Enhancement of spontaneous transmitter release at neonatal mouse neuromuscular junctions by the glial cell line-derived neurotrophic factor (GDNF)

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1. The acute effects of neurotrophic factors on the frequency of spontaneous transmitter release (miniature endplate potentials (MEPPs)) from motor nerve terminals has been examined in skeletal muscles of neonatal mice aged between 9 and 20 days. The following factors were tested at a concentration of 50 ng ml⁻¹: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), ciliary neuronotrophic factor (CNTF), leukaemia inhibitory factor (LIF), insulin-like growth factors 1 and 2 (IGF-1 and IGF-2), and glial cell line-derived neurotrophic factor (GDNF). In some experiments, the responses to 2 μM LaCl₃ and 10 mM K⁺, or to 2—5 nM purified α-latrotoxin (α-LTX) were also measured.

2. Neither BDNF, NT-3, NT-4, LIF, IGF-1 or IGF-2 – singly or in combination – caused any significant change in MEPP frequency. GDNF, however, produced a highly significant, 2-fold increase in neurotransmitter release that was reproduced in fourteen muscles.

3. Potentiation of MEPP frequency in GDNF was of the same order as that induced by tetanic stimulation or substitution of the bathing medium with hypertonic saline; but substantially less than that induced either by lanthanum ions or α-latrotoxin.

4. The data suggest that concentrations of GDNF that produce maximal enhancement of motoneurone survival in vitro and in vivo also produce acute, non-saturating enhancement in transmitter release at immature mammalian neuromuscular synapses. Taken together with other reports, these findings suggest that GDNF may mediate both functional and structural plasticity of neonatal neuromuscular junctions.

Keywords: Nerve growth, Neuromuscular junction, Synaptic transmission
of connections suggested by these studies, we have screened a panel of neurotrophic factors on isolated neuromuscular preparations from mice, at an intermediate stage during the maturation of neuromuscular synapses; that is, when elimination of polyneuronal innervation is almost complete (Parson et al. 1997). Of the neurotrophic factors we tested, enhanced spontaneous neurotransmitter release was observed only in the presence of GDNF. Taken together with other studies, the present results support a role for specific neuronal cell-derived factors (in addition to factors acting upon neuroglia; Trachtenberg & Thompson, 1996, 1997) in the functional and structural plasticity of neuromuscular synapses during postnatal development.

Some of the data presented have been communicated previously in an abstract (Thomson et al. 1997).

METHODS

Mice aged 9–20 days were killed by cervical dislocation, a Schedule I method in accordance with UK Home Off ice guidelines. Intracellular recordings were made using standard techniques from isolated preparations of either flexor digitorum brevis (FDB; innervated by spinal motor nuclei) or levator auris (LA; innervated by the facial nucleus) bathed in mammalian physiological saline, composition (mÒ): NaCl, 137; KCl, 5; CaClµ, 2—8; MgClµ, 1; NaHµPOÚ, 0·2; NaHCO×, 23; ª_glucose, 5; bubbled to equilibration with 95 % Oµ—5 % COµ. Most experiments were done on FDB because the fibres are short (< 300 ìm in length) and isopotential, facilitating recording of focal synaptic responses. A number of the experiments involving administration of BDNF were carried out on LA, in the light of reports suggesting a physiological role for trkB and BDNF in the facial nucleus which innervates LA (Klein et al. 1993; but see Silos-Santiago et al. 1997). Spontaneous MEPPs were recorded at room temperature (about 20 °C) before and after administration of growth factors (human recombinant; purchased from Alomone Labs, Jerusalem, Israel) at a concentration of 50 ng ml—1. The frequency of MEPPs was first estimated in each muscle fibre by counting the number of occurrences of MEPPs in a single oscilloscope sweep of 10–50 s duration. The number of MEPPs in twenty successive sweeps of between 50 ms and 50 s duration were then counted before and after adding growth factor to the bathing medium. Between five and ten fibres were sampled before and after growth factor administration in each experiment. Some of the data were digitized via a CED 1401 + interface into a personal computer running Spike-2 acquisition and analysis software (Cambridge Electronic Design). In some experiments the bathing fluid was replaced with mammalian saline buffered with Hapes instead of bicarbonate—COµ and containing either: (a) 2 µM Laµ³ ions and elevated Kµ (10 mÒ; Curtis et al. 1986); or (b) 2—5 nÒ purified α-latrotoxin (α-LTX; Lelianova et al. 1997) in nominally Caµ²⁺ free solution; or (c) normal mammalian saline made hypertonic with added sucrose (100 mÒ). In some experiments, MEPPs were recorded in low Caµ²⁺ (0·5 mÒ Caµ²⁺, 4 mÒ Mgµ²⁺) solutions before and after tetanic stimulation for 1 min at 50 Hz. All these procedures were undertaken as positive controls to measure against the effects of the neurotrophic factors. Continuous impalements were maintained during these treatments in only a minority of fibres. Thus most of the comparisons of effects of the neurotrophic factors are based on the mean or median frequencies measured in samples of different fibres impaled before and after adding growth factors to the bathing medium. Non-parametric statistical methods were mostly used to analyze the data, which were plotted using Jandel SigmaPlot (SPSS Inc., Chicago).

Figure 1. Effect of GDNF on MEPP frequency

Intracellular recordings from eight different muscle fibres in a 13-day-old mouse FDB muscle before (left) and after (right) adding 50 ng ml—1 human recombinant GDNF to the bathing solution. Doublet or triplet MEPP responses were uncommon in 1 s oscilloscope sweeps made before and after adding GDNF, but were observed more frequently after adding GDNF. The apparent differences in MEPP amplitude between these samples was coincidental. Overall, there was no significant effect of GDNF on MEPP amplitude or rise time (see text). Calibrations: top three sets of records, 2 mV, 0·5 s, lowest pair of records, 2 mV, 20 ms.
RESULTS

Figure 1 shows examples of MEPPs recorded from four fibres before and (in a different set of four fibres in the same muscle) after administration of GDNF to the medium bathing an isolated FDB muscle from a 13-day-old mouse. In this experiment, a small but significant increase in the median frequency of MEPPs was observed in the two samples of fibres obtained before and after adding GDNF to the bathing medium. This finding was reproduced in all fourteen experimental muscles tested in this group. In one experiment, GDNF was added to the bathing medium, and median MEPP frequency increased reversibly: it reduced on returning to normal bathing medium and was increased again after reapplying the factor. GDNF had no significant effects on MEPP amplitude. The mean MEPP amplitude from four muscles before GDNF treatment was 0.97 ± 0.27 mV (mean ± s.e.m., n = 21 fibres). Thirty minutes after GDNF administration, the mean MEPP amplitude was 1.21 ± 0.5 mV (n = 21 fibres; P > 0.05, Student's paired t test). Likewise, GDNF did not significantly alter MEPP rise time, which was overall 1.10 ± 0.16 ms, or time from peak to half-decay, which was overall 2.91 ± 0.46 mV (AC-coupled records).

The distributions of MEPP frequencies from ninety-two muscle fibres in control solutions, and the same number of fibres after adding GDNF to the bathing medium, showed many more fibres with MEPP frequencies greater than 0.2 s⁻¹ in the GDNF-treated group. The median and distributions of MEPP frequencies from fourteen muscles were statistically highly significantly different (control median, 0.16 s⁻¹; interquartile (i.q.) range 0.07–0.35; GDNF median, 0.43 s⁻¹, i.q. range 0.13–1.13; P < 0.01, Mann–Whitney U test; P < 0.01, Kolmogorov–Smirnov test). There was no significant increase in the control MEPP frequency with age over the range and in the samples we tested (Fig. 2), but the frequency of MEPPs before and after adding GDNF was significantly correlated, with a slope of 1.5–4 (95% confidence limits). We thus conclude that GDNF increases MEPP frequency in most muscle fibres by about a factor of two.

Other growth factors produced no clear or statistically significant effects. For example, we tested BDNF on ten LA muscles and two FDB muscles. Median MEPP frequency increased in two of the LA muscles and in one of the FDB muscles, but decreased in the other nine muscles. Other factors were tested on two FDB muscles each, also with no overt or consistent effects. The data are summarized in

Figure 2. GDNF increases MEPP frequency by about a factor of two

There was no apparent change in sensitivity of nerve terminals to GDNF over the postnatal period covered. Mean MEPP frequencies before (A) and after adding GDNF (B) were not significantly correlated with the postnatal age of the mice. However the mean frequencies after GDNF were significantly greater and correlated with the frequency before adding GDNF. Note the difference in scale on the ordinate. C, data showing correlation of median frequencies before and after adding GDNF in each of the 14 muscles studied, together with the linear regression and 95% confidence limits (dotted lines). D, for each muscle the mean MEPP frequency after applying GDNF (G) was divided by the mean frequency before GDNF (C) and the ratio (G:C) was calculated. The box-and-whisker plot shows the distribution of the G:C ratios (horizontal line within box, median; box limits, 25–75% interquartile ranges; whiskers, 5%–95% ranges; dots = outliers) and suggests that GDNF potentiated MEPP frequency by a factor of 1.5 to 5, with an average (median) effect of a 2-fold increase in MEPP frequency.
Fig. 3. A cocktail comprising all the growth factors (at 50 ng ml⁻¹ each), including GDNF, caused a slight increase in mean MEPP frequency in one preparation, but decreased it in another (data not shown). Thus whilst we have no compelling evidence to suggest any synergistic effects of other factors with GDNF, we cannot rule out the possibility that other factors might in fact antagonize the effects of GDNF, or indeed reduce MEPP frequency.

As positive controls, the effects of GDNF on MEPP frequency were compared with the effects of other substances known to potentiate spontaneous transmitter release. La³⁺ ions at 2 µM in Hepes-buffered solution containing 10 mM K⁺ enhanced spontaneous release in some, but not all, muscle fibres as reported by Curtis et al. (1986). Thus the distribution of MEPP frequency in La³⁺ solutions was positively skewed, but the median frequencies were 1·5 to 9·8 times as great as in control solutions (Fig. 3).

Much larger increases in MEPP frequency were induced by brief (1–5 min) exposure to purified α-latrotoxin (2–5 nM). Figure 4A shows a modest increase in MEPP frequency during exposure to GDNF. By contrast, Fig. 4B shows the result of an experiment in which MEPP frequency increased by at least three orders of magnitude during exposure to 5 nM α-LTX. The median increase in twenty-one fibres was a factor of about 200 compared with controls (Fig. 3). As expected, after the initial increase, the frequency of MEPPs declined steeply in α-LTX (Fig. 4B), presumably as the intraterminal pools of synaptic vesicles became depleted; but the residual frequencies remained substantially above either control or GDNF-treated preparations. The bathing medium was only nominally Ca²⁺ free; thus some recycling of synaptic vesicles may have continued in the α-LTX-treated terminals (Pease et al. 1986; Ceccarelli et al. 1988).

We also tested the effect of prolonged tetanic stimulation of neuromuscular junctions, in one preparation from a 12-day-old mouse. Stimulation at 50 Hz for 60 s produced an increase in MEPP frequency from less than 1 s⁻¹ before to about 3 s⁻¹ immediately after the train of stimuli (Fig. 4C). This experiment was carried out in a solution containing reduced Ca²⁺ and elevated Mg²⁺ (Nussinovitch & Rahamimoff, 1988) to facilitate continuous recording from muscle fibres. Similar post-tetanic potentiation of MEPP frequency was seen in five other fibres in the same muscle. Potentiations of MEPP frequency by factors of two to ten were also observed in six fibres in another neonatal muscle after adding 100 mM sucrose to the bathing medium.

**DISCUSSION**

Statistically highly significant effects on MEPP frequency were seen with bath application of GDNF in 9- to 15-day-old mouse FDB muscle preparations, of a similar order to the effects of tetanic stimulation or treatment with hyperosmotic saline. It is perhaps surprising that none of the other factors tested, including BDNF and NT-3, had any effect on MEPP frequency, especially in the light of the
positive effects reported for amphibian neurone—myocyte co-cultures (Lohof et al. 1993; Liou et al. 1997). The concentrations of GDNF and the other factors we used were in the range reported to be maximal in enhancing embryonic motoneurone survival in vitro (Henderson et al. 1994) but it is possible that higher concentrations may be more effective at postnatal neuromuscular synapses. Furthermore, some factors like CNTF appear to produce a delayed response, requiring signalling via the cell nucleus (Stoop & Poo, 1996). Thus we cannot yet rule out a possible role for the growth factors we tested in addition to GDNF, since our tests were acute and carried out using isolated nerve—muscle preparations, which were of course disconnected from motoneurone cell bodies. Studies of acute or chronic administration of these other factors in vivo, or to isolated preparations with an intact spinal cord still connected to the muscles, might be of interest in establishing any physiological effects on neurotransmitter release. The increases in MEPP frequency produced by GDNF were substantially less than the maximum the neonatal synapses were capable of producing, since La ions or purified α-latrotoxin stimulated far greater increases than GDNF. Nonetheless, the effects of GDNF were consistent, and evidently indicate that this factor may act locally on motor nerve terminals to modulate neurotransmitter release.

The mechanism of action of GDNF on neonatal terminals merits further investigation. Upregulation of transmitter release by exogenous factors is normally mediated either by direct actions on L/P-type Ca$^{2+}$ channels or Ca$^{2+}$

![Figure 4. Responses to GDNF are submaximal](image)

Continuous intracellular recordings of MEPPs following treatment of FDB muscles from 13-day-old mice, with the following. A, GDNF which in this fibre increased MEPP frequency by about a factor of 5. Record duration, 5 min; B, α-latrotoxin, which enhanced MEPP frequency several 100-fold within 1—2 min. C, tetanic stimulation at 50 Hz for 1 min, which produced a 3- to 5-fold potentiation of MEPP frequency, comparable to the effects of GDNF. EPSPs were partly blocked by the low Ca$^{2+}$, high Mg$^{2+}$ bathing solution. Horizontal bars in these panels indicate duration of growth factor/toxin administration or stimulation.
GDNF receptors are present in motor nerve terminal membranes during and after the normal period of synapse elimination in mice (Parson et al. 1997) and are linked to intracellular mechanisms that regulate neurotransmitter release.


