enzyme in a small proportion of human ALS cases (~15–25% of genetic cases) (Rosen et al. 1993) has led to the creation of a number of different SOD1 transgenic mouse models of the disease (Bruijn et al. 1997; Gurney et al. 1994; Jonsson et al. 2004, 2006; Ripp ps et al. 1995), which develop similar forms of the human disease with features including spasticity (Dentel et al. 2013; Modol et al. 2014). Embryonic and neonatal in vitro preparations of spinal motoneurons from these mutants have shown an increased intrinsic excitability of the motoneurons, which may contribute to an eventual excitotoxicity (Kuo et al. 2004, 2005; Pieri et al. 2003; van Zundert et al. 2008). Recent work, however, has found this hyperexcitability to be restricted to the less vulnerable S-type motoneurons (Leroy et al. 2014) and in vivo recordings from motoneurons of adult mutants, suggest the neurones appear to compensate for this early excitability, displaying relatively normal intrinsic properties (Meehan et al. 2010a; Delestre et al. 2014). The spinal motoneurons are, however, embedded within spinal circuitry. Changes in this circuitry producing an imbalance between excitatory and inhibitory synaptic inputs to the motoneurons could therefore provide a possible alternative substrate for an excitotoxic increase in excitability. Anatomical studies using the SOD1 ALS mutants are consistent with such an imbalance favoring excitation (Carunchio et al. 2008; Chang and Martin 2009, 2011a,b; Sunico et al. 2011; Wootz et al. 2013).

In the present experiments we used the G127X SOD1 ALS mouse model, an ALS model with a prolonged asymptomatic phase and fulminant symptom onset, which is particularly suitable to evaluate electrophysiological changes at symptom onset (Moldovan et al. 2012). Here we could directly explore PACdT of the Ia EPSP in this model using intracellular recording in vivo both at presymptomatic (immediately before symptom onset) and symptomatic time points.

To be able to investigate PACdT in transgenic mouse models, it was first necessary to validate that it was, in fact, possible to accurately record PACdT in adult mice in vivo as this had not been done before. We were then able to confirm that, in mice, PACdT was both qualitatively and quantitatively similar to that which has been observed in larger animals, in which PACdT has been investigated traditionally (i.e., cat and human). We then showed that PACdT is reduced in the G127X SOD1-mouse model of ALS at both presymptomatic and symptomatic time points.

Finally, little is known regarding possible reductions of PACdT in situations affecting the activity levels of pathways involving spinal motoneurons in which spasticity is not present. The most obvious condition would be that of aging. This is also of interest given that a number of abnormalities observed in the ALS SOD1 models are consistent with an accelerated maturation and/or aging (Das and Svendsen 2015; Gordon et al. 2004; Quinlan et al. 2011). PACdT in the elderly has only been explored indirectly in humans using the stretch reflex and H reflex in two studies so far, which have yielded conflicting results. The first, using the stretch reflex for the soleus muscle, found a reduction in PACdT (Robertson and Koceja 2003), whereas the second, using the H reflex for the flexor carpi radialis muscle, found a reduction in frequency depression at 2 Hz but not at 1 Hz (Trompetto et al. 2014).

The efficacy of these reflexes, however, may also be influenced by a number of other systemic factors known to be affected in aging. These include an increase in passive muscle stiffness (Rosant et al. 2007), a decreased sensitivity of muscle spindles to muscle stretch (Miwa et al. 1995), a reduction in presynaptic inhibition (Butchart et al. 1993), and a decreased excitability of spinal motoneurons (Morales et al. 1987) resulting in H reflexes of decreased amplitude and longer duration and being polyphasic with a longer latency (Sabbahi and Sedgwick 1982). We therefore decided to also measure PACdT in aged mice by direct recordings of the Ia EPSP and confirmed that PACdT was, in fact, also reduced in lumbar motoneurons in aged mice. Surprisingly, the reduction in PACdT in the aged mice was similar in magnitude to that which we observed in the G127X ALS model, raising questions as to its causal role in the development of hyperreflexia and/or glutamate-induced excitotoxicity.

METHODS

Ethical approval. The experiments were performed at the University of Copenhagen. The experimental protocol was approved by the Danish National Animal Experiment Committee (Permission No. 2012-15-2934-00501) and was in accordance with European Union Regulations. All surgery and experiments were performed under anesthesia and were acute, ensuring no suffering.

Control mice. Experiments on wild-type (WT) mice were performed on 19 adult C57BL/6j mice. Thirteen of these mice were used at ~100–220 days old (9 female, 4 male) and six at ~580 days of age (4 female, 2 male). We will refer to this first group as adult mice and the second as aged mice.

C57BL/6j mice age ~150 times faster than humans and thus are generally considered to be adult by 3–6 mo, middle-aged by 10–14 mo, and old at 18–24 mo (~56–69 yr in humans) after which point the survival rate drops drastically (Flurkey et al. 2007). All of the younger mice were used to investigate the general features of PACdT in mice and as controls for the aged mice whereas only mice around 220 days old (7 of the 13 mice) were used as age-matched controls for the G127X mice.

G127X SOD1 mice. Mice transgenically expressing G127insTGGG (G127X) mutant human SOD1 (Jonsson et al. 2004) were backcrossed on C57BL/6j mice for more than 25 generations in Umea, Sweden (Jonsson et al. 2004). Homozygote mice from the original line 716 expressing 19 copies of the human SOD1 G127X were then bred as homozygotes at our own institution. The mutant SOD1 itself lacks enzyme activity, and it is rapidly degraded resulting in low levels of the mutant enzyme in the spinal cord (Jonsson et al. 2004), which reduces the problems of overexpression artifacts associated with the more commonly used G93A SOD1 mutant. The G127X strain has a relatively late symptom onset at around 250 days, which is followed by a rapid progression of ~1 wk. Experiments were therefore performed on five mice (4 males, 1 female) at ~210 days, a time point close to but before obvious symptom onset. A further two mice were used at a symptomatic time point (2 males, mean age 229 days). This low number is due to the rapid progression of...
symptoms and our humane endpoints for this model, combined with the problem that the in vivo experiments are difficult to perform at the symptomatic stage due to respiratory failure under anesthesia. The data obtained from these mice, however, are included with the caveat that they represent only two mice. Symptomatic in this case was defined as a slowing of movement, a hunched gait, and extreme weakness or paralysis in at least one limb.

Electrophysiology experiments. A brief anesthesia was induced with isoflurane. A longer lasting anaesthesia was then induced and maintained with intraperitoneal injections of Hypnorm and midazolam, mixed one part Hypnorm (fentanyl-citrate 0.315 mg/ml and fluanisone: 10 mg/ml), one part midazolam (5 mg/ml), and two parts sterile water. An induction dose of 10 ml/kg was given with supplemental doses of 0.5 ml every 20 min. All mice received a single dose of atropine (0.02 mg ip) at the start of the surgery. A tracheal cannula was inserted to allow for later artificial ventilation and intraperitoneal cannulas were inserted for drug delivery during the experiment. The main tibial nerve branch and the common peroneal (CP) nerve branch were dissected, and a hemilaminectomy was performed at vertebral levels T12-L1, exposing the spinal cord. This uncovers segments L3-L5 of the mouse spinal cord (Harrison et al. 2013). In the mouse spinal cord, motoneurons for both the tibial and the CP nerves are located mostly within L3-L4 with the motoneurons in the CP nerve lying slightly more rostral to those of the tibial nerve (but with a large overlap). We used the incoming volleys recorded from the cord dorsum electrodes and extracellular antidromic field potentials to confirm our segments and electrode tracks were therefore made both rostrally and caudally within both segments so as to include populations of both.

Mice were then placed in a Narishige stereotactic frame with the head secured in a head holder. In the recording frame vertebral clamps were attached on the vertebrae above and below the laminectomy to secure the spinal column in this region. The electrocardiogram (ECG) was monitored using clips placed on the ear and rear foot. The temperature was monitored using a rectal probe and maintained at 37°C using a heat pad underneath and a heat lamp above the mouse, controlled by the output from the temperature probe. Mice were then paralyzed using the neuromuscular blocking agent Pavulon (diluted 1:10 with saline, administered as an initial dose of 0.1 ml followed by 0.05-ml doses per hour). Anesthetic was continuously given at the same rate as during the surgical procedure (0.05 ml of the mixture/20 min). Expired carbon dioxide levels were measured using a Capstar CO₂ analyzer (ITTC Life Science).

For the intracellular recordings, an Axoclamp 2B intracellular amplifier was used in bridge mode. Signals were then further amplified and filtered (at 10 kHz) with custom-made modules (University of Copenhagen). Finally, the signals were digitized (at 20 kHz) using a 1401 analog to digital converter [Cambridge Electronic Design (CED), Cambridge, UK] and recorded using the Signal software (CED).

Intracellular recordings were performed as described in Meehan et al. (2010b). Briefly, with the use of an electronic microdrive, a glass microelectrode (filled with 2 M potassium acetate) was inserted into the spinal cord. Antidromic field potentials from stimulation (0.05-ms pulse) of the peripheral nerves were used as guides to locate the motoneurons. Identification of motoneurons was made by the presence of an all or none antidromic action potential following stimulation of the peripheral nerves (Fig. 1A). Antidromic spikes were distinguished from orthodromic spikes by their latency relative to the incoming volley (cord dorsal potential) recorded from electrodes placed on the dorsal lateral surface of the spinal cord immediately lateral to the spinal region where the glass electrode was lowered. The stimulus intensity was then reduced until only the lower threshold monosynaptic EPSP was visible (Fig. 1A).
Postactivation Depression in Aging and ALS

1199

...nance of the effects of PActD from the classical presynaptic inhibition of the Ia afferent, which has been demonstrated to have a much shorter time course. The resulting EPSP (from this delayed stimulation) will be referred to as the test EPSP. The same stimulator was used for the conditioning volley and test pulse, and both were triggered by the CED controlled by the Signal software, which allowed us to alternate between the different time intervals.

Only motoneuron penetrations with membrane potentials more hyperpolarized than $-45 \text{ mV}$ were accepted for analysis. Averages were obtained from multiple trials (usually $>10$) using the Signal software. The ECG was also recorded and trials were excluded from averages if the ECG occurred at the same time point as the control or test EPSP. Trials were also excluded if there was a clear difference in resting membrane potential between control and test EPSPs due to obvious mechanical movement. The size of the test EPSP was then compared with that of the control EPSP (Fig. 1Bi) and expressed as a percentage of the control EPSP to gain a measure of the PActD (i.e., the higher the percentage the lower the PActD).

In a few cells (4 in control mice and 3 in G127X mice), the antidromic action potential was at such a low stimulus threshold that a small hyperpolarizing current was necessary to prevent invasion of the antidromic action potential to visualize the Ia EPSP. To verify whether this would affect the PActD, we recorded the PActD at resting $V_m$ in another 13 motoneurons and then injected constant current to record the same PActD in the same motoneurons at a more hyperpolarized $V_m$. This did not systematically affect the level of PActD (paired $t$-test, $P = 0.18$, data not shown).

In some cells we also delivered a short positive square current pulse through the microelectrode to evoke single action potentials in the cells. Averages of multiple trials were then used to measure the duration of the postspike afterhyperpolarization (AHP). Different electrophysiological data were also obtained in some of the mice for another project and the mice were then overdosed and transcardially perfused with saline followed by 2% paraformaldehyde.

Statistics. All statistical tests were performed using the GraphPad Prism software. D’Agostino and Pearson omnibus normality tests were used to confirm normality. For data passing this test, parametric statistics were used, either a $t$-test (for 2 groups) or an ANOVA followed by Tukey’s multiple comparisons test (for 3 groups). For data not passing the normality tests, nonparametric statistics were used, either a Mann-Whitney test (for 2 groups) or a Kruskal-Wallis test followed by Dunn’s multiple comparisons test (for 3 groups).

Statistical significance was accepted at the $P \leq 0.05$ level. On all graphs asterisks are used to indicate the following significant differences: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$. Unless indicated on the graphs no significant difference was found.

RESULTS

With the use of the above protocols and the strict criteria for acceptance of both cells and trials, it was possible to obtain stable averages allowing detection of PActD of the homonymous (previously activated) Ia EPSP in all of the control motoneurons (example shown in Fig. 1B). This occurred with no obvious change in membrane potential (between the control and test EPSPs) and no detectable change in the magnitude of the incoming volley (example shown in Fig. 1Bi). Data were obtained from 74 motoneurons in the control adult mice (100–220 day old), from 37 motoneurons in the aged mice (580 days old), and from 46 motoneurons in the G127X mice. We will initially address the data obtained in the control adult mice (100–210 day old) allowing us to first characterize the normal features of PActD in adult healthy mice.

The time course of PActD in mice. To determine the time course of the PActD, we tested the depression with intervals between the conditioning and test stimuli ranging from 0.5 to 5 s (Fig. 2A) in 12 motoneurons from 5 of the control mice (100–220 day old). From these recordings, it can be seen that both the magnitude (at the different intervals) and the time course of the effect are comparable to those results obtained from cats, illustrated by Hultborn et al. (1996) (Fig. 2A, A and B). For “between group comparisons,” the magnitude of the PActD at the 0.5-s interval was used, as this was considered to be the time when presynaptic inhibition was over but before the PActD started to decline.

Control EPSP size and membrane potential do not influence PActD magnitude. To determine whether the initial control EPSP size had an influence on the magnitude of PActD, we plotted the obtained PActD values by size of the conditioning EPSP (73 cells, 13 adult WT mice; $R^2 = 0.0002$; Fig. 2C). We also tested whether the magnitude of PActD was affected by membrane potential. The membrane potential during the recording of the PActD protocol was confirmed by checking the extra-axonal zero potential. Linear regression analysis confirmed that PActD was not influenced by the membrane potential (66 cells, 13 adult WT mice; $R^2 = 0.03384$; data not shown).

PActD is strictly homosynaptic. In the mouse the tibial and the CP nerve branches are not strict collections of flexors and extensors, respectively. There were therefore cases in which stimulation of the tibial nerve caused monosynaptic EPSPs in CP motoneurons and vice versa. In these cases, the effects of conditioning stimulation trains from heteronymous nerves could be tested. As in larger animals, the PActD effect appeared to be restricted to the homonymous nerve. This is illustrated in a recording from a CP motoneuron in Fig. 3A. From this it can be seen that there is a clear depression of the test Ia EPSP elicited by stimulation of the CP nerve if the stimulating train was chosen to be restricted to the homonymous nerve (Fig. 3Ai). This was not the case, however, if the conditioning train of EPSPs was elicited by the stimulation of the heteronymous tibial nerve (Fig. 3Ai). If the CP test pulse is given 100 ms after the conditioning train elicited by the tibial nerve, however, then the test EPSP is clearly depressed, consistent with presynaptic inhibition (Fig. 3Aii).

Similarly, it can be seen in Fig. 3B in a tibial motoneuron that if both the train of EPSPs and the test EPSP are evoked

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A

Control EPSP

Test EPSP

Stimulus artefact

1 mV calibration pulse

Control EPSP

Test EPSP after 0.5 s.

Control EPSP

Test EPSP after 1 s.

Control EPSP

Test EPSP after 2 s.

Control EPSP

Test EPSP after 3 s.

Control EPSP

Test EPSP after 4 s.

B

% of control EPSP

Train

Interval (seconds)

200 ms, 20 Hz

C

% of control EPSP

Control EPSP size (mV)
from the heteronymous CP nerve, then the test pulse at 0.5 s after the train is not depressed, yet a brief depression during the train (consistent with presynaptic inhibition) is still observed. This is also observed in CP motoneurons with train and test EPSPs both from stimulation of the heteronymous tibial nerve (but not tested in the cell illustrated in 3A), although fewer examples could be tested with this combination due to the heteronymous stimulation trains more often facilitating large disynaptic inhibitory postsynaptic potentials in CP motoneurons.

Thus the depression we see at 0.5 s after the pulse in Fig. 3Ai is consistent with being the previously described PActD, as, unlike presynaptic inhibition, it is restricted to the homonymous synapse. The lack of PActD of heteronymous EPSPs in motoneurons was confirmed in 10 motoneurons.
PActD in the G127X SOD1 mice. Given that the mutant SOD1 G127X has no enzymatic function (Jonsson et al. 2004) and that it was necessary for us to breed the G127X mice as homozygotes, we decided to use the data obtained from the C57BL/6j mice as WT nontransgenic controls (as this was background strain for the G127X). Given that PActD is possibly affected by age, we used only the adult control mice of a similar age to the G127X mice as aged-matched controls (7 mice, 5 female, 2 male, ~220 days).

PActD was measured at the 0.5-s time interval in five presymptomatic G127X mutants (mean age 209 days) and two symptomatic mice (mean age 229 days). Examples representative of the mean values are illustrated in Fig. 4A. The distributions of the cells in these groups can be seen in Fig. 4B with significantly reduced levels of PActD seen in cells in both presymptomatic and symptomatic G127X mice compared with WT mice. (P = 0.0002; WT: mean 77.57%, SD 5.312, 33 cells, 7 mice; G127X presymptomatic: mean 84.05%, SD 6.487, 28 cells, 5 mice; G127X symptomatic: mean 82.71%, SD 4.624, 18 cells, 2 mice). Post hoc tests confirmed this to be due to significant differences between the WT and both the presymptomatic and symptomatic G127X mice but with no significant difference between the two G127X groups.

From Fig. 4B it can also be seen that while the magnitude of PActD in some motoneurons in presymptomatic G127X mice falls within the normal range seen in the WT, there is clearly a population falling outside of this range. Different populations of fast vs. slow motoneurons have been shown to differ in their vulnerability in ALS (Hegedus et al. 2007, 2008, 2009). We therefore decided to investigate whether the most vulnerable fast motoneurons were the more affected. The feature that most easily distinguishes between the two types of motoneurons is the duration of their AHP (Burke 1967; Gardiner and Kernell 1990). We measured the AHP duration at half amplitude as illustrated in Fig. 4C. As the exact range of AHP duration for each motoneuron type has not been established for mice yet, we simply plotted the magnitude of PActD by AHP duration for 20 WT motoneurons and 37 of the G127X motoneurons (including both presymptomatic and symptomatic; Fig. 4D). Linear regression analysis showed no clear relationship between AHP duration and the levels of PActD. Furthermore, the reduction of PActD in the G127X motoneurons appeared to occur irrespective of the duration of the AHP.

There were no significant differences between the PActD observed in motoneurons from male vs. female WT mice (P = 0.29). Due to the low number of motoneurons obtained in female G127X mice, statistical analysis by sex was not possible for the mutant mice. The general distribution, however, did not appear to differ between males and females (data not shown).

To investigate if there were other abnormalities with respect to the monosynaptic EPSP that may influence the effect of the observed reduction in PActD in the G127X mice, we measured the latency of the EPSPs, from the stimulus artifact, their amplitude, and their width at half height. In a small number of cells these measurements were not possible due to contamination of the EPSP averages with antidromic M spikes at the start and/or later synaptic activity. The data for EPSP latency are shown in Fig. 4E. The latency was significantly longer for symptomatic G127X mice (P = 0.0041; WT: mean 1.236 ms, SD 0.183, 32 cells, 7 mice; G127X symptomatic: mean 1.244 ms, SD 0.144, 28 cells, 5 mice; G127X symptomatic: mean 1.439 ms, SD 0.301, 18 cells, 2 mice). Post hoc tests confirmed this to be due to significant differences between the symptomatic G127X mice and both WT and presymptomatic mice but with no significant difference between these latter two groups. To determine if this was due to changes in synaptic delay or Ia afferent axonal conduction time, we measured the latency from the stimulus artifact to the onset of the cord dorsum potential following stimulation of the tibial nerve. The results are shown in Fig. 4F. It can be seen that there was no change in Ia axonal conduction time in either the presymptomatic or symptomatic G127X mice when the distribution is compared with WT, although the low numbers prevent a valid statistical analysis.

The data for EPSP width at half height are shown in Fig. 4G. EPSPs from the symptomatic G127X mice were significantly wider than EPSPs in both WT and presymptomatic G127X mice (P = 0.0001 level; WT: mean 2.68 ms, SD 0.543, 28 cells, 7 mice; G127X presymptomatic: mean 2.39 ms, SD 0.592, 27 cells, 5 mice; G127X symptomatic: mean 3.797 ms, SD 0.290, 18 cells, 2 mice). There was no significant difference with respect to EPSP width, however, between the WT and presymptomatic mice.

With respect to EPSP amplitude it must be noted that, as our experiments were initially not intended to measure and compare absolute EPSP size between groups (just the percentage of depression), the stimulation intensity used for the PActD protocol was not kept uniform across cells but instead was set to just below threshold for antidromic activation of the individual motoneuron. Nonetheless, we have measured the absolute amplitudes of the depressed EPSP at the 0.5-s interval and present the data with this caveat in mind. Scatterplots of the data can be seen in Fig. 4H. A significant difference was found (P = 0.0269, WT: mean 3.502 mV, SD 1.717, 32 cells, 7 mice; G127X presymptomatic: mean 4.542 mV, SD 1.604, 28 cells, 5 mice; G127X symptomatic: mean 3.476 mV, SD 0.8563, 18 cells, 2 mice). This was due to the EPSP amplitude from the presymptomatic G127X mice being significantly larger than in WT.

PActD in aged mice. PActD was then measured in 37 motoneurons in 6 aged mice (~580 days) and compared with the values seen in the normal adult mice (~100–220 days group). Examples representative of the mean values are illustrated in Fig. 5A. The distribution of the cells in these groups can be seen in Fig. 5B with reduced levels of PActD visible in the aged mice. PActD was significantly reduced in aged mice (P = 0.0004; adult: mean 79.12%, SD 4.99, 74 cells, 13 mice; aged: mean 83.38%, SD 7.110, 37 cells, 6 mice). There was no significant difference between the PActD recorded in motoneurons in male vs. female aged mice (P = 0.7) with both groups having similar means values (83%, data not shown).

Again to investigate if there were other abnormalities with respect to the monosynaptic EPSP that may influence the effect of our observed reduction in PActD, we measured the latency of the EPSPs (measured from the stimulus artifact) and their width at half height in the aged mice. Once more, these measurements were not possible in a small number of cells due to contamination of the EPSP averages with antidromic M spikes at the start or by later synaptic activity.

The data for EPSP latency are shown in Fig. 5C. A significant increase in latency in the aged mice was observed (P <
Interestingly, the increase in latency observed in the symptomatic G127X mice was not significantly different from that which we observed here in the aged mice (P = 0.516; see earlier means and numbers). To determine if this increase in latency in aged mice was due to changes in synaptic delay or Ia afferent axonal conduction time, we measured the latency from the beginning of the stimulus artifact to the onset of the cord dorsum potential following stimulation of the tibial nerve. The results for the

Fig. 4. Postactivation depression in mice with amyotrophic lateral sclerosis. A: examples (representative of the means) of superimposed control and test EPSPs from the aged matched wild-type (left) and the presymptomatic (middle) and symptomatic (right) G127X groups, showing how the PActD is decreased on average in the G127X mice. B: scatterplot showing the mean magnitude of PActD (with SD) for the 3 groups (wild type, presymptomatic, and symptomatic) mice tested. The magnitude is expressed as the size of the test EPSP as a percentage of the control EPSP. The horizontal spread of data points reflects values close to each other. The vertical range of the data points indicates the spread of PActD magnitude within each group. From this a significant reduction in PActD can be observed in both the presymptomatic and symptomatic G127X mice. C: measurement of the postspike afterhyperpolarization (AHP). Dashed lines are used to show how the AHP duration and size was calculated. The duration of the AHP was measured at half height, as the return to baseline was not always easy to detect. D: plot of the magnitude of PActD (in terms of the size of the test EPSP expressed as a percentage of the size of the control EPSP) with respect to AHP duration measured in ms for the WT (black dots) and G127X (both groups, open dots) motoneurons. Linear regression analysis revealed that there is no relationship between the magnitude of PActD and AHP duration, as can be appreciated from the spread of the data points. Therefore, no motoneuron subtype specific difference in the reduction of PActD can be observed in the G127X mice. E: scatterplot showing the mean latency of the incoming volley recorded at the spinal cord (measured from the stimulus artifact) with the standard deviation for wild-type and G127X mice. There are no significant differences between the groups. F: scatterplot showing the mean width of the EPSP (measured at half height) with the standard deviation for wild-type and G127X mice. There is no change in the results obtained from the presymptomatic group when compared with wild type, but a significant widening of the EPSPs at the symptomatic stage can be observed. H: scatterplot showing the mean amplitude (with SD) of the depressed EPSP at the 0.5-s interval after the stimulus train. There is a slight but significant increase in the EPSP amplitude in the presymptomatic G127X mice but not in the symptomatic mice. *P ≤ 0.05, **P ≤ 0.001, and ***P ≤ 0.001.
mice used in this study are presented in Fig. 5D. From this it can be seen that the increase in latency of the EPSP was in fact (at least partially) due to a significant increase in conduction time of the Ia afferents in the aged mice (\(P = 0.0007\); adult: mean 0.453 ms, SD 0.282, 13 mice; aged: mean 0.59 ms, SD 0.643, 6 mice) although the low numbers implicate that caution must be taken using statistical analysis.

The data for EPSP width at half height are shown in Fig. 5E. No significant differences were found (\(P = 0.0648\); adult: mean 2.745 ms, SD 0.657, 63 cells, 13 mice; aged: mean 0.59 ms, SD 0.643, 6 mice) although the low numbers implicate that caution must be taken using statistical analysis.

**DISCUSSION**

The results of these experiments demonstrate a number of key points. First, we demonstrate that it is possible to record PActD in adult mice in vivo and that the characteristics of PActD recorded in the mice are similar to that recorded in larger animals: Both the magnitude and the time course are similar to those seen in cats (Hultborn et al. 1996), and it was also restricted to the homonymous nerve consistent with being
the same phenomenon. This further reinforces the feasibility and validity of using mouse models to study this phenomenon. Next, using the G127X transgenic mouse model of ALS, we found that PActD was reduced both immediately before and after symptom onset. Finally, we also found a surprisingly similar magnitude of reduction during normal aging, a condition not normally associated with spasticity.

**Implications for hyperreflexia.** Although spasticity was not the specific focus of the current experiments, it was the fact that it is present in ALS and that patients do indeed show a hyperreflexia that lead us to originally hypothesize that PActD may be affected in this disease. It seems logical, therefore, to discuss the potential implications that our observed reduction in PActD in the G127X mice would have for the monosynaptic reflex itself. At first glance, a reduction in the depression of the EPSP from 22% (78% of control EPSP size) in WT mice to 16% (84% of control EPSP size) in the G127X mice may seem so small as to be negligible. First, however, we must remember that the comparisons were made at the 0.5-ms time interval as this allowed us to distinguish PActD from presynaptic inhibition. If we observe the relationship between time interval and PActD shown in Fig. 2B we can extrapolate that, in fact, the effect at even shorter time intervals is likely to be even greater. Furthermore, the increased amplitude of the EPSP would activate and be further amplified by the increased persistent inward currents (see section below) that we have observed in motoneurons in this precise model at a similar time point (Meehan et al. 2010a).

More importantly, however, to understand the implications of the reduction one first needs to appreciate the known precise relationship between the depressed EPSP amplitudes and the actual effect on the reflex itself. Hultborn et al. (1996) have demonstrated this relationship using simultaneous recording of both the homonymous monosynaptic EPSP and the monosynaptic reflex (MSR) recorded from the ventral roots in cat. In their illustrated examples (Fig. 6 of the article), at the 0.5-ms time interval (the same used in our experiments for our between group comparisons) the EPSP was ~80% of the size of the control EPSP. The corresponding MSR, however, was only ~20% percent of the control values suggesting that the small depression in EPSP size has profoundly larger effects on the MSR itself. When the EPSP returned to 90% of control values (at the 2-s interval), the resulting MSR was then 60–80%. PActD and the MSR then increased in parallel as the time intervals between stimuli increased. The authors explain this as being due to the fact that a relatively large EPSP is necessary to bring the motoneuron to threshold to be recruited into the reflex. When motoneurons are close to this threshold, only relatively small changes at the EPSP level are needed to recruit many more motoneuron, therefore, having a larger effect on the MSR.

From Fig. 2B it can be seen that our recordings of PActD at different time intervals are roughly consistent with those observed in cat by Hultborn et al. 1996. Therefore, we can extrapolate that the reductions we observed are within the range in which subtle changes would have a relatively larger effect on the MSR. In fact, from this we can predict that the reduction in the depression of the EPSP that we detect (from 78% of control EPSP size in WT to 84% in the G127X mice) would most likely change the depression of the reflex from being 20–40% of the undepressed reflex to being 40–60%.

We argue that when viewed in isolation our observed reduction in PActD would have a large impact on the size of the reflex itself. The question therefore remains whether this change does, in fact, occur in isolation or whether it is compensated for, enhanced by, or indeed is itself compensatory to other changes elsewhere in the reflex. In an in vitro adult sacral spinal cord preparation, G93A SOD1 mice do show a decrease in the monosynaptic reflex recorded from the ventral roots evoked by stimulation of dorsal roots but this could be due to motoneuron loss (Jiang et al. 2009). From this perspective, an increased effectiveness of the Ia input to remaining functional motoneurons would be compensatory. Unpublished data from our laboratory suggest that in the G127X mutants there is not extensive cell loss at the presymptomatic time point at which our recordings were made; however, we have no knowledge regarding the integrity of the neuromuscular junction at this time point.

With symptom onset G93A SOD 1 mice actually show an increased fatigueability of the MSR with prolonged (>1 min) stimulation at 1 Hz or faster (Schomburg et al. 2011). It is also worth noting that, while spasticity is a common feature of ALS in human and has been demonstrated in the G93A SOD1 mouse model, no specific measures of spasticity have been made on the G127X SOD1 mutant so it is possible there may be mutant strain differences.

Finally, when discussing the implications for spasticity, it must be acknowledged that the observation that PActD is consistently reduced across a range of spastic disorders provides a correlation not necessarily a cause and effect relationship. It would undoubtedly contribute to a hyperreflexia of the stretch reflex but the overall contribution of this to “spasticity” is difficult to dissociate from the numerous other changes occurring in the different spasticity disorders. These include alterations in the drive to motoneurons from other sources such as local interneurones (Kapitza et al. 2012), changes in the motoneurons such the appearance of constitutively active serotonin receptors (Murray et al. 2010), increases in passive tension in the muscle fibers (Olsson et al. 2006), and general joint stiffness (Mirbagheri et al. 2001).

**Implications for excitotoxicity.** As mentioned in the Introduction, an increase in excitation of spinal motoneurons could also contribute to an excitotoxicity of the motoneurons with the large, fast motoneurons being particularly vulnerable due to their low calcium buffering capabilities (Palecek et al. 1999). As recent investigations of adult spinal motoneurons in SOD1 ALS models (Delestree et al. 2014; Meehan et al. 2010a) have not observed the same increased intrinsic excitability of the motoneurons that was observed in embryonic and neonatal in vitro preparations, attention has therefore focused again onto changes in the synaptic inputs to the motoneurons as a source of increased excitation.

The reduction in PActD we observed in the G127X mutants was seen before symptom onset and did not appear to reduce further with disease progression. This would appear to suggest that a reduction in PActD is therefore, by itself, unlikely to explain the progression of this disease. This conclusion must be treated with caution, however, if one examines the distribution of data points for the symptomatic mice compared with the presymptomatic mice in Fig. 4B. From this it can be seen that despite the mean being similar for both presymptomatic and symptomatic groups there are clearly less cells at the upper
extreme (i.e., the most affected) now in the symptomatic group. The most logical explanation would be cell loss (or those cells atrophying, making our penetrations biased to least affected cell populations). That the most affected cells (with respect to PActD) may be lost first would be consistent with the glutamate hypothesis of excitotoxicity.

It is also important to acknowledge that a similar mean reduction of PActD may play a greater role if other factors change with disease progression that will interact with the reduction in PActD to render it more influential. There is, in fact, considerable evidence that this is the case. There is, for example, growing evidence that changes in the local spinal circuitry involving motoneurons may change in SOD1 ALS mice as the disease progresses, involving a reduction in inhibition to motoneurons resulting in an imbalance between excitation and inhibition. This has been shown to be due to a number of different causes. One appears to be the loss of inhibitory projections to motoneurons (Chang and Martin 2009), caused by a loss of Renshaw cells projecting to motoneurons. There is also evidence for a reduction in the excitatory drive to inhibitory interneurons that project onto motoneurons (Casas et al. 2013; Wootz et al. 2013). Finally, there is also a reduction in glycine receptors on the most vulnerable (large) motoneurons resulting in smaller mini inhibitory postsynaptic currents (Chang and Martin 2011a,b). All of this would not only contribute to a more hyperexcitable stretch reflex but by increasing the activation of motoneurons would lead to a greater calcium influx in to the motoneurons.

As mentioned in the introduction, recent evidence suggests that many of the basic intrinsic properties of the motoneurons themselves are relatively normal in both the adult G127X and the G93A SOD1 mutants (Delestree et al. 2014; Meehan et al. 2010a). There was, however, evidence for a hyperexcitability of spinal motoneurons related to an increase in persistent inward currents (PICs) (Meehan et al. 2010a). Such currents are partially mediated by t-type calcium channels located on dendrites, with kinetics such that they would preferentially be activated by sub (spike) threshold inputs like the Ia EPSPs. Increased PICs would amplify the less depressed EPSPs, which, in turn, would then be likely to activate more voltage-gated ion channels mediating the PICs. It is unknown whether PICs would further increase with disease progression. It seems plausible to suggest, however, that the combination of decreased inhibition with symptom onset with increased PICs would increase the effect of our observed reduction in PActD, thus possibly contributing to a hyperreflexia in this disease and an overall increased excitation of spinal motoneurons.

Genetic manipulation reducing VGlut2 receptors has been shown to reduce motoneurons from cell death; however, it did not affect disease onset or duration (Wootz et al. 2010). Whether increased activity of the motoneurons necessary results in excitotoxicity in ALS has also recently been questioned by the rather surprising observation that manipulations aiming to increase the excitability of spinal motoneurons were, in fact, neuroprotective in the G93A SOD1 model while decreasing excitability paradoxically enhanced disease progression (Saxena et al. 2013).

Implications for the stretch reflex in aging. By contrast, all evidence seems to suggest that EPSPs in the aged animal would be likely to be less effective. In aged mice we observed that the conduction velocity of the Ia afferents was significantly reduced. This is consistent with recordings in aged cats (Boxer et al. 1988). The trend towards an increase in EPSP half-width that we observed in aged mice is also consistent with that which has been observed in aged cats, shown to be a consequence of a decreased rise time (Boxer et al. 1988; Chase et al. 1985). This may be consistent with our observations given that the EPSPs were of the same amplitude. A decrease in the rate of rise would make the EPSPs less effective at generating action potentials (Fetz and Gustafsson 1983). Taken together, all this is consistent with the observation that the H reflex in elderly humans has a smaller amplitude, a higher threshold, a longer latency, and a longer duration and is polyphasic (Sabbahi and Sedgwick 1982). Furthermore, there is a reduced sensitivity of muscle spindles in aging that would further reduce the stretch reflex. Thus, a reduction in PActD in aged mice may, in fact, be an attempt to compensate for all these changes rather than to produce hyperexcitability of the reflex loop.

Possible cellular mechanisms of PActD and its reduction. Given that PActD is restricted to homonymous (previously activated) synapses and occurs with no change in membrane potential, conductance, input resistance, or membrane time constant, it has been assumed that the underlying mechanisms are restricted to the presynapse (Hultborn et al. 1996; Lev-Tov and Pinco 1992). This has been hypothesized to be due to a reduction in the probability of vesicle release (Lev-Tov and Pinco 1992). Our results are indeed consistent with this. Precisely why it should be restricted to Ia afferent synapses on homonymous motoneurons is unclear but this has now been confirmed in mouse, cat and human (Hammar et al. 2002; Hultborn et al. 1996; Lamy et al. 2005) and therefore must be of some significance. Observations of field potentials evoked by stimulation of the same Ia afferents recorded in different regions of the spinal cord (in and outside of motor nuclei) have shown that PActD of field potentials from the same afferents differ with respect to location of the target neurone, being significantly larger within motor nuclei than in intermediate regions. This suggests that the magnitude of the PActD is highly dependent on the target neuron (Hammar et al. 2002).

Insights into potential mechanisms underlying PActD may potentially be gained from observations of a similar phenomenon at glutamatergic synapses in the hippocampal CA1 area, referred to as “postburst depression,” which has a similar time course and magnitude as PActD (Andersson and Hanse 2010). Postburst depression is associated with a reduction in the probability of vesicle release due to a decrease in the pool of primed vesicles (Andersson and Hanse 2010; Andersson and Hanse 2011). Astrocyte activity has been shown to be crucial for postburst depression by imposing a delay on the recovery of primed vesicles (Andersson and Hanse 2010, 2011).

The ability of astrocytes to influence transmitter release in the hippocampus is not an entirely new idea. Ca$^{2+}$ elevation in astrocytes has been shown to moderate transmitter release probability (Perea and Araque 2007). Furthermore, there is now evidence that astrocytes may provide both a tonic and phasic inhibition of excitatory events in the spinal cord also (Carlsten and Perrier 2014). This may all be highly relevant for the present study given the accumulating evidence for an involvement of astrocytes in the pathophysiology of ALS (Benkler et al. 2013; Boilée et al. 2006; Díaz-Amarilla et al. 2011; Fritz et al. 2013; Haidet-Phillips et al. 2011; Phatnani et
Astrocytes are involved in the uptake of neurotransmitters around synapses on motoneurons using EAAT2 (GTL-1) glutamate transporters (Sattler and Rothstein 2006). The loss of the EAAT2 (GTL-1) glutamate transporter has been observed in human ALS cases (sporadic and familial) (Rothstein et al. 1992, 1995) and in SOD1 mutant mice models (Bendotti et al. 2001) therefore providing a possible mechanism by which astrocyte dysfunction may affect synaptic efficacy in ALS.

**Sex differences in ALS.** Due to the slow breeding of our mutant we were unable to obtain a large number of recordings in female mice to allow us to systematically compare male and female mutants thus any conclusion regarding sex are to be treated with caution. In general, however, we did not observe any significant differences in PActD between motoneurons from male and female mice within any groups (WT, G127X, or aged). Possible sex differences may be of interest given that progesterone has been shown to be positively correlated with better prognosis in this disease in humans (Monachelli et al. 2011). In G93A SOD1 mice disease onset is earlier and there is faster disease progression in males (Cervetto et al. 2013). Consistent with a neuroprotective role, progesterone treatment protects against motoneuron cell death and delays disease progression in the G93A SOD1 mouse (Kim et al. 2013). With respect to sex differences regarding synaptic inputs to motoneurons in the G93A SOD1 mouse, cholinergic (C) boutons have been shown to be enlarged on spinal motoneurons of male but not female G93A SOD1 mice (Herron and Miles 2012). It would therefore be of interest to explore possible PActD differences between males and females more systematically as the disease progresses.

**Motor activity, PActD, and aging.** As described in the Introduction, a reduction in PActD is commonly observed in disorders in which the descending drive to motoneurons is impaired such as in stroke or spinal cord injury. The immediate consequence of such disorders is a sudden reduction in motor activity levels. Thus it is possible that such reduced activity itself may cause a reduction in PActD as a compensatory mechanism. Consistent with this, physical exercise in spinal cord transected rats has been shown to return reduced levels of PActD to control levels (Skinner et al. 1996). Such training also leads to a reduction in spasticity in spinal cord injured rats (Bose et al. 2012) consistent with reduced activity levels being the causative factor in the both the reduction of PActD and spasticity. This hypothesis is further reinforced by human experiments in which a reduction in PActD is seen when human feet/ankle joints are immobilized with a cast for 2 wk (Lundbye-Jensen and Nielsen 2008). Thus the reduced levels of motor activity seen in aging may partially explain the reduction of PActD in the aged mice. This cannot, however, explain the reduction in PActD observed in the G127X mice as the reduction was also observed at presymptomatic time points, where motor activity is, presumably, not affected. It could be speculated, therefore, that the reasons for the reduction in PActD in aging and in ALS may be very different.

Finally, there are, however, a number of similarities between aging and ALS, including oxidative stress (Federico et al. 2012; Nunomura et al. 2012), mitochondrial dysfunction (Bricic and Trifunovic 2010; Cozzolino and Carri 2012; Martin 2012; Shi et al. 2010; Terzioglu and Larsson 2007), and deficits at the neuromuscular junction (Dupuis and Loeffler 2009; Jang and Van 2011; Nishimune 2012). All of these may influence vesicle release either directly, in the case of mitochondria, via local calcium buffering and ATP production, or indirectly, in the case of the neuromuscular junction via homeostatic mechanisms modulating quantal release. It is, therefore, perhaps, not surprising that some of the changes that we have observed in features of the EPSPs (increased latency and width) in the symptomatic G127X mice are similar to those observed in the aged mice. The similarity between aging and ALS have led some to suggest that ALS may be considered to be “premature aging.” While our results may appear to be consistent with this, to address this would require a more systematic analysis at additional age points in both WT and mutants. There are, however, also many differences between EPSPs in aging and ALS including the possible causes for the reduction in PActD which requires further investigation.

**Conclusions.** The phenomenon of PActD appears to function in fundamentally similar ways in mice as in larger animals with a similar magnitude and duration of effect. PActD is reduced in both aging and ALS although we hypothesize that the causes may be very different. We postulate that the reductions in PActD we observed at the synaptic level would potentially have a large effect on the reflex level, but whether this does or not would depend highly on other factors known to change in ALS and aging.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: A.H., N.C.P., and C.F.M. conception and design of experiments in which a reduction in PActD is seen when a reduction was also observed at presymptomatic time points, where motor activity is, presumably, not affected. It could be speculated, therefore, that the reasons for the reduction in PActD in aging and in ALS may be very different.

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