Long term effect of low frequency chronic electrical stimulation on the fast hind limb muscles of dystrophic mice

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SUMMARY  Low frequency chronic electrical stimulation can have a beneficial effect on dystrophic muscles. The present study was undertaken to assess the long term effect of such stimulation on the fast hind limb muscles of dystrophic mice. The relationship between the changes induced by stimulation and the initial condition of the dystrophic muscles, as well as other factors which might contribute to this relationship, were examined. The stimulation induced an increase in the force output of weak dystrophic muscles and a speeding of their time course of contraction and relaxation, as well as an increase in their fatigue resistance. In relatively strong dystrophic muscles, the stimulation induced similar changes in contractile speed and fatigue characteristics, but it led to a slight decrease in force output. Our results suggest that the stimulation promotes the growth and differentiation of the small regenerating fibres known to be present in the diseased muscles and, in addition, induces an increase in the mitochondrial content of the muscle fibres. Our results indicate that these effects are not permanent.

It has previously been reported that low frequency electrical stimulation has a beneficial effect on dystrophic muscles. Experiments carried out on dystrophic mice have shown that chronic electrical stimulation at 8–10 Hz induced a functional improvement as well as an increase in the total number of fibres in the fast muscles of the hind limbs to which it was applied.7 In addition, it was found that the slowing of the time course of contraction and relaxation caused by the disease process in these muscles was reduced following stimulation and their resistance to fatigue was increased. Further examination of the same preparations showed that both the level of glycolytic enzymes, as well as that of their different isozymes, and the membrane properties characteristic of adult fast muscles had been restored following stimulation.3 In dystrophic chickens, a similar pattern of electrical stimulation applied chronically to the posterior latissimus dorsi muscle caused a reduction in the rate of deterioration of its fibres as well as in the level of AChE activity, usually elevated in that particular muscle.1 Finally, and perhaps most importantly, electrical stimulation at 5–8 Hz was shown to produce a significant increase in the maximum voluntary force developed by the Tibialis anterior (TA) muscle in patients with Duchenne muscular dystrophy (DMD).4

In view of these findings, it seems important to discover if the changes induced by such stimulation are permanent. In only one of these studies were the subjects re-examined six months after the end of the stimulation treatment.4 This examination indicated an overall deterioration in the physical characteristics of the patients. However, as the assessment was based on the overall strength of several muscle groups, the contribution of the stimulated muscle, per se, was not clear.

In a previous study,5 it was reported that functional improvement in dystrophic mice following electrical stimulation was only apparent when the muscles were severely affected by the disease process. It could be that the improvement in function induced by chronic stimulation is due to an improved growth of the regenerated fibres that are known to be present in dystrophic muscles, particularly during the early stage of disease.
Long term effect of low frequency chronic electrical stimulation

of the disease, and in a weaker muscle the number of such fibres may be greater. However, this is not necessarily so, and other factors may contribute to the different response of "weak" and "strong" muscles to electrical stimulation.

This study was undertaken to assess the long term effect of low frequency chronic electrical stimulation of the fast hind limb muscles of dystrophic mice (dy²), and to define further the relationship between the initial condition of the diseased muscles and the changes induced by electrical stimulation.

Methods

Dystrophic mice of the strain C57Bl/6J dy² aged 3–6 months were used in these experiments.

1) Experimental protocols

Using chloral hydrate anaesthesia (IP, 1 ml/100 g body weight of a 4–5% solution) and sterile precautions, Teflon coated stainless steel wires were implanted either side of the lateral popliteal nerve in one hind limb, the other one serving as a control. The Teflon coating was removed from both ends of the wires over a length of 0.5 mm. A fine silk thread was used to secure the wires, through small loops, to the lateral head of the gastrocnemius muscle. The wires were led under the skin and externalised at the neck of the animal, where the ends were attached to two small hooks and sewn to the skin. The animals were left to recover and stimulated daily via the implanted electrodes at 10 Hz for 30 minutes in each hour for 6–8 hours each day, or for one minute every second minute for a similar period of time. No differences were noted between these regimes of stimulation. The stimulation parameters were such that not only contraction of the TA muscle could be felt, but flexion of both the ankle and the digits could be clearly observed.

Five dystrophic animals implanted with electrodes were left unstimulated for control experiments (DIC). Thirty one dystrophic mice were subjected to the stimulation treatment for periods of time ranging from five to 21 days (DS). In 19 of these mice, the force output, contractile properties, weight and fatigue characteristics of both the operated and contralateral TA and EDL muscles were examined upon termination of the stimulation treatment (Short term). In 12 animals, a "rest" period of four weeks was allowed between the last day of chronic electrical stimulation and the final experiment (Long term). At various intervals after the initial operations, the animals were anaesthetised with chloral hydrate (as above). The tendons of the Tibialis anterior (TA) and Extensor digitorum longus (EDL) muscles were freed and attached to fine silk threads. The sciatic nerve was dissected and cut centrally. Small rigid pins were put through the proximal and distal ends of the tibia and the legs were then secured to a rigid table. Contractions were elicited by stimulating the sciatic nerve with supramaximal stimuli. The tendons were attached to strain gauges to record isometric contractions. These were displayed on an oscilloscope screen, from which they were photographed, as well as on a Devices pen recorder. To test fatigability, the muscles (TA only) were stimulated by trains of 10 pulses at 40 Hz, repeated every second for three minutes. The changes in tension were expressed as the ratio of the tension developed after three minutes to the initial tension × 100. This is referred to as the Fatigue Index (FI). When the recordings were completed, the muscles were excised and weighed, then quickly frozen in isopentane precooled in liquid nitrogen.

2) Statistical analysis

Comparison between the values of the different parameters studied obtained from the contralateral untreated muscles in the different experimental groups was made using a one way analysis of variance (ANOVA). If significance was established (P < 0.05, 2 tails) the values were further compared using the Newmann-Keuls multiple comparison test. Comparison between the values obtained from the operated muscles and their respective unoperated contralateral counterparts within each experimental group studied was made using a paired Student t-test (2 tails). In some instances, the raw data obtained from the operated muscles in the different experimental groups were directly compared using a one way analysis of variance. If necessary, the Newmann-Keuls multiple comparison test was used.

Results

The values of the different parameters studied obtained from the untreated contralateral TA and EDL muscles in the various groups of stimulated mice (that is, short term and long term) are summarised in table 1, where they are compared to those obtained from the corresponding unoperated contralateral muscles of implanted control (dystrophic) mice. Both in the stimulated and implanted control animals, the force output and weight of the untreated contralateral TA and EDL muscles were within the same range. These unoperated contralateral muscles will therefore be used, within each animal, to assess the changes induced by the treatment applied to their operated counterparts (that is, electrical stimulation or electrode implantation).

For other parameters, such as the time course of contraction (TTP) and relaxation (1/2 Relax), and the Fatigue Index (FI), however, some differences were observed. For instance, the TTP and half relaxation of the contralateral (untreated) EDL muscle in the "short term stimulated mice" were found to be significantly different (P < 0.05, both cases) from those of the contralateral untreated EDL muscle in the implanted mice. A similar trend could be observed with respect to the TA muscle, but the differences were not significant. The FI of the contralateral TA muscle in the short term stimulated mice, on the other hand, was significantly different (P < 0.05) from that of the corresponding TA muscle in the implanted control animals. Interestingly, it was found that in muscles contralateral to the "long term stimulated" ones, the values of these parameters were not significantly different from those of the "implanted control"
between the muscles. The smaller the initial force output of the stimulated TA muscle within each animal studied was first examined as a function of the length of time during which stimulation was applied (fig 1a). It was found that there was no correlation between the changes induced by chronic electrical stimulation and its duration. However, when the changes in force output seen in the stimulated TA muscles were examined as a function of the initial force developed by the muscles (taken to be that of the contralateral control muscle within each individual animal studied), a much clearer pattern emerged (fig 1b). The smaller the initial force output of the dystrophic muscle, the more likely it was to respond favourably to electrical stimulation. On the other hand, those muscles which initially developed relatively large forces appeared to be adversely affected by the stimulation treatment. The degree of association between both variables in this relationship (that is, % change and initial force output) was tested using the Pearson’s test. The correlation factor between the two variables was found to be significant

categorical properties, force output and fatigue characteristics of the untreated TA and EDL muscles in dystrophic
mice from the different experimental groups studied

<table>
<thead>
<tr>
<th>Gp</th>
<th>n</th>
<th>Twitch tension (g)</th>
<th>TTP (ms)</th>
<th>1/2 Relax tension (g)</th>
<th>Tetanic tension (g)</th>
<th>Muscle weight (mg)</th>
<th>Fl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIC</td>
<td>5</td>
<td>14-30 (2-60)</td>
<td>35-60 (4-02)</td>
<td>54-60 (9-47)</td>
<td>46-20 (6-39)</td>
<td>28-44 (1-67)</td>
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<tr>
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<td>19</td>
<td>16-93 (1-26)</td>
<td>30-05 (1-22)</td>
<td>41-68 (3-60)</td>
<td>59-95 (4-06)</td>
<td>31-73 (1-15)</td>
<td>50-98 (4-97)†</td>
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<td>Gp1</td>
<td>7</td>
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<td>31-71 (2-33)</td>
<td>42-29 (4-69)</td>
<td>46-14 (1-79)</td>
<td>29-46 (2-32)</td>
<td>57-67 (9-17)</td>
</tr>
<tr>
<td>TA muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gp2</td>
<td>8</td>
<td>16-64 (1-03)</td>
<td>30-63 (1-72)</td>
<td>46-75 (6-59)</td>
<td>57 (1-56)</td>
<td>32-53 (1-63)</td>
<td>45-88 (7-99)</td>
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<td>4</td>
<td>22-41 (3-65)</td>
<td>26 (1-41)</td>
<td>30-50 (3-56)</td>
<td>90 (5-40)</td>
<td>34-10 (0-92)</td>
<td>51-15 (9-01)</td>
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<tr>
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<td>32-25 (1-46)</td>
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<td>51-25 (1-97)</td>
<td>32-43 (1-23)</td>
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<td>46-86 (9-67)</td>
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<tr>
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<td>31-40 (9-98)</td>
<td>48-20 (7-17)</td>
<td>58-10 (1-89)</td>
<td>33-42 (2-25)</td>
<td>36-10 (9-35)</td>
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</table>

Twitch tension. Time to peak (TTP). Half relaxation (1/2 Relax). Tetanic tension, Weight and Fatigue Index (Fl) of the untreated contralateral TA and EDL muscles, respectively, from Dystrophic implanted control (DIC) and Dystrophic Stimulated (DS) Short term (St) and Long term (Lt) mice. Values are given as mean SE (mean). Anova: (*) P < 0.05; (***) P < 0.005. Newman-Keuls multiple comparison test: † different from DIC, P < 0.05; (‡) difference between DS(S)t and DS(L)t, P = 0.05. Also summarised, but not included in the comparison, are the values of the parameters studied in the contralateral TA and EDL muscles for each of the subgroups defined in the text.

1) Short term effect of chronic electrical stimulation
a) TA muscle The changes (% in maximum force output of the stimulated TA muscle within each animal studied were first examined as a function of the length of time during which stimulation was applied (fig 1a). It was found that there was no correlation between the changes induced by chronic electrical stimulation and its duration. However, when the changes in force output seen in the stimulated TA muscles were examined as a function of the initial force developed by the muscles (taken to be that of the contralateral control muscle within each individual animal studied), a much clearer pattern emerged (fig 1b). The smaller the initial force output of the dystrophic muscle, the more likely it was to respond favourably to electrical stimulation. On the other hand, those muscles which initially developed relatively large forces appeared to be adversely affected by the stimulation treatment. The degree of association between both variables in this relationship (that is, % change and initial force output) was tested using the Pearson’s test. The correlation factor between the two variables was found to be significant.

Fig 1 Changes (%) in force output of stimulated dystrophic TA muscles expressed (a) as a function of the length of time (days) during which stimulation was applied; (b) as a function of the maximum force (g) initially developed by the muscles. Each point represents an individual experiment.
Long term effect of low frequency chronic electrical stimulation

(P < 0.02). The animals were divided into three groups according to the maximum force their TA muscle (that is, their contralateral TA muscle) initially developed. In Gp1, animals were selected whose TA muscles developed forces below the mean (range 35 to 50 g) tetanic tension produced by all the 19 TA muscles. Gp2 comprised animals whose TA muscles developed maximum forces within this average (range 51 to 75 g), and Gp3 comprised animals whose TA muscles produced relatively large forces in comparison with this average (range 76 to 105 g). When this arbitrary grouping was performed, it was found that the weakest muscles (Gp1) were also the slowest, while the relatively "strong" dystrophic TA muscles (Gp3) were associated with a relatively faster speed of contraction and relaxation (table 1).

We found that, indeed, the stimulation treatment led to a significant increase (P < 0.05) in the force output of the TA muscles from Gp1, that is, the weakest muscles (fig 2a). On the other hand, the stimulation did not affect the force output of the muscles from Gp2, but it produced a small (17%) decrease in the force output of those dystrophic muscles which initially developed large forces (Gp3). This difference was not significant. The implantation of electrodes, per se, had no effect on the force output of the treated muscles, or on any of the parameters studied. There were no differences in weight between operated and contralateral muscles within any of the groups of stimulated animals. There were no differences in contractile speed and FI between operated and contralateral muscles within each of these groups. Since stimulation might have a slight effect contralaterally with respect to the implanted muscles, a direct comparison of the stimulated muscles from each of these groups with the implanted control muscles was made (see Methods). It could then be seen that the contractile speed, particularly the time course of relaxation, became slightly, but not significantly, faster in the stimulated muscles. Similarly, there was a tendency for their fatigue resistance to be enhanced (fig 2b).

b) EDL muscle The changes observed in the force output of dystrophic EDL muscles following chronic electrical stimulation showed a similar pattern to that seen in dystrophic TA muscles. There was no correlation between the changes in force output induced by the stimulation and the length of time during which the stimulation was applied (fig 3a). There was a highly significant correlation (P < 0.005) between the changes in force output induced by the stimulation and the force developed by the untreated contralateral EDL muscle within each individual animal (fig 3b). The animals were divided into two groups according to whether the maximum force initially developed by the EDL muscle was below (Gp1) or above (Gp2) the population's average. Again, relative differences in the contractile speed of the muscles in each of these subgroups could be seen (table 1). It should be noted that these differences were also noticeable in the corresponding subgroups from the "long term" stimulated muscles. It was found that the stimulation treatment led to a significant improvement in the force output of the weakest muscles (P < 0.02), while it produced a small, but significant (P < 0.01) decrease in the force output of the "strongest" dystrophic muscles (fig 4a). As for TA muscle, these changes in force output were not associated with any changes in weight. A direct comparison (fig 4b) of the data obtained from the stimulated EDL muscles in the various groups, with that obtained from the implanted...
control muscles revealed a significant decrease in the time course of contraction (ANOVA, P < 0.05) and relaxation (ANOVA, P < 0.02) in all stimulated muscles. While it might appear that these parameters were more affected in the stimulated muscles from the relatively "strong" dystrophic mice, it has to be remembered that these muscles were relatively faster to begin with (table 1). There were no differences in contractile speed between operated and contralateral muscles within each group of stimulated animals. The implantation of electrodes, per se, had no effect on any of the parameters studied in this muscle.

These results indicated that chronic electrical stimulation at low frequency brings about changes in dystrophic muscles. The question was whether these changes were permanent.

2) Long term effect of electrical stimulation

This question was addressed by studying TA and EDL muscles which had been subjected to the same stimulation treatment, then left unstimulated for a period of four weeks before the final experiment.

a) TA muscle

It was found that four weeks after the

![Graph](image-url)
End of the stimulation treatment, the maximum force developed by the treated muscles from Gp1 was similar to that of their contralateral control muscles, that is, that the increase in force output that had presumably occurred was no longer apparent (fig 2). As in the previous series of experiments, the maximum force developed by the stimulated TA muscles from Gp2 was similar to that developed by their contralateral control muscles after this period of "rest". As we were not able to predict the total amount of force a dystrophic muscle would develop from the clinical examination of the sick animal, there is no data available corresponding to Gp3. We do not know, therefore, whether the slight decrease in force output that had been seen in the relatively strong dystrophic muscles following stimulation would also be observed under these particular conditions. Fig 2b shows that the values of both the 1/2 Relaxation time and the FI in the long term stimulated TA muscles were similar to those obtained from the implanted control muscles. Thus, the small changes seen in the values of these properties in the short term stimulated dystrophic TA muscles were no longer apparent under these particular conditions.

b) EDL muscle After a similar period of "rest", the stimulated EDL muscles from the weakest group of animals (Gp1) developed significantly greater force (P < 0.05) than that of their untreated contralateral muscles. In contrast, the stimulated muscles from the relatively "strong" dystrophic animals (Gp2) developed slightly, but significantly (P = 0.05), smaller force output than their contralateral control muscles (fig 4a). In both groups, the contractile speed of the long term stimulated EDL muscles differed from that of implanted control muscles (fig 4b). The difference was significant on the time course of relaxation (Newmann-Keuls test; P < 0.05 and P < 0.025, respectively), but not on the time course of contraction. These values were not significantly different from those of the short term stimulated muscles examined previously. However, a tendency towards a return to "normality" could be seen.

Discussion

Our results indicate that chronic electrical stimulation at low frequency (10 Hz) brings about an increase in the force output of the weakest TA and EDL muscles from the dystrophic mice studied, while it induces a slight, but significant, decrease in the force output of relatively strong dystrophic muscles. They also indicate that the percentage change in force output observed in the dystrophic stimulated muscles bears no relationship to the duration of the stimulation. Finally, our results show that the stimulation has no effect on the weight of these muscles, while it increases their contractile speed and their resistance to fatigue independently of whether or not their force output improves.

That such stimulation can improve the performance of dystrophic muscles agrees with reports in the literature on a similar preparation15 or on children suffering from DMD.14 The lack of correlation between the extent of these changes and the length of time during which stimulation was applied differs from the finding of Scott et al44 who reported that in young children suffering from DMD, the stimulation treatment had to be applied for at least six weeks before any effect could be seen. Our finding, however, is similar to that of Luthert et al41 who suggested that this lack of correlation could be because the mice used in their study were not a homogeneous group. This argument may also apply to our study, since the age of the animals used ranged from three to six months, the severity of their affliction was variable and the range of tension developed by the control, unstimulated TA and EDL muscles of individual animals in our study was even greater than in their study. The lack of effect of the stimulation on the weight of dystrophic muscles is similar to that reported by Luthert et al40 on a similar preparation. They suggested that this observation could simply be explained in view of the large amount of connective tissue and degenerating fibres present in the diseased muscles.16 The speeding of the time course of contraction and relaxation of the muscles following stimulation agrees with that reported for the dystrophic mouse. A similar trend was observed for the time course of relaxation of the stimulated TA muscle of children suffering from DMD. The increase in the fatigue resistance of the dystrophic TA muscle following stimulation was similar to that previously reported for the EDL muscle in dystrophic mice.15 Such an increase in fatigue resistance was not seen in the stimulated TA muscle of children suffering from DMD.14 Unlike muscle from adult dystrophic mice, however, the muscles of normal children or from children suffering from DMD already have a great ability to maintain tension.13,14,15

While our results confirm the relationship between the initial weakness of the dystrophic muscle and its likeliness to respond favourably to stimulation, first emphasised by Vrbouva and Ward,13 they also suggest that the performance of relatively strong dystrophic muscles might be unfavourably influenced by the stimulation treatment. This is important because, according to these authors, the TA and the EDL muscles in dystrophic mice do not deteriorate at the same rates. Thus, it is possible that while the stimulation could be beneficial to one muscle, it could be detrimental to the other one. In our study, seven out of 19 animals had a TA muscle whose force output, initially below the average tension developed by all
muscles studied in that group, improved following stimulation. Only two of these animals also had a weak EDL muscle whose performance improved following stimulation, and five of these animals had relatively strong EDL muscles whose force output decreased slightly following the treatment. This decrease in force output was, however, small and since it was not accompanied by a proportional decrease in muscle weight, it is possible that it is due to the replacement of contractile proteins by mitochondria, as suggested by the increased resistance to fatigue of these muscles. This property is known to reflect the oxidative capacity, that is, the mitochondrial volume of the muscle fibres. In view of the poor performance dystrophic muscles already have, compared with normal muscles, this small decrease in force output should perhaps be considered. It should be noted that our data also indicate that in most of the dystrophic mice studied, the TA muscle was more severely affected than the EDL muscle. From our results, it also appears that the contractile speed of the dystrophic EDL muscle is more readily affected by the stimulation than that of the dystrophic TA muscle. Finally, our results show that there is a marked contralateral effect of the stimulation on both the contractile speed and the fatigue resistance of the muscles. The extent of the changes induced in the untreated contralateral muscles are similar to those induced in the stimulated muscles. Such an effect of the stimulation, applied to the nerve via implanted electrodes, as carried out in the present experiments, has not been reported previously, at least in dystrophic animals. The stimulated animals did not appear to use their contralateral limb preferentially during the stimulation periods and thus, it is possible that this contralateral effect of the stimulation is related to some cross-reflex activity.

Four weeks after the end of the stimulation treatment, the changes that had been seen in the stimulated dystrophic TA muscle were no longer apparent. These changes, therefore, are not permanent. Interestingly, the response of the dystrophic EDL muscle was slightly different from that of the TA muscle as after a similar period of “rest”, the changes in force output and contractile properties seen in the stimulated EDL muscles of both subgroups are still present. We realise that subgroup one contains only two samples. However, we feel that the difference in force output between operated and untreated contralateral control muscles is large enough to be reliable. Our data suggest that while outlasting the changes induced by the stimulation in the dystrophic TA muscle by at least four weeks, the changes induced in the dystrophic EDL muscle are unlikely to be permanent.

The TA muscle deteriorates more severely than the EDL muscle (and present study) and the changes it undergoes following stimulation disappear more quickly than those induced in the EDL muscle. This is interesting since both muscles have a similar function and fibre type composition. Their anatomical position, however, is different as the TA muscle covers only one joint (ankle) while the EDL muscle covers both the knee and the ankle joints. Since in these dystrophic mice the ankle joint is incapable of dorsiflexion while the knee retains some flexibility, the TA muscle may be subjected to more stretch than the EDL muscle. Thus, it is possible that mechanical stretch might contribute to the deterioration of the diseased muscles and might also influence the response of the muscle to stimulation.

It is known that low frequency electrical stimulation can induce the changes normally undergone by mammalian skeletal muscle during postnatal development, in particular, the increase in the time course of contraction and relaxation that has been shown to occur in developing fast skeletal muscles. The dystrophic muscles whose force output improved following stimulation, that is, the weakest muscles, have a relatively slower speed of contraction and relaxation. It is known that, at least during the early stages of the disease, the sick muscles contain regenerating muscle fibres. The latter, like immature muscle fibres contract and relax slowly. Thus, the slower contractile speed of the weakest dystrophic muscles reflects the presence of these fibres. It is possible that stimulation will induce not only the growth of these fibres, but also their differentiation. This would account for both the increase in force output and for the changes in contractile properties seen in these muscles in this study and for the increase in the number of fibres previously reported to occur in these muscles. The relatively faster contractile speed of the stronger dystrophic muscles suggests that they might not contain such fibres and it is possible that in these muscles, the stimulation acts on the remaining relatively healthy fibres. This, however, seems unlikely since electrical stimulation of a similar kind applied to normal fast muscles from adult rabbits or rats does not lead to such changes in contractile speed. On the contrary, low frequency electrical stimulation of normal adult fast muscles produces a slowing of the time course of contraction and relaxation which results from the transformation of fast muscle fibres into fibres with morphological, biochemical and histochiochemical properties similar to those of slow muscle fibres. Thus, in these muscles too it is likely that the stimulation acts on a population of regenerating or immature muscle fibres.

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Long term effect of low frequency chronic electrical stimulation

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J Dangain and G Vrbova

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