Effect of fluoxetine on disease progression in a mouse model of ALS


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Koschnitzky JE, Quinlan KA, Lukas TJ, Kajtaz E, Kocevar EJ, Mayers WF, Siddique T, Heckman CJ. Effect of fluoxetine on disease progression in a mouse model of ALS. J Neurophysiol 111: 2164–2176, 2014. First published March 5, 2014; doi:10.1152/jn.00425.2013.—Selective serotonin reuptake inhibitors (SSRIs) and other antidepressants are often prescribed to amyotrophic lateral sclerosis (ALS) patients; however, the impact of these prescriptions on ALS disease progression has not been systematically tested. To determine whether SSRIs impact disease progression, fluoxetine (Prozac, 5 or 10 mg/kg) was administered to mutant superoxide dismutase 1 (SOD1) mice during one of three age ranges: neonatal [postnatal day (P)5–11], adult presymptomatic (P30 to end stage), and adult symptomatic (P70 to end stage). Long-term adult fluoxetine treatment (started at either P30 or P70 and continuing until end stage) had no significant effect on disease progression. In contrast, neonatal fluoxetine treatment (P5–11) had two effects. First, all animals (mutant SOD1<sup>G93A</sup> with control: nontransgenic and SOD1<sup>WT</sup>) receiving the highest dose (10 mg/kg) had a sustained decrease in weight from P30 onward. Second, the high-dose SOD1<sup>G93A</sup> mice reached end stage ~8 days (~6% decrease in life span) sooner than vehicle- and low-dose animals because of an increased rate of motor impairment. Fluoxetine increases synaptic serotonin (5-HT) levels, which is known to increase spinal motoneuron excitability. We confirmed that 5-HT increases spinal motoneuron excitability during this neonatal time period and therefore hypothesized that antagonizing 5-HT receptors during the same time period would improve disease outcome. However, ciproheptadine (1 or 5 mg/kg), a 5-HT receptor antagonist, had no effect on disease progression. These results show that a brief period of antidepressant treatment during a critical time window (the transition from neonatal to juvenile states) can be detrimental in ALS mouse models.

amyotrophic lateral sclerosis; motoneuron excitability; fluoxetine; Prozac; antidepressant

THE PROGRESSION of clinical symptoms in amyotrophic lateral sclerosis (ALS) patients is due to the progressive detachment and death of spinal and bulbar motoneurons. In these patients motoneuron hyperexcitability is a pathological feature (Kanai et al. 2006; Tamura et al. 2006; Vucic and Kiernan 2006a, 2006b; Vucic et al. 2007, 2008), and at therapeutic doses riluzole, still the only FDA-approved drug to treat ALS, is known to decrease motoneuron excitability through inhibition of persistent inward currents (PICs) and glutamate release (Coderre et al. 2007; Del Negro et al. 2005; Harvey et al. 2006c; Lamanaukas and Nistri 2008; Manzt et al. 1992; Martin et al. 1993; Miles et al. 2005; Theiss et al. 2007; Zona et al. 2002).

At the same time, many ALS patients are prescribed selective serotonin reuptake inhibitors (SSRIs) (Andersen et al. 2005), which increase extracellular monoamine levels by blocking serotonin reuptake transporters (SERTs) on presynaptic terminals (Nichols and Nichols 2008). In the spinal cord, increased monoamines, specifically serotonin (5-HT) and norepinephrine, increase motoneuron excitability. 5-HT can increase excitability by reducing the action potential threshold, increasing the hyperpolarization-activated inward current ($I_h$), decreasing the afterhyperpolarization potential (AHP), and increasing input resistance (Bayliss et al. 1995; Berger et al. 1992; Harvey et al. 2006a, 2006b; Hounsgaard et al. 1988; Hsiao et al. 1997, 1998; Inoue et al. 1999; Ladewig et al. 2004; Larkman and Kelly 1992, 1997; Li et al. 2007; Takahashi and Berger 1990; White and Fung 1989; Wu et al. 1991). In addition, 5-HT facilitates PICs by binding to postsynaptic 5-HT<sub>2</sub> metabotropic receptors that activate a G protein cascade (Fone et al. 1991; Harvey et al. 2006a, 2006b; Heckman et al. 2008, 2009; Li et al. 2007; Perrier and Hounsgaard 2003; Perrier et al. 2000; Sorkin et al. 1991).

Although the actions of riluzole and SSRIs appear to have opposite effects on motoneuron excitability, no systematic studies have been performed to determine whether antidepressants affect disease progression in ALS patients. We therefore tested the effect of the SSRI fluoxetine (Prozac) in an ALS mouse model expressing the human superoxide dismutase 1 (SOD1) gene with a glycine to alanine mutation at codon 93 (SOD1<sup>G93A</sup>) (Gurney et al. 1994).

To test whether fluoxetine administered at the start of clinical symptoms would affect disease progression, fluoxetine treatment began at postnatal day (P)70 and continued until end stage. Starting treatment at P70 corresponds to the onset of tremor in this mouse model; however, progressive motoneuron detachment from the neuromuscular junction and death have already occurred (Hegedus et al. 2007, 2008; Pun et al. 2006). This time period was also chosen because three well-controlled studies using clinician interviews and DSM-IV criteria reported the prevalence of depression in ALS patients at 10% (Ganizin et al. 1999; Rabkin et al. 2000, 2005), double the rate in otherwise healthy individuals (Narrow et al. 2002). Furthermore, it is recommended by the European Federation of Neurological Societies to treat depression in ALS patients with amitriptyline, a tricyclic antidepressant, or SSRIs such as fluoxetine (Andersen et al. 2005). In addition to depression,
ALS patients also suffer from pseudobulbar affect (Gallagher 1989), fatigue (Barthlen and Lange 2000; Francis et al. 1999; Lou et al. 2003; Thomas and Zijdewind 2006), and muscle weakness (Gourie-Devi et al. 2003), which are thought to be due to changes in the serotonergic system and are often also treated with antidepressants (Brooks 2007; Ferraro et al. 2000, 2001, 2002, 2005; Schiffer et al. 1985; Szczudlik et al. 1995).

In a second set of experiments, fluoxetine treatment was started at P30, which is well before overt clinical symptoms such as tremor and paralysis are apparent, and continued until end stage was reached. Starting treatment at P30 corresponds with a spike in endoplasmic reticulum stress and the earliest signs of neuromuscular junction detachment in fast fatigable motor units in this mouse model (Hegedus et al. 2007, 2008; Pun et al. 2006; Saxena et al. 2009). In addition, SSRIs are widely prescribed for depression and anxiety disorders in otherwise healthy individuals (i.e., before ALS symptoms would be detected) (Nichols and Nichols 2008). It is therefore likely that some ALS patients had taken an SSRI before being diagnosed.

Finally, although never tested in humans, pathological changes in motoneuron electrical properties are present in ALS mouse models during the transition between neonatal and juvenile states. Specifically, voltage-sensitive currents in bulbbar and spinal motoneurons are upregulated and can produce hyperexcitability in some preparations (Carunchio et al. 2010; Kuo et al. 2004, 2005; Pieri et al. 2009; van Zundert et al. 2008). However, during this neonatal time period spinal motoneuron excitability may not be increased because of a concurrent increase in input conductance and changes in morphology (Bories et al. 2007; Pambo-Pambo et al. 2009; Quinlan et al. 2011). Therefore during the neonatal time period pathological changes are present, but normal excitability may be maintained. To determine the effects of altering motoneuron excitability during this time period, we conducted two sets of experiments. We 1) artificially increased excitability with fluoxetine or 2) decreased excitability with the antihistamine and 5-HT2 receptor antagonist cyproheptadine.

MATERIALS AND METHODS

All procedures were approved by the Northwestern University Animal Care and Use Committee.

Mouse Housing and Genotyping

Mice were group housed in barrier facilities on a 12:12-h light-dark cycle. Food and water were provided ad libitum. The background for all mice was B6SJL. For the fluoxetine studies, nontransgenic female mice were bred with transgenic male mice expressing either the wild-type human SOD1 gene (SOD1 WT) or the human SOD1 gene with a glycine to alanine mutation at amino acid 93 (SOD1 G93A) (high SOD1 expression line). For the cyproheptadine study only SOD1 G93A males were used for breeding. Tail clippings were genotyped for the human SOD1 gene with standard PCR techniques (Rosen et al. 1993). The primers for amplification were SOD1P7: CAT CAG CCC TAA TTC ATC TGA and SOD1P8: CGC GAC TAA CAA TCA AAG TGA. All breeding and genotyping was done by the Siddique laboratory.

Drug Administration

Cyproheptadine was administered to neonatal mice from P5 to 11 (Cypro P5-11), through once daily injections (1 or 5 mg/kg body wt ip) or saline vehicle injections (0.1 ml/10 g body wt). Each pup in the litter was given the same drug dose, and care was taken to minimize the disruption of the litter. These cyproheptadine concentrations were chosen because of their clinical relevance (Kilic et al. 2011; Semenova and Ticku 1992; Singh and Goel 2010; Yi et al. 2011).

Fluoxetine (Sigma, St. Louis) was administered to neonatal mice from P5 to P11 (Fluox P5-11), adult mice from P30 to end stage (Fluox P30), or adult mice from P70 to end stage (Fluox P70). In the Fluox P5-11 study, fluoxetine was administered through a once-daily injection (5 or 10 mg/kg body wt ip) or saline vehicle injection (0.1 ml/10 g body wt). Again, each pup in the litter was given the same drug dose, and care was taken to minimize the disruption of the litter. For the long-term fluoxetine treatments (Fluox P30 and P70), fluoxetine was delivered in the water with target concentrations of 5 or 10 mg/kg body wt per day. Fluoxetine was added to water bottles for a final concentration of 25 or 50 mg/l (for 5 and 10 mg/kg body wt per day doses, respectively) based on preliminary measurements of average water consumption (5 ml) and average adult weight (22.5 g). The water was replaced weekly to reduce the effects of fluoxetine instability (Binsmait et al. 2001). These fluoxetine concentrations were chosen because of their clinical relevance (Balu et al. 2009; Nuñez et al. 2006; Yirmiya et al. 2000; Zhang et al. 2000).

Behavioral Testing

Behavioral testing was performed every 5 days between 7 and 10 AM by persons blind to the drug condition and genotype. Testing was started at P35 for the Fluox and Cypro P5-11 and Fluox P30 studies. Testing was started at P50 for mice that began fluoxetine treatment at P70. Near end stage, mice were tested every day or every other day. Weight, tremor, rotarod performance, and end stage were monitored for all mice. Tremor was determined on a five-point scale: 0 (no tremor), 1 (very small amplitude, induced by manually stroking the limb, constrained to 1 or 2 limbs), 2 (medium amplitude, induced or present at rest, constrained to 1 or 2 limbs), 3 (large amplitude, induced or present at rest, spread to >2 limbs), 4 (very large amplitude, present in all limbs). Tremor onset was defined as the first of three consecutive observations where the mouse scored a 1 or higher.

Rotarod training occurred on three consecutive days before rotarod testing commenced. During testing, the rotarod was set to 4 rpm and increased 4 rpm every 30 s up to a maximum rotation of 20 rpm at 120 s. The rotarod then maintained a rotational speed of 20 rpm for 60 s, for a total of 180 s. Mice were run three times with a minimum of 5 min between each run. The time of the longest run and the age at which the mice failed to complete the entire protocol (180 s) on all three runs were recorded. End stage was defined as when the mice could no longer maintain themselves on the rotarod for >5 s, about one-third of a turn on the rotarod. This corresponded to gross motor deficits and the inability to maintain body weight.

Voluntary locomotion was monitored every 10 days for both neonatal studies (Fluox and Cypro P5-11) and the Fluox P30 study. Each mouse was placed in an empty cage with a 2 × 3 grid on the bottom. Line crossings were recorded for 3 min to determine whether the drugs altered their general locomotor and exploratory behavior. The cage was cleaned after each mouse. Water consumption was monitored for the Fluox P70 study to determine whether fluoxetine treatment altered drinking behavior (recorded from P50 to P110).

Tissue and Serum Samples

To ensure that detectible levels of fluoxetine were still present at end stage, tissue and serum samples were taken from a subset of SOD1 G93A mice that had started fluoxetine treatment at P70. At end stage, these mice were quickly killed with an overdose of isoflurane. They were immediately decapitated, and trunk blood samples were collected into a BD Vacutainer sodium heparin tube (2 ml). The head

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was submerged in ice-cold dissecting solution containing (mM) 2.5 KCl, 11 glucose, 4.0 MgSO4·7H2O, 1.0 Na2HPO4, 15 HEPES, and 0.10 CaCl2, 2H2O. The brain without the cerebellum was removed and immediately frozen in liquid nitrogen. The sample was then placed into a −80°C freezer (Sanyo Scientific, San Diego CA) for later analysis.

The blood samples were left for 1 h at room temperature and then transferred into a 2-ml polypropylene centrifuge tube and centrifuged for 5 min at 5,000 rpm on a bench-top Minipin plus (Eppendorf, Westbury, NY). The supernatant was collected, and the process was repeated to isolate the serum. The serum sample was then moved into a new centrifuge tube and placed in a −4°C freezer for later analysis.

Measurement of Fluoxetine and Its Metabolite Norfluoxetine

A three-step method was devised to measure the fluoxetine and norfluoxetine in mouse brain. Brains were thawed and weighed after transfer into a tared 1.7-ml conical tube. Two volumes (0.6 ml) of extraction buffer (0.01 M ammonium formate, pH 5.0) were added, and the brain was homogenized with a motorized plastic homogenizer (15 s at room temp). The homogenate was centrifuged at 14,000 rpm for 20 min. The supernatant was saved and the sample homogenized again with 0.6 ml of extraction buffer. After centrifugation, the combined supematants were applied to a weak cation exchanger (WCX) solid-phase extraction (SPE) cartridge (Supelco no. 52737U) and allowed to flow under gravity. The flow through was saved for subsequent chromatography on a reversed-phase SPE cartridge. The WCX cartridge was washed with 2 ml of the extraction buffer and then eluted with 2 × 0.5 ml of 0.25 M ammonium formate pH 3.0. The eluant was concentrated on a Speed Vac to near dryness and then transferred into a 2-ml polypropylene centrifuge tube and then placed into a −80°C freezer for later analysis.

The eluant was concentrated on a Speed Vac to near dryness and then redissolved in 100 μl of the extraction buffer. After centrifugation, the combined supernatants were applied to a weak cation exchanger (WCX) solid-phase extraction (SPE) cartridge (Supelco no. 52737U) and allowed to flow under gravity. The flow through was saved for subsequent chromatography on a reversed-phase SPE cartridge. The WCX cartridge was washed with 2 ml of the extraction buffer and then eluted with 2 × 0.5 ml of 0.25 M ammonium formate pH 3.0. The eluant was concentrated on a Speed Vac to near dryness and then resuspended to a volume of 100 μl of 5% methanol. This eluant contains the 5-HT and a small percentage (1–2%) of the fluoxetine/norfluoxetine from the brains of treated mice.

The flow through from the WCX cartridge was adjusted to −pH 2 with 10% formic acid and applied to a C18 SPE cartridge (Nest Group no. SMMS318V) that was preequilibrated with 100% acetonitrile followed by 0.1% trifluoroacetic acid-water. After washing with 2 ml of 0.1% trifluoroacetic acid, the column was eluted with 70% acetonitrile (2 × 0.5 ml). The eluates were combined and evaporated to dryness on a Speed Vac. The residue was redissolved in 100 μl of 10% acetonitrile-water. This eluant contains 5-hydroxyindoleacetic acid (5-HIAA) and a small amount (1–2%) of norfluoxetine/fluoxetine from the treated mice.

The pellet from the original tissue lysate was rehomogenized in 0.8 ml of 90% methanol and centrifuged again. The supernatant was analyzed directly for fluoxetine and norfluoxetine.

Samples were analyzed on a HPLC–mass spectrometry system (Agilent 1100) that has an ion-trap mass spectrometer detector. Chromatography was done on a Waters Atlantis T3 column (2.1 × 150 mm) at a flow rate of 150 μl/min. The elution solvents were 10 mM ammonium formate, pH 3.5 (A) and acetonitrile containing 0.1% formic acid (B). The gradient program was 12% B for 2 min followed by a gradient to 62% B in 12 min and hold for 8 min. Detection of the compounds was done with the selected reaction monitoring (SRM) method. The parent MH+ and transition ions were 5-HT (177→160), 5-HIAA (192.5→146), norfluoxetine (296→134), and fluoxetine (310→148). Peak areas of the transitions were recorded and the samples compared with standards run with each group of samples. Typically, seven or eight concentrations of standards were used to construct calibration curves that were linear over the measured range. The ion accumulation time was set maximally to 100 ms and the four transitions monitored simultaneously at a scan speed of 4,000 m/z s. Only those transitions that occurred in the standard retention time windows were used for area calculations. Recovery factors were determined by spiking standards into control tissue samples that had been depleted of 5-HT and 5-HIAA. Recovery of 5-HT and 5-HIAA was 80%, while recovery of fluoxetine was 15.9% and that of norfluoxetine was 11.5%. Recovery of fluoxetine and norfluoxetine in the mouse brain was limited by the compound solubility in the extraction solution (90% methanol).

Experimental Paradigms and Statistics

Fluox P70-end stage study. All groups were sex balanced. Nontransgenic littermates (n = 10 per drug level) and transgenic SOD1 WT mice (n = 10 per drug level) were used as drug controls. SOD1 G93A mice (n = 14 per drug level) were used to test the effect of the drug on disease progression. SOD1 G93A littermate controls were used for each drug level.

Fluox P30-end stage study. All groups were sex balanced. Nontransgenic littermates (n = 10 per drug level) and transgenic SOD1 WT mice (n = 10 per drug level) were used as drug controls. SOD1 G93A mice (n = 14 per drug level) were used to test the effect of the drug on disease progression. SOD1 G93A littermate controls were used for each drug level.

Fluox P5-11 study. All groups were sex balanced. Nontransgenic littermates (n = 10 per drug level) and transgenic SOD1 WT mice (n = 10 per drug level) were used as drug controls. SOD1 G93A mice (n = 20 per drug level) were used to test the drug’s effect on disease progression. Each drug concentration was given to an entire litter; therefore drug concentration was not littermate controlled between SOD1 G93A animals.

Cypro P5-11 study. All groups were sex balanced. Nontransgenic litter mates (n = 10 per drug level) were used as drug controls. SOD1 G93A mice (n = 20 per drug level) were used to test the effect of the drug on disease progression. Each drug concentration was given to an entire litter; therefore drug concentration was not littermate controlled between SOD1 G93A animals.

Statistics. For each study, there were no differences between nontransgenic and transgenic SOD1 WT mice. Therefore, these two groups were combined (control group). The four studies (Cypro P5-11, Fluox P5-11, Fluox P30, and Fluox P70) were analyzed independently of each other.

In the cyproheptadine and fluoxetine neonatal injection studies (Cypro P5-11 and Fluox P5-11), a repeated-measure (RM) ANOVA was performed on the average weight per mouse for each litter from P5 to P11 with a between-subject factor of drug concentration.

In all studies, RM ANOVAs were used for water consumption, weight, tremor severity, quadrant crossing, and rotarod performance with the within-subject factor of age and between-subject factors of genotype and drug concentration with sex as a covariate. The Greenhouse-Geisser correction was used because the sphericity assumption was not met. For SOD1 G93A mice only, univariate ANOVAs were used to compare tremor onset and age when rotarod performance was first impaired with the between-subject factor of drug concentration with sex as a covariate. The Kaplan-Meier (K-M) log rank survival test was used to analyze end stage with the factor (drug concentration) compared pairwise and pooled over strata (sex) to test the effect of drug concentration.

Intracellular Whole Cell Recordings

Whole cell patch-clamp recordings were made with glass electrodes (resistance 2–5 MΩ) from visualized motoneurons in acute transverse slices (350 μm) from the lumbar spinal cord of mice (P6-10) during bath application of citalopram (2–10 μM) and α-methyl 5-HT (0.1–0.3 μM). After drug application, it was occasionally necessary to apply a hyperpolarizing holding current to maintain the motoneuron close to the previous resting potential for consistency. PICs were measured in voltage-clamp mode from cells with series resistance ≤ 15 MΩ as described previously in Quinlan et al. (2011). Depolarizing current ramps and steps were used in current-clamp mode to measure firing threshold voltage.
action potential properties including overshoot and width at half-peak, afterhyperpolarization after single action potentials (AHP), the slope of the firing frequency-current relationship, and the current at which firing commences (I-ON) and ceases (I-OFF). Other cell properties including resting potential, input resistance, and sag and rebound potentials (the hallmarks of the $I_h$ current) were also recorded in current clamp. All data were acquired with custom WinFluo software (University of Strathclyde), all analyses were made in Spike2 (Cambridge Electronic Design), and statistics were tested in SPSS.

**RESULTS**

**Fluoxetine: P70 to End Stage (Fluox P70)**

Fluox P70: fluoxetine did not alter SOD1<sup>G93A</sup> disease progression. Fluoxetine treatment started at P70 and continuing until end stage had no effect on disease progression. Fluoxetine administration had no effect on rotarod performance or weight as the SOD1<sup>G93A</sup> mice aged (Fig. 1, A and B). There were also no significant differences in tremor onset, the onset of rotarod decline, or end stage (Table 1 and Fig. 1C).

Fluox P70: fluoxetine did not alter water intake. Water consumption was monitored in the Fluox P70 study to determine whether the presence of fluoxetine in the water altered fluid intake. In agreement with another study using similar methods (Miller et al. 2008), there was no difference in water consumption between fluoxetine drug levels (monitored from P50 to P110) (Fig. 1D).

Fluox P70: fluoxetine was present in the CNS at end stage. To make sure that fluoxetine was still detectable at end stage, tissue and serum concentrations of fluoxetine and norfl uoxetine, the active metabolite, were taken at end stage in some SOD1<sup>G93A</sup> mice. It is likely that SOD1<sup>G93A</sup> mice had lower water consumption near end stage because of decreased mobility, and therefore it was expected that fluoxetine and norfluoxetine levels would be lower than previously reported for these concentrations. Combined concentrations of fluoxetine and norfluoxetine at end stage in SOD1<sup>G93A</sup> mice were about one-fourth to one-half of the concentrations previously reported (Durand et al. 1999; Miller et al. 2008). Combined fluoxetine/norfluoxetine serum concentrations for the 5 and 10 mg/kg groups were 48.1 ± 18 (n = 4) and 223.4 ± 128 (n = 5) ng/ml, respectively. Tissue concentrations from the whole brain were 6,387.2 ± 5,301 (n = 7) and 13,286.0 ± 4,999.9 (n = 9) ng/g, respectively.

**Table 1. Fluoxetine P70 to end stage**

<table>
<thead>
<tr>
<th>Fluox P70</th>
<th>Tremor Onset</th>
<th>RR Onset Decline</th>
<th>End Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN ± SD</td>
<td>DPN ± SD</td>
<td>DPN ± SD</td>
<td>DPN ± SD</td>
</tr>
<tr>
<td>Vehicle</td>
<td>89 ± 12</td>
<td>118 ± 17</td>
<td>133 ± 10</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>88 ± 11</td>
<td>116 ± 12</td>
<td>129 ± 12</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>87 ± 14</td>
<td>109 ± 16</td>
<td>163 ± 12</td>
</tr>
</tbody>
</table>

Means ± SD (n = 14 per drug level) are presented for tremor onset, rotarod (RR) onset of decline, and end stage. Data are divided into drug levels (vehicle, 5 mg/kg, and 10 mg/kg). There was no significant effect of drug level on any of these parameters. Univariate ANOVAs were used to compare tremor onset and age when rotarod performance was first impaired with between-subject factor of drug concentration with sex as a covariate. The Kaplan Meier (K-M) log rank survival test was used to analyze end stage with the factor (drug concentration) compared pairwise and pooled over strata (sex) to test the effect of drug concentration. DPN, postnatal day; Fluox, fluoxetine.
Fluoxetine: P30 to End Stage (Fluox P30)

Fluox P30: fluoxetine did not alter SOD1<sup>G93A</sup> disease progression. Fluoxetine treatment started at P30 and continuing until end stage had no effect on disease progression. Fluoxetine administration had no effect on rotarod performance or weight as the SOD1<sup>G93A</sup> mice aged (Fig. 2, A and B). There were also no significant differences in tremor onset, the onset of rotarod decline, or end stage (Table 2 and Fig. 2C).

Fluox P50: fluoxetine did not alter gross motor activity. Quadrant crossing was monitored in the Fluox P30 study to determine whether fluoxetine altered gross motor activity. Fluoxetine administration had no significant effect on quadrant crossing (monitored from P40 to P110), indicating that fluoxetine did not affect activity in a time period before severe paralysis was present in the SOD1<sup>G93A</sup> animals (data not shown).

Fluoxetine: P5 to P11 (Fluox P5-11)

Fluox P5-11: neonatal fluoxetine did not alter disease onset in SOD1<sup>G93A</sup> mice. Fluoxetine administered between P5 and P11 did not alter tremor onset or the onset of rotarod decline (Table 3). However, there was a small but significant difference in the time from tremor onset to the onset of rotarod decline between the three drug levels (mean ± SD): vehicle (32.7 ± 17 days), low dose (34.8 ± 15 days), high dose (22.7 ± 15 days) [ANOVA: Drug level, F(2,55) = 3.3, P < 0.05]. These data suggest that the rate of disease progression was increased in the high-dose group, although paired post hoc tests did not reach statistical significance.

Fluox P5-11: neonatal fluoxetine increased the rate of disease progression. Although there was no difference in the onset of motor impairment, fluoxetine dose had a significant effect on how quickly rotarod performance declined (analyzed between P80 and P160) [RM ANOVA: Age × Drug level, F(5,322) = 2.8, P < 0.05]. This within-subject difference was specific to the SOD1<sup>G93A</sup> mice [RM ANOVA: Age × Genotype × Drug level, F(5,322) = 2.61, P < 0.05] (Fig. 3A). There was also a between-subject interaction of drug level on rotarod performance for SOD1<sup>G93A</sup> mice [RM ANOVA: Ge-

### Table 2. Fluoxetine P30 to end stage

<table>
<thead>
<tr>
<th>Fluox P30</th>
<th>Tremor Onset DPN ± SD</th>
<th>RR Onset Decline DPN ± SD</th>
<th>End Stage DPN ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>95 ± 15</td>
<td>119 ± 14</td>
<td>130 ± 8</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>95 ± 21</td>
<td>117 ± 9</td>
<td>128 ± 10</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>93 ± 13</td>
<td>114 ± 9</td>
<td>132 ± 12</td>
</tr>
</tbody>
</table>

Means ± SD (n = 14 per drug level) are presented for tremor onset, rotarod onset of decline, and end stage. Data are divided into drug levels (vehicle, 5 mg/kg, and 10 mg/kg). There was no significant effect of drug level on any of these parameters. Univariate ANOVAs were used to compare tremor onset and age when rotarod performance was first impaired with between-subject factor of drug concentration with sex as a covariate. The K-M log rank survival test was used to analyze end stage with the factor (drug concentration) compared pairwise and pooled over strata (sex) to test the effect of drug concentration.

### Table 3. Neonatal fluoxetine P5 to P11

<table>
<thead>
<tr>
<th>Fluox P5-11</th>
<th>Tremor Onset DPN ± SD</th>
<th>RR Onset Decline DPN ± SD</th>
<th>End Stage DPN ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>90 ± 16</td>
<td>122 ± 9</td>
<td>133 ± 11</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>87 ± 14</td>
<td>122 ± 9</td>
<td>134 ± 10</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>96 ± 15</td>
<td>118 ± 8</td>
<td>126±† 6</td>
</tr>
</tbody>
</table>

Means ± SD (n = 20 per drug level) are presented for tremor onset, rotarod onset of decline, and end stage. Data are divided into drug levels (vehicle, 5 mg/kg, and 10 mg/kg). There was no significant effect of drug level on tremor onset or rotarod onset of decline. There was a significant difference in end stage, with the high-dose group reaching end stage ~8 days sooner than both vehicle (K-M log rank: χ² = 7.4, P ≤ 0.01) and low-dose (K-M log rank: χ² = 11.4; P ≤ 0.001) groups. ANOVAs were used to compare tremor onset and age when rotarod performance was first impaired with between-subject factor of drug concentration with sex as a covariate. The K-M log rank survival test was used to analyze end stage with the factor (drug concentration) compared pairwise and pooled over strata (sex) to test the effect of drug concentration.

*Significant difference between vehicle and high dose (P < 0.05); †significant difference between low and high dose (P < 0.05).
not type × Drug level, $F(2,128) = 4.44, P < 0.05$. Post hoc tests show that the high-dose SOD1<sup>G93A</sup> group had significantly worse rotarod performance compared with both vehicle ($P < 0.05$) and low-dose ($P < 0.05$) SOD1<sup>G93A</sup> groups when collapsed across this age range (see Fig. 3A for significance at each time point). In conjunction with rotarod performance, end stage was significantly earlier by ~8 days in the high-dose group compared with vehicle (K-M log rank: $\chi^2 = 7.4, P \leq 0.01$) and low-dose (K-M log rank: $\chi^2 = 11.4; P \leq 0.001$) groups (Fig. 3B and Table 3). This is equal to a 6% decrease in life span of the typical SOD1<sup>G93A</sup> mouse.

**Fluxo P5-11:** neonatal fluoxetine affected weight in both SOD1<sup>G93A</sup> and control animals. It has been previously shown that neonatal fluoxetine exposure can decrease normal weight gains in rats (Silva et al. 2010) that can last into adulthood (Karpova et al. 2009). Neonatal fluoxetine administration did not alter the average weight per animal between P5 and P11 (Fig. 4A). However, there was a small but sustained decrease in weight in all adult animals receiving the highest fluoxetine
dose. When weight was analyzed presymptomatically (P35–70), there was a significant between-subject effect of drug level on weight [RM ANOVA: Drug level, F(2,127) = 14.03, P < 0.0001]. Post hoc tests show that the high-dose group (regardless of genotype) had significantly lower weights compared with both vehicle (P < 0.0001) and low-dose (P < 0.0001) groups when collapsed across this age range (see Fig. 4B for significance at each time point).

Similar results were obtained when weight was analyzed postsymptomatically between P80 and P160. There was a significant between-subject effect of drug level on weight [RM ANOVA: Drug level, F(2,127) = 18.4, P < 0.0001]. Post hoc tests show that the high-dose group (regardless of genotype) had significantly lower weights compared with both vehicle (P < 0.0001) and low-dose (P < 0.0001) groups when collapsed across this age range (see Fig. 4C for significance at each time point).

**Fluox P5-11:** Neonatal fluoxetine did not alter gross motor activity. Quadrant crossing was monitored in the Fluox P5-11 study to determine whether fluoxetine altered gross motor activity. There were no significant differences in quadrant crossing between fluoxetine levels (monitored from P40 to P110), indicating that fluoxetine did not affect activity in a time period before severe paralysis was present in the SOD1G93A animals (data not shown). Therefore, the low weights in the high-dose fluoxetine group are not due to changes in activity levels.

**Serotonin can increase motoneuron excitability during this neonatal period.** To verify that 5-HT can increase motoneuron excitability during this neonatal period, in vitro spinal cord slices from P6–10 mice were made from both nontransgenic and SOD1G93A mice. Electrophysiological measurements were made before and after α-methyl 5-HT was bath applied. Coapplication of citalopram, a selective and potent SSRI, was necessary to inhibit fast 5-HT reuptake by SERTs, enabling us to use very low α-methyl 5-HT concentrations (0.1–0.3 μM). Because of the low endogenous levels of 5-HT in the slice preparation, citalopram alone likely has no effect on motoneuron excitability.

There were no differences between nontransgenic and SOD1G93A mice in the response to α-methyl 5-HT; therefore the data were collapsed across genotypes (Table 4). Coapplication of α-methyl 5-HT and citalopram caused a significant depolarization of the resting membrane potential [paired t-test: t(9) = −2.77, P < 0.05]. The AHP measured after a single action potential was also decreased [paired t-test: t(5) = 4.32, P < 0.01] (Fig. 5Ca). This is due to an increase in Jh, which was seen as an increase both in sag as a percentage of the voltage change [paired t-test: t(4) = −2.92, P < 0.05] and in the rebound as a percentage of voltage change [paired t-test: t(4) = −3.78, P < 0.05] when hyperpolarizing voltage steps were applied (Fig. 5A). In addition, there were changes in the PIC, a voltage-gated conductance that has a major impact on excitability. After coapplication of α-methyl 5-HT and citalopram, the PIC was fully activated at more hyperpolarized potentials (PIC peak voltage) [paired t-test: t(7) = 2.38], increasing the intrinsic excitability of the neuron (Fig. 5D). We have therefore confirmed that, in this neonatal age range, 5-HT has an acute excitatory effect on motoneuron excitability.

<table>
<thead>
<tr>
<th>Control</th>
<th>α-Methyl 5-HT + Citalopram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SE</td>
<td>Mean ± SE (n)</td>
</tr>
<tr>
<td>RMP, mV</td>
<td>−59 ± 2</td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
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</tr>
<tr>
<td>PIC amplitude, pA</td>
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</tr>
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<tr>
<td>V threshold, mV</td>
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</tr>
<tr>
<td>AHP, mV</td>
<td>7.7 ± 1.3</td>
</tr>
</tbody>
</table>

Means ± SE for n animals are presented. Results include motoneurons from both SOD1G93A mice and their nontransgenic littermates. Motoneurons were visually identified in spinal cord slices from P6–10 animals. Measurements were taken before (control) and after bath coapplication of α-methyl serotonin (5-HT) (0.1–0.3 μM) and citalopram (2–10 μM). Significance was determined by a Student’s paired t-test. AHP, afterhyperpolarization potential; I-ON, current at onset of firing; Ith, hyperpolarization-activated inward current; PIC, persistent inward current; Rbd, rebound; RMP, resting membrane potential; V, voltage. Significant values: *P < 0.05, †P < 0.01.

**Cyproheptadine: P5 to P11 (Cypro P5-11)**

**Cypro P5-11:** cyproheptadine did not alter SOD1G93A disease progression. Neonatal cyproheptadine was administered between P5 and P11 to test whether a 5-HT receptor antagonist, as opposed to increasing 5-HT receptor activation with fluoxetine, had a beneficial effect on disease progression. Cyproheptadine, however, did not improve rotarod performance or prevent weight loss as the SOD1G93A mice aged (Fig. 6, A and B). There were also no significant differences in tremor onset, the onset of rotarod decline, or end stage (Table 5 and Fig. 6C).

**Cypro P5-11:** cyproheptadine did not alter gross motor activity. Quadrant crossing was monitored in the Cypro P5-11 study to determine whether cyproheptadine altered gross motor activity. There were no significant differences in quadrant crossing between cyproheptadine levels (monitored from P40 to P110), indicating that cyproheptadine did not affect activity in a time period before severe paralysis was present in the SOD1G93A animals (data not shown).

**DISCUSSION**

**Summary**

Long-term adult fluoxetine treatment (started at either P30 or P70 and continuing until end stage) did not affect disease progression (Tables 1 and 2 and Figs. 1 and 2). In contrast, neonatal fluoxetine treatment (P5-11) had two effects. First, all animals receiving the highest dose had a sustained decrease in weight from P30 onward (Fig. 4, A and B). Second, the high-dose SOD1G93A mice reached end stage sooner because of an increased rate of motor impairment (Table 3 and Fig. 3). Fluoxetine increases synaptic 5-HT levels, which is known to increase spinal motoneuron excitability in adults. We confirmed, using spinal cord slices, that 5-HT increases spinal motoneuron excitability during this neonatal time period (Table 4 and Fig. 5) and therefore hypothesized that antagonizing 5-HT receptors during the same time period would improve disease outcome. Neonatal cyproheptadine, a 5-HT receptor antagonist, did not alter disease progression (Tables 5 and 6 and Figs. 5–7). We hypothesized that cyproheptadine reduced motoneuron excitability and thereby reduced disease progression. Unexpectedly, we found that neonatal cyproheptadine actually increased disease progression (Tables 5 and 6 and Figs. 5–7). This is most apparent in the high-dose group, where all animals receiving the highest dose died within 10 days of birth, versus 20 days in the control, high-dose fluoxetine, or low-dose cyproheptadine groups (Fig. 7A). There were no significant differences in weight, motor impairment, or survival between the high-dose cyproheptadine and high-dose fluoxetine groups, or between the low-dose cyproheptadine and control groups.

**Table 4. In vitro whole cell electrophysiology**

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Means ± SE for n animals are presented. Results include motoneurons from both SOD1G93A mice and their nontransgenic littermates. Motoneurons were visually identified in spinal cord slices from P6–10 animals. Measurements were taken before (control) and after bath coapplication of α-methyl serotonin (5-HT) (0.1–0.3 μM) and citalopram (2–10 μM). Significance was determined by a Student’s paired t-test. AHP, afterhyperpolarization potential; I-ON, current at onset of firing; Ith, hyperpolarization-activated inward current; PIC, persistent inward current; Rbd, rebound; RMP, resting membrane potential; V, voltage. Significant values: *P < 0.05, †P < 0.01.
Tremor onset and the onset of rotarod decline did not differ between groups, indicating that the advancement in end stage was due to an increase in the rate of motor impairment, not a change in the onset of clinical symptoms.

This short time window for neonatal fluoxetine treatment (P5-11) corresponds with a developmental increase in the PIC amplitude (Quinlan et al. 2011). In addition, the PIC during this time period is pathologically elevated in the high-expressor SOD1G93A mouse line (Kuo et al. 2005; Pieri et al. 2009; Quinlan et al. 2011; van Zundert et al. 2008). In some studies, this resulted in an increase in motoneuron excitability (Kuo et al. 2005; Pieri et al. 2009; van Zundert et al. 2008); however, in the Quinlan et al. (2011) study the pathological increase in the PIC is matched by a similar increase in the input conductance of the SOD1G93A motoneurons, resulting in no net increase in excitability.

Whether or not motoneurons are hyperexcitable during this time period, adding fluoxetine increases excitability by increasing synaptic 5-HT levels. We confirmed that 5-HT increases spinal motoneuron excitability during this time period with acute coapplication of α-methyl 5-HT and citalopram to in vitro slices. Coapplication of α-methyl 5-HT and citalopram hyperpolarized the PIC peak voltage and also depolarized the resting membrane potential and increased $I_B$ in neonatal spinal motoneurons from both nontransgenic and SOD1G93A mice. The increase in motoneuron excitability (and probable increase in calcium entry) during administration of fluoxetine to neonates may play a role in regulating postnatal gene expression, thus causing more permanent shifts in motoneuron physiology.

By shifting motoneuron properties during this time, the motoneurons may play a role in regulating postnatal gene expression, thus causing more permanent shifts in motoneuron physiology.

A recent review by van Zundert et al. (2012) highlighted the importance of early pathological changes in ALS disease models including pathological increases in motoneuron excitatory voltage-sensitive conductances (Carunchio et al. 2010; Kuo et al. 2004, 2005; Pieri et al. 2009; van Zundert et al. 2008, 2012). In addition, motoneuron hyperexcitability is also a pathological feature late in ALS disease progression (Kanai et al. 2006; Tamura et al. 2006; Vucic and Kiernan 2006a, 2006b; Vucic et al. 2007, 2008). We therefore hypothesized that further increasing motoneuron excitability at any age would be detrimental to SOD1G93A mice because it would enhance these pathological features, increasing metabolic demands and exacerbating any excitotoxic effects.

As we had hypothesized, neonatal fluoxetine treatment from P5 to P11 had a negative effect on disease progression in the SOD1G93A mice. Mice in the high-dose fluoxetine group had greater motor impairment during rotarod testing and reached end stage criteria sooner than the vehicle and low-dose groups. Tremor onset and the onset of rotarod decline did not differ between groups, indicating that the advancement in end stage was due to an increase in the rate of motor impairment, not a change in the onset of clinical symptoms.

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By shifting motoneuron properties during this time, the motoneurons may play a role in regulating postnatal gene expression, thus causing more permanent shifts in motoneuron physiology.
tomeurons may thus be rendered more vulnerable later in life. However, we did not directly test whether prolonged fluoxetine administration in vivo affected motoneuron excitability or initiated compensatory mechanisms. Furthermore, a more detailed study would be needed to identify whether subtle differences exist between SOD1G93A and nontransgenic motoneuron responses to 5-HT.

However, neonatal cyproheptadine treatment produced no beneficial results. Cyproheptadine is a 5-HT2 receptor antagonist that has been shown to decrease motoneuron excitability (Barbeau and Rossignol 1990; D’Amico et al. 2013; Murray et al. 2010; Thompson and Hornby 2013; Yamazaki et al. 1992). Cyproheptadine therefore either 1) does not decrease motoneuron excitability in neonatal mice, 2) was not at a concentration high enough to produce an effect, or 3) decreased excitability but did not affect disease progression. We believe options 1 and 2 are unlikely because cyproheptadine has previously been shown to block the effects of 5-HT application on neonatal rat motoneurons in vitro (Wang and Dun 1990), and the doses for this study were chosen based on their ability to inhibit 5-HT-modulated effects in vivo (Kilic et al. 2011; Semenova and Ticku 1992; Singh and Goel 2010; Yi et al. 2011). We therefore believe that these cyproheptadine doses were sufficient to inhibit motoneuron excitability. Instead, this study suggests that either baseline levels of 5-HT do not generate enough hyperexcitability to induce a detrimental state or the fluoxetine treatment affected more than motoneuron excitability.

As an alternative possibility, neonatal fluoxetine treatment may have affected the development of monoaminergic pathways. In particular, neonatal fluoxetine treatment has been shown to affect the development of the serotonergic system itself. In rats, daily subcutaneous injections (P1-21) decreased the size of serotonergic neurons in the dorsal and median raphe nucleus and the number of serotonergic terminals in the dentate gyrus (Silva et al. 2010). Other studies also suggest that neonatal SSRI treatment (>14 days) can alter the serotonergic system, resulting in decreased 5-HT levels (Feenstra et al. 1996; Hilakivi et al. 1987; Vijayakumar and Meti 1999), tryptophan hydroxylase (a rate-limiting enzyme in 5-HT production), and SERT expression (Macciag et al. 2006). To minimize these changes, fluoxetine was only administered for 7 days during the time period (P5-11) when both the Na and Ca PIC are known to be upregulated in the SOD1G93A mouse model (Quinlan et al. 2011). Despite this, adult weights were significantly lower in the high-dose fluoxetine group regardless of genotype. These data correspond with the low weight gains seen in rats by Silva et al. (2010) and Karpova et al. (2009). Additional studies need to be performed to test whether neonatal fluoxetine treatment similarly affected the serotonergic system.

Hypothesizing that early alterations in the serotonergic system affect disease progression is intriguing given the large amount of data identifying changes in this system in ALS patients. These data have been reported in a detailed review by Sandyk (2006). As a whole, the data suggest that ALS patients have lower 5-HT levels because of a decrease in the production and/or release of 5-HT (Bertel et al. 1991; Monaco et al. 1979).}

**Table 5. Neonatal cyproheptadine P5-11**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Tremor Onset</th>
<th>RR Onset Decline</th>
<th>End Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPN ±SD</td>
<td>DPN ±SD</td>
<td>DPN ±SD</td>
</tr>
<tr>
<td>Vehicle</td>
<td>92 ±25</td>
<td>119 ±10</td>
<td>132 ±9</td>
</tr>
<tr>
<td>1 mg/kg</td>
<td>91 ±18</td>
<td>119 ±11</td>
<td>129 ±11</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>89 ±22</td>
<td>118 ±11</td>
<td>133 ±8</td>
</tr>
</tbody>
</table>

Means ± SD (n = 20 per drug level) are presented for tremor onset, rotarod onset of decline, and end stage. Data are divided into drug levels (vehicle, 5 mg/kg, and 10 mg/kg). There was no significant effect of drug level on any of these parameters. Univariate ANOVAs were used to compare tremor onset and age when rotarod performance was first impaired with between-subject factor of drug concentration with sex as a covariate. The K-M log rank survival test was used to analyze end stage with the factor (drug concentration) compared pairwise and pooled over strata (sex) to test the effect of drug concentration. Cypro, cyproheptadine.
but not in 5-HT metabolism (Ohsugi et al. 1987). Dupuis et al. (2010) have further shown that low platelet 5-HT levels were predictive of lower survival rates. 5-HT receptors are also differentially expressed with regard to receptor subtype and regional specification (Forrest et al. 1996; Manaker et al. 1988), although these changes appear to be secondary physiological adaptations.

Given the relationship between ALS and the serotonergic system, the negative data in the chronic adult fluoxetine studies (Fluo P30 and Fluo P70) become difficult to interpret. This is especially true because chronic adult SSRI treatment can also cause decreases in 5-HT levels, tryptophan hydroxylase (Caccia et al. 1992; Thompson et al. 2004; Trouvin et al. 1993), and SERT expression (Dygalo et al. 2006; Lesch et al. 1993; Neumaier et al. 1996; Oliva et al. 2005; Walther et al. 2003). However, the effect of this downregulation is complicated because chronic SSRI treatment can also desensitize the 5-HT$_{1A}$ autoreceptors, which provide negative feedback to serotonergic neurons (Hensler 2003), and increase 5-HT$_{2}$ receptor expression (Laakso et al. 1996). Furthermore, chronic fluoxetine treatment increases the production of neurotrophic factors. Specifically, chronic fluoxetine treatment increases the production of brain-derived neurotrophic factor (BDNF) (De Foubert et al. 2004), which may protect motoneurons by promoting cell survival (Lewin and Barde 1996) and decreasing glutamate mediated excitotoxicity (Mattson et al. 1995). Our studies found no impact on disease progression when fluoxetine was given for prolonged periods in the adult state, which may be attributed to these mixed effects on motoneuron excitability or production of additional neurotrophic factors. These competing factors may also shed some light on why an earlier study showed that chronic treatment with fluoxetine was given for neonatal periods in the adult (BDNF) (Kurkinen et al. 2001). These data also show that SSRI treatment in adults had no effect on disease progression and support the idea that acute neonatal interventions may affect ALS disease progression and secondary changes related to chronic treatment.

In summary, neonatal fluoxetine treatment impaired motor performance in the SOD1$^{G93A}$ mouse model, resulting in early death, but chronic fluoxetine treatment during adulthood had no effect. These data suggest that acute neonatal interventions can affect ALS disease progression and support the idea that developmental processes may contribute to later disease. The data also show that SSRI treatment in adults had no effect on disease progression, supporting the use of antidepressants in ALS patients.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


