Supporting Information

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SI Text

TGF-β2 is a potential therapy for motor neuron disease (1); therefore the observations made on the rat diaphragm (main text) have been partially replicated here with mice,as a prelude to investigating the actions of TGF-β2 in mice with motor neuron disease.

Motor neuron disease begins with the failure of nerve terminals (2), at least in hSOD1 mice that carry a disease-causing transgene (3). Administration of TGF-β2 to hSOD1 mice causes an acute restoration of full motor function without preventing the ongoing deterioration of the mouse (1). This improvement is unlikely to be caused by TGF-β2's trophic and anti-apoptotic actions (1, 4), because other anti-apoptotic factors do not produce acute therapeutic benefits. This reasoning, coupled with the speed of action of TGF-β2, raises the possibility that TGF-β2 alters synaptic function in SOD1 mice, possibly by changing presynaptic quantal size (QSpre). If so, manipulation of the TGF-β2 pathway may provide symptomatic relieve for patients with motor neuron disease, in a manner analogous to L-dopa treatment of Parkinson's patients.

SI Results

The effects of TGF-β2 on neurotransmission in the murine diaphragm were broadly similar to those in the rat. In mice, 1 ng/mL of TGF-β2 increased the mean amplitude of miniature endpotentials (MEPPs) by 33% (Fig. S1*A*), and its effect on evoked postsynaptic potential (EPP) amplitude was ≈50% smaller (Fig. S1*B*), as occurred in rats (main text).

The magnitude of the effect of TGF-β2 on murine neuromuscular junction (NMJs) was smaller than in the rat. The lesser magnitude, in combination with the large between-fiber variability, made it more difficult to measure changes in quantal content (QC) accurately. The effect of TGF-β2 on vesicle use therefore was examined by preloading the vesicles with FM1-43, which accumulates in synaptic vesicles. The rate of destaining of the nerve terminal after nerve stimulation gives a sensitive measure of the rate of vesicle use (5, 6). TGF-β2 significantly slowed the destaining associated with stimulation at 20 Hz: More than twice as long a time was required to release half of the stain (Fig. S2).

SI Discussion

Murine Observations. The effect of TGF-β2 on neurotransmission at the murine NMJ mirrored the observations of increased postsynaptic potential size in rats, providing additional verification of the initial rat data. The magnitude of the effect of TGF-β2 on MEPPs was smaller in mice than in rats but still was biologically significant, with TGF-β2 being sufficient to reduce synaptic vesicle use by more than one half (Fig. S2).

The FM1-43 technique was used initially because of its greater sensitivity, but it also has the advantage of using a more physiological pattern of nerve simulation. Single-evoked stimuli have a short time course, and the level of negative feedback therefore is small and determined largely by recent MEPPs. With a 20-Hz stimulus train, there is time for ACh levels to rise in the synaptic cleft and for the level of negative feedback to increase. The fact that TGF-β2 has a larger effect on vesicular conservation with a stimulus train than with single-evoked stimuli thus provides further evidence that TGF-β2 acts to increase QSpre, with consequent enhancement of negative feedback. Whatever TGF-β2's mechanism of action may be, the FM1-43 observations show that physiological levels of TGF-β2 (1 ng/mL), as used here, produce a large conservation of vesicle use.

Action of Drugs. Atropine, vesamicol, and bafilomycin did not affect MEPP amplitude, EPP amplitude, or QC when applied alone (Fig. 5) (main text). This result is consistent with previous studies but requires explanation, because these observations reflect the way the drugs were used and/or the specific characteristics of the synapses being examined.

Vesamicol. The ACh in vesicles at the NMJ does not exchange freely with cytoplasmic ACh (7–9); the vesicles seal once they have reached a certain set point of filling (10). Once filled, the vesicle remains filled until it is released. Consequently, inhibitors of vesicular filling, such as vesamicol and bafilomycin, have little or no action on vesicles that are already filled and sealed within the timescale of the experiments here.

In the current study, about half the vesicles in the readily releasable pool would have been released spontaneously during the preincubation with the drugs. If TGF-β2 alters the filling of vesicles during this period, then vesamicol should block all loading, as discussed in the main text, so that vesicles recycled in the presence of vesamicol would be empty and undetectable electrophysiologically, whereas prefilled vesicles would not load with additional ACh in the presence of TGF-β2. Our observations are consistent with this reasoning (Fig. 5).

Vesamicol also blocks the filling of vesicles when TGF-β2 is absent, but this effect would not be apparent in the electrophysiological data. When vesamicol is applied alone, the presynaptic terminals contain a mixture of empty, newly recycled vesicles and normal filled, yet-to-be-released vesicles. Empty vesicles are not detectable by current or voltage clamp (10) and consequently will not be incorporated into any measure of MEPP amplitude or miniature excitatory postsynaptic current. The experiments were of sufficiently short duration for the nerve terminals to continue to release prefilled vesicles, particularly because the preparations were stimulated only minimally (i.e., enough to collect 1–2 EPPs per NMJ and a maximum of 10 NMJs per muscle). Consequently, the lack of effect of vesamicol on MEPP amplitude is to be expected, because the measured amplitudes would arise only from the release of vesicles that were filled before the experiment began.

Vesamicol can change MEPP/EPP amplitudes and QC under certain circumstances (11). For instance, if the terminals are stimulated by a train, then QC declines rapidly, because the proportion of empty vesicles being released increases rapidly. Conversely, if subblocking levels (<500 nM) of vesamicol are used, vesicle filling will be blocked only partially, resulting in the subsequent release of partially filled vesicles. This scenario will be evidenced by a decrease in MEPP amplitude, which, if present, is detectable in our system (Fig.1*A*), because partially filled vesicles cause small MEPPs. Finally, at very high concentrations (>10 μM), vesamicol can partially inhibit AChRs, reducing both MEPP and EPP amplitudes (4). For this reason, before beginning the study, we ascertained that the dose of vesamicol used was fully effective, with the normality of the MEPPs in presence of vesamicol alone (Fig. 5 and main text) serving as proof.

Bafilomycin. Bafilomycin alone had no effect on MEPP amplitude (Fig. 5 and main text), a result that is consistent with previous reports of its action on cholinergic synapses (12). The issues here are analogous to those outlined for vesamicol, because filled cholinergic vesicles are stable for some hours in the presence of bafilomycin, with the action of the drug limited to inhibition of filling of emptied vesicles.

As a cautionary note, we point out that many types of synaptic vesicles, such as glutamatergic vesicles, are in equilibrium with cytoplasmic neurotransmitter. Consequently, bafilomycin will al-

ter the contents of previously filled glutamatergic vesicles (13); therefore the experimental strategy used here cannot be applied uncritically to noncholinergic synapses.

Atropine. Atropine blocks the cholinergic negative feedback on the release of synaptic vesicles by inhibition of presynaptic muscarinic AChRs. This inhibition affects the frequency at which vesicles are spontaneously released, and our data show an increase from 2.3 ± 0.2 (n = 23) to 3.6 ± 0.6 Hz (n = 16; $P < 0.05$). However, at the modest concentrations used here, atropine does not alter MEPP amplitude (14), so the contents of the vesicles are unaltered.

During evoked transmission for single stimuli, atropine had no effect on EPP amplitude (P = 0.7). Muscarinic receptors can affect the temporal dispersal of vesicle release (15), and consistent with this effect we found EPP rise time (0–100%, 0.99 ± 0.05 ms) and time to return to 50% peak potential (2.79 ± 0.09 ms) were slightly prolonged (1.33 ± 0.08 and 3.21 ± 0.12 ms, respectively; both $P < 0.005$) in the presence of atropine. In the absence of effects on MEPP or EPP amplitude, QC is unaltered. Hence, the ability of atropine to affect QC in the presence but not in the absence of TGF-β2 (Fig. 5) is consistent with TGF-β2 acting to increase QSpre.

SI Materials and Methods

Animals. Adult C57/Bl6 male mice (26–37 g) were killed by cervical dislocation, in accordance with Schedule 1 of the U.K. Animals (Scientific Procedures) Act, 1986.

Electrophysiological and FM1-43 Destaining Recordings. The MEPP and EPP amplitudes were recorded, as described in the main text. The measurement of synaptic vesicle use with FM1-43 was as described previously (5, 6). Briefly, the diaphragm was incubated in 2 μM FM1-43, and the phrenic nerve was stimulated at 10 Hz to load the vesicles, then incubated for 1 h in TGF-β2 (1 ng/mL). Vesicle exocytosis was measured by monitoring the destaining of terminals during 10 min of 20-Hz stimulation, with images acquired at 10-s intervals using a Retiga EXi (Q-Imaging) digital camera. Tubocurarine (5 μM) was included in the incubation medium to prevent movement of the fibers during recording. Control experiments show tubocurarine has no effect on destaining rates under these conditions (6).

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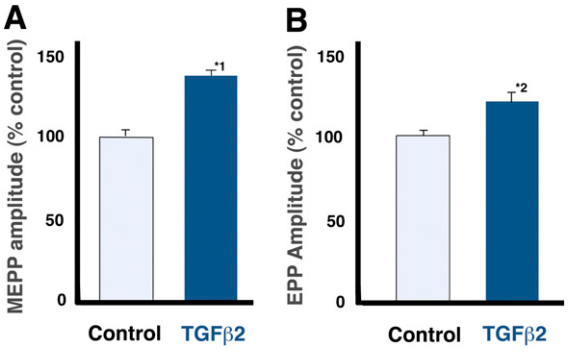

Fig. S1. TGF-β2 regulates MEPP and EPP amplitude. *(A)* TGF-β2 (1 ng/mL, dark blue bar) increased the amplitude of MEPPs relative to controls (light blue bars). The bars are mean ± SEM of 39 control and 52 TGF-β2–treated NMJs. ($*^1P = 0.0002$). *(B)* The amplitudes of the EPPs also were increased by TGF-β2 ($*^2P = 0.001$).

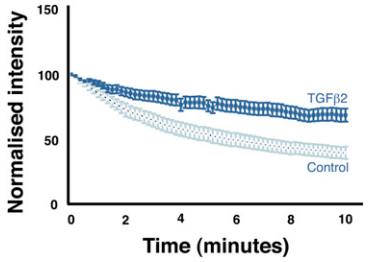

Fig. S2. TGF-β2 reduced exocytosis of FM1-43–loaded vesicles. TGF-β2 slowed the destaining of FM1-43–stained nerve terminals during a 20-Hz, 10-min stimulation. The rate of destaining was significantly different between the TGF-β2-treated preparations (dark blue) and control (light blue) ($P = 0.003$, two-way ANOVA). Each data point is the mean ± SEM of seven control and 17 TGF-β2–treated preparations.