Treatment with an antibody directed against Nogo-A delays disease progression in the SOD1\textsuperscript{G93A} mouse model of Amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal, neurodegenerative disorder in which motor neurons in the spinal cord and motor cortex degenerate. Although the majority of ALS cases are sporadic, mutations in Cu–Zn superoxide dismutase-1 (SOD1) are causative for 10–20\% of familial ALS (fALS), and recent findings show that a hexanucleotide repeat expansion in the C9ORF72 gene may account for >30\% of fALS cases in Europe. SOD1\textsuperscript{G93A} transgenic mice have a phenotype and pathology similar to human ALS. In both ALS patients and SOD1\textsuperscript{G93A} mice, the first pathological features of disease manifest at the neuromuscular junction, where significant denervation occurs prior to motor neuron degeneration. Strategies aimed at preventing or delaying denervation may therefore be of benefit in ALS. In this study, we show that Nogo-A levels increase in muscle fibres of SOD1\textsuperscript{G93A} mice along with the elevation of markers of neuromuscular dysfunction (CHRNA1/MUSK). Symptomatic treatment of SOD1\textsuperscript{G93A} mice from 70 days of age with an anti-Nogo-A antibody (GSK577548) significantly improves hindlimb muscle innervation at 90 days, a late symptomatic stage of disease, resulting in increased muscle force and motor unit survival and a significant increase in motor neuron survival. However, not all aspects of this improvement in anti-Nogo-A antibody-treated SOD1\textsuperscript{G93A} mice were maintained at end-stage disease. These results show that treatment with anti-Nogo-A antibody significantly improves neuromuscular function in the SOD1\textsuperscript{G93A} mouse model of ALS, at least during the earlier stages of disease and suggest that pharmacological inhibition of Nogo-A may be a disease-modifying approach in ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder in which motor neurons degenerate, resulting in progressive muscle paralysis and death, typically within 2–5 years of diagnosis. Although ALS is predominantly a sporadic disease, ~10\% of cases are familial ALS (fALS) and until recently, mutations in the superoxide dismutase (SOD1) gene were thought to be the most common cause of fALS, accounting for 10–20\% of cases (for review 1). However, a hexanucleotide repeat expansion in the C9ORF72 gene has recently been identified in more than half of fALS cases in Finland and may indeed account for >30\% of all fALS cases in Europe (2).

However, despite such advances in our understanding of the genetic causes of ALS, there is still neither a cure nor effective treatment for this devastating disease (3). Thus, Riluzole, which has limited therapeutic benefits and only modest effects on survival, remains the only licenced drug for ALS (4,5). Therefore, the identification of an effective disease-modifying therapy for ALS remains an imperative.

The SOD1\textsuperscript{G93A} mouse model of ALS has been widely used to investigate the pathological mechanisms of disease and to also undertake pre-clinical experiments assessing the effectiveness of a variety of therapeutic agents (for review 6). Studies in SOD1\textsuperscript{G93A} mice have revealed a number of pathological processes that contribute to the disease including deficits in...
axonal transport, excitotoxicity and perturbed protein metabolism due to proteasome dysfunction (3,7,8). Furthermore, it has also been established that ALS is a non-cell autonomous disease in which a number of cell types play an important role in disease progression, including not only motor neurons but also astroglia and microglia within the CNS (9), for review see 10. Furthermore, events in the periphery, at the neuromuscular junction (NMJ), also play a significant role in disease progression. Indeed, the very earliest manifestation of disease in both the SOD1G93A mouse model and ALS patients occur at the NMJ, where significant levels of denervation can be observed long before the onset of motor neuron degeneration (11,12). It is therefore possible that strategies to maintain innervation of skeletal muscles in order to retain target-derived trophic support may be a particularly effective approach to delay disease progression and prolong survival in ALS.

Neurite outgrowth inhibitor or Nogo, also known as Reticulon-4, was first identified as a myelin-associated neurite outgrowth inhibitor in the central nervous system (13-15). Alternatively spliced transcript variants encoding at least five different isoforms have now been identified, the major isoforms being Nogo-A, -B and -C. Nogo-A has two known inhibitory domains, one of which, the N-terminus, is specific to Nogo-A. The other domain, a 66 amino acid loop is shared by all three isoforms (Nogo-66) (16). Several experimental approaches have been used to investigate the effects of targeting Nogo-A to promote axonal regeneration following spinal cord injury (17,18). In 2002, analysis of mouse Nogo isoform expression in SOD1G86R transgenic mice found increased levels of Nogo-A and decreased Nogo-C at early asymptomatic stages (19). The characteristic altered expression of Nogo isoforms was confirmed in both post-mortem and biopsy samples from diagnosed sporadic and familial ALS patients, suggesting for the first time that Nogo may play a central role in ALS pathogenesis. Furthermore, genetic ablation of Nogo-A has been shown to reduce muscle denervation and extend survival of SOD1 mice. Conversely, ectopic muscle Nogo-A overexpression leads to loss of neuromuscular contacts in wild-type (WT) muscle, suggesting that Nogo-A may play a role in destabilization of the NMJ and subsequent dying back of motor axons in ALS (20). These findings led to the suggestion that muscle Nogo-A expression could serve as a diagnostic marker, particularly during the early stages of the disease (15).

Irrespective of whether Nogo-A is a specific marker of ALS, it clearly plays a role in the loss of muscle innervation, the earliest pathological manifestation of ALS. Thus, strategies that aim to prevent or reduce Nogo-A expression may be effective in maintaining muscle innervation, thereby delaying progression of disease symptoms. Various strategies have previously been employed to test the effects of inhibition of Nogo-A, particularly in the context of developing new treatment strategies for spinal cord injury that aim to enhance plasticity and neurite growth, by blocking Nogo-A or its downstream effectors. For example, genetic ablation of the Nogo receptor (Nogo-R), through which Nogo-66 exerts its effects, has been shown to result in improved axonal regeneration following injury to the optic nerve in Nogo-R knockout mice (21). Alternatively, inhibition of Nogo-A with a function-blocking antibody has been shown to stimulate axonal sprouting and improve the functional outcome following spinal cord injury in monkeys (22). Furthermore, Nogo-A blocking antibodies have been shown to improve function and recovery in models of stroke in the rat (23). A Phase I clinical trial applying anti-Nogo-A antibody to subjects with acute spinal cord injury has been successfully completed (24).

In view of the established role of Nogo-A in muscle denervation in ALS, together with the beneficial effects of neutralization of Nogo-A following CNS injuries, Ozanezumab, a humanized monoclonal antibody directed against Nogo-A, is being developed for the treatment of ALS and has just completed a phase I clinical study in ALS subjects (submitted). In this study, we report the results of blockade of Nogo-A activity on disease phenotype and progression in the SOD1G93A mouse model of ALS. SOD1G93A transgenic mice were treated with a murine antibody directed against Nogo-A (GSK577548: the murine parental antibody for Ozanezumab), from an early symptomatic stage of the disease, when a proportion of the muscle fibres are already denervated and which may represent a similar stage to that at which the large majority of ALS patients first present to their physician.

RESULTS

Elevation of Nogo-A levels in muscle fibres of treated SOD1G93A mice

The level of Nogo-A expression in the gastrocnemius muscle of WT and SOD1G93A mice was assessed at both the RNA and protein levels (see Fig. 1). Using immunohistochemistry, we were able to show that Nogo-A protein levels were low but detectable in the muscle fibres of WT animals at both the 90 and 120 day time points (Fig. 1A). In contrast, elevated levels of Nogo-A were detected in the muscle fibres of the SOD1G93A mice in both the vehicle- (PBS) and anti-Nogo-A-treated groups at both time points. Quantifying these results using laser scanning cytometry confirmed the elevated expression of Nogo-A in the SOD1G93A mice when compared with WT animals, and showed marginally lower Nogo-A levels in the anti-Nogo-A-treated animals at 90 days, although no difference in expression was seen at 120 days (Fig. 1B). By staining sections with a specific anti-idiotypic antibody to GSK577548, we were able to confirm the presence of the drug between the muscle fibres in the tissue, and confirm co-localization of drug with Nogo-A (Fig. 1C).

Consistent results were observed with the RNA levels of Nogo-A and markers of neuromuscular dysfunction (Fig. 1D). Nogo-A RNA levels were 5-fold higher in the muscles of SOD1G93A mice than their WT littermates at 90 days, and this increased further to a 9-fold difference at 120 days. Similar increases were observed for markers of neuromuscular dysfunction (CHRNA1 and MUSK), with increasing expression noted up to 120 days. While similar accumulations of Nogo-A and neuromuscular markers were seen in the anti-Nogo-A-treated group, the magnitude of the accumulation was smaller at the 90 day time point, although this effect was lost by 120 days. Significantly reduced accumulation of Nogo-A and CHRNA1 RNA was detected at 90 days in the anti-Nogo-A-treated group when compared with WT littermates.

Treatment with an anti-Nogo-A antibody improves muscle innervation in SOD1G93A mice

The extent of innervation of the slow-twitch soleus muscles and the fast-twitch extensor digitorum longus (EDL) muscles of
SOD1\textsuperscript{G93A} and WT littermates treated with anti-Nogo or vehicle was established at 90 days of age, a late stage of disease progression. Figure 2 shows examples of EDL muscle sections from WT (Fig. 2A), vehicle-treated SOD1\textsuperscript{G93A} (Fig. 2B) and antibody-treated SOD1\textsuperscript{G93A} mice (Fig. 2C), stained for silver cholinesterase. In WT mice, the majority of endplates were innervated and stained intensely for acetylcholinesterase (AChE) (Fig. 2A). In contrast, in SOD1\textsuperscript{G93A} mice, many endplates were denervated and stained weakly for AChE, indicating a reduction in the level of this activity-dependent enzyme (Fig. 2B). There is a clear improvement in the level of innervation in antibody-treated SOD1\textsuperscript{G93A} mice (Fig. 2C) The extent of denervation of each muscle was calculated by expressing the number of denervated endplates as a percentage of the total number of endplates counted per muscle (>200 endplates were examined per muscle). The results are summarized in Figure 2D for EDL and in Figure 2E for soleus muscles of vehicle and anti-Nogo-A antibody-treated 90 day old WT and SOD1\textsuperscript{G93A} mice.

The number of denervated endplates in muscles of WT mice treated with the anti-Nogo-A antibody was similar to that in vehicle-treated muscles. Thus, 11.16 ± 1.06% of endplates were denervated in anti-Nogo-A-treated WT EDL compared with 8.65 ± 1.89% in vehicle-treated WT EDL muscles ($P = 0.33$; Fig. 2D). In contrast, in vehicle-treated SOD1\textsuperscript{G93A} mice, 28.47 ± 1.36% of endplates were already denervated by 90 days. However, in SOD1\textsuperscript{G93A} mice treated with anti-Nogo-A, there was a significant improvement in innervation, and only 18.24 ± 1.75% of endplates were denervated ($P = 0.0025$).

In the soleus muscles of WT mice, the number of denervated muscle fibres was also similar in vehicle and anti-Nogo-A antibody-treated mice and at 90 days of age, 8.19 ± 0.54% of endplates were denervated in soleus muscles of anti-Nogo-A-treated WT mice and 8.99 ± 3.38% in vehicle-treated WT mice ($P = 0.84$; Fig. 2E). In soleus muscles of SOD1\textsuperscript{G93A} mice, although significantly more muscle fibres were denervated compared with WT mice ($P = 0.03$), the extent of denervation was not as great as that observed in EDL muscles of SOD1\textsuperscript{G93A} mice and the beneficial effects of treatment with the Nogo-A antibody were not as dramatic. Thus, 23.31 ± 3.11 and 18.41 ± 2.19% of endplates were denervated in soleus muscles of SOD1\textsuperscript{G93A} mice treated with vehicle and anti-Nogo-A antibody, respectively. Thus, unlike EDL, treatment with the anti-Nogo-A antibody did not result in a significant improvement in innervation of soleus muscles of 90 day old SOD1\textsuperscript{G93A} mice, possibly because the level of denervation in soleus muscles of vehicle-treated SOD1\textsuperscript{G93A} mice at this stage is not as advanced as that in EDL muscles.

**Treatment with an anti-Nogo-A antibody improves muscle force and motor unit survival in 90 day SOD1\textsuperscript{G93A} mice**

**Muscle force**

In order to establish whether the improvement in muscle innervation observed in 90 day old SOD1\textsuperscript{G93A} mice treated with an anti-Nogo-A antibody was reflected in a corresponding improvement in neuromuscular function, we next examined...
muscle force and motor unit survival. Following weekly treatment with either vehicle (saline) or anti-Nogo-A from 70 days of age, 90 day old WT and SOD1G93A mice were prepared for in vivo physiological analysis of isometric force of tibialis anterior (TA) and EDL muscles. Examples of typical traces of TA twitch and tetanic force of vehicle-treated WT, vehicle-treated SOD1G93A and antibody-treated SOD1G93A mice are shown in Figure 3A–C, respectively. Treatment with either vehicle or the anti-Nogo-A antibody (data not shown) had no effect on the maximum force produced by TA and EDL muscles in WT mice. However, as can be seen in Figure 3D, in 90 day old SOD1G93A mice, there was a significant decrease in maximum tetanic force in the TA muscles. Thus, in vehicle-treated WT mice, the maximum tetanic force of TA muscles is 105.18 ± 3.81 g compared with only 44.44 ± 5.02 g in vehicle-treated SOD1G93A mice (P ≤ 0.001, Fig. 3D). However, in SOD1G93A mice treated with the anti-Nogo-A antibody, TA was significantly stronger than in vehicle-treated SOD1G93A mice, and the maximum tetanic force was 72.22 ± 3.45 g, which represents a 26% increase in muscle force (P = 0.005).

Similarly, in EDL muscles, both vehicle- and antibody-treated SOD1G93A mice showed a significant decrease in tetanic force compared with vehicle-treated WT mice (Fig. 3E). However, in anti-Nogo-A antibody-treated SOD1G93A mice, EDL produced a maximum tetanic force of 19.84 ± 1.71 g, which is significantly more than in vehicle-treated mice, in which EDL produced 15.15 ± 1.37 g. Treatment of SOD1G93A mice with the anti-Nogo-A antibody therefore resulted in a 20% increase in EDL muscle force compared with that of vehicle-treated SOD1G93A EDL (P = 0.014).

Thus, at 90 days of age, although there was a significant decrease in the force of TA and EDL muscles in SOD1G93A mice treated with either vehicle or anti-Nogo-A antibody compared with WT littermates, treatment with the antibody resulted in a significant improvement in muscle force in SOD1G93A mice at 90 days of age.

Motor unit survival
The number of functional motor units innervating the EDL muscle was established in mice of each experimental group at 90 days of age. Representative examples of motor unit traces

Figure 2. Treatment with an anti-Nogo-A antibody preserves hindlimb muscle innervation in SOD1G93A mice at 90 days of age. The photomicrographs show examples of EDL muscle sections stained with silver cholinesterase from (A) WT animals and (B) vehicle-treated SOD1G93A mice and (C) anti-Nogo-A antibody-treated SOD1G93A mice at 90 days of age. The number of endplates that were contacted by a nerve terminal was determined in muscles of each animal. The results are summarized in the bar charts which show the number of denervated endplates expressed as a percentage of the total number of endplates counted in (D) EDL and (E) soleus muscles of 90 day old mice. n = min 5 animals per group. Error bars = SEM **P < 0.01, scale bar = 10 μm.
from EDL muscles of WT and SOD1<sup>G93A</sup> mice are shown in Figure 4A for 90 day old animals and the mean motor unit survival in each experimental group is summarized in Figure 4B.

Treatment of WT mice with the anti-Nogo-A antibody had no effect on the number of motor units innervating EDL, and 35 ± 0.25 and 36 ± 0.37 motor units were recorded in vehicle and antibody-treated WT EDL muscles, respectively (P = 0.185). Motor unit survival in SOD1<sup>G93A</sup> mice was therefore compared with that in WT vehicle-treated mice. As can be seen in Figure 4B, in EDL muscles of vehicle-treated SOD1<sup>G93A</sup> mice, 22 ± 1.28 motor units survived at 90 days compared with 26 ± 1.33 motor units in SOD1<sup>G93A</sup> mice treated with the anti-Nogo-A antibody. Thus, there is a significant decrease in motor unit survival in EDL of both vehicle- and antibody-treated SOD1<sup>G93A</sup> mice. However, the loss of motor units is significantly greater in vehicle-treated mice, where 37% fewer motor units survive compared with WT, than in SOD1<sup>G93A</sup> mice treated with antibody, where 25.7% fewer motor units survive compared with WT. This represents a significant 11.4% improvement in motor unit survival in SOD1<sup>G93A</sup> mice treated with anti-Nogo-A antibody (P = 0.03).

**Treatment with an anti-Nogo-A antibody improves motor neuron survival in 90 day old SOD1<sup>G93A</sup> mice**

The lumbar spinal cord of each mouse was dissected and serial cross-sections (20 µm) were cut on a cryostat and processed for morphological analysis of motor neuron survival by staining with gallocyanin, a Nissl stain. Examples of cross-sections of lumbar spinal cords from WT and SOD1<sup>G93A</sup> mice treated with either vehicle or anti-Nogo-A antibody are shown in Figure 5A. Motor neurons within the sciatic motor pool within the ventral horn are shown in the high-power inserts. The mean motor neuron survival was established for each experimental group.
group and the results are summarized in Figure 5B. As previously reported (25), by 90 days of age, there is already a significant reduction in motor neuron survival in vehicle-treated SOD1G93A mice compared with WT control mice ($P \leq 0.001$). However, in 90 day old SOD1G93A mice treated with anti-Nogo-A antibody, there was a significant increase in motor neuron survival compared with vehicle-treated SOD1G93A mice, so that $329 \pm 8.28$ motor neurons survived in antibody-treated mice compared with $272 \pm 12$ ($P = 0.003$) in untreated mice. Thus, treatment of SOD1G93A mice with an anti-Nogo-A antibody results in a 21% increase in motor neuron survival in 90 day old mice, which closely reflects the improvement in motor unit survival observed in EDL muscles (Fig. 4B).

**Figure 5.** Treatment with anti-Nogo-A antibody improves motor neuron survival in 90 day old SOD1G93A mice. Examples of cross-sections of spinal cord of 90 day old vehicle-treated WT mice and SOD1G93A mice treated with vehicle or anti-Nogo-A antibody, showing motor neurons within the ventral horn. The location of the sciatic motor pool is shown within the dotted circles, which is enlarged within the magnified inserts. The number of motor neurons in the sciatic motor pool of each spinal cord was determined and the results are summarized in the bar chart. $n = 5$ per group. Error bars represent SEM. **$P < 0.01$, scale bars $= 200 \mu m$.**

Treatment with the anti-Nogo-A antibody prevents the transformation in the phenotype of fast-twitch muscles in 90 day old SOD1G93A mice

Previous studies have established that as a result of motor neuron degeneration in SOD1G93A mice, fast-twitch muscles such as TA and EDL undergo a dramatic transformation in their phenotype and exhibit both functional and molecular characteristics of slow-twitch muscles (25–27). This is likely to be the result of selective death of a sub-population of motor neurons that innervate fast-twitch type IIb muscle fibres and which are known to be particularly vulnerable to cell stress in ALS (28). In view of the improvement in motor unit and motor neuron survival observed in antibody-treated 90 day old SOD1G93A mice, we next examined whether the disease-related transformation in muscle phenotype in TA muscles of SOD1G93A mice was prevented. Thus, TA muscles were stained for the oxidative enzyme, succinate dehydrogenase (SDH) which is normally expressed at low levels in fast-twitch TA muscle fibres of WT mice, but which has been shown to increase in fast-twitch fibres during disease progression in SOD1G93A mice.

As can be seen in the representative sections shown in Figure 6, TA muscles from WT mice treated with either vehicle (Fig. 6A) or the anti-Nogo-A antibody (Fig. 6B), reveal a mosaic pattern of lightly and darkly stained muscles fibres, with the majority of fibres staining lightly for SDH, indicating a low oxidative capacity and the presence of few oxidative Type I muscle fibres. In contrast, in vehicle-treated SOD1G93A mice, even by 90 days, the phenotype of TA has begun to change, and a large number of fibres stain darkly for SDH, indicating an increase in oxidative capacity in these fibres (Fig. 6C). However, in TA muscles of antibody-treated SOD1G93A mice, far fewer muscle fibres stain intensely for SDH (Fig. 6D) and the overall pattern of SDH staining is very similar to that observed in WT TA muscles, with only a slight increase in the number of fibres staining darkly for SDH.
This histochemical evidence that treatment with the anti-Nogo-A antibody prevented the transformation in the phenotype of fast-twitch muscles was confirmed by the results from functional, physiological analysis of the contractile characteristics of TA and EDL. In SOD1G93A mice, fast-twitch muscles take longer to contract and relax than normal, as a result of the transformation in their muscle fibre phenotype. In TA muscles treated with anti-Nogo-A antibody, this decrease in the speed of contraction was prevented and even at late stage disease there was an improvement in the contractile characteristics of TA in antibody-treated SOD1G93A mice compared with vehicle-treated SOD1G93A mice. Thus, in 120 day old vehicle-treated SOD1G93A mice, the time taken to reach maximum twitch contraction (time to peak; TTP) in TA muscles (40.58 ± 2.32 ms) was significantly greater than in WT mice (30.82 ± 1.42 ms). In contrast in antibody-treated SOD1G93A mice, TA TTP (29.14 ± 1.52 ms) was significantly faster than in vehicle-treated SOD1G93A mice (P < 0.001). Likewise, the time taken to reach the half relaxation (1/2RT) in TA muscles of antibody-treated SOD1G93A mice (65.35 ± 5.16 ms) was significantly faster than in vehicle-treated SOD1G93A mice (106.24 ± 8.7 ms; P = 0.002), which in turn was significantly slower than 1/2RT in WT mice (62.57 ± 4.45 ms). At 90 days, although the changes in TA contractile characteristics in SOD1G93A mice are not so pronounced at this earlier stage of the disease, the 1/2RT of antibody-treated TA muscles of SOD1G93A mice (49.49 ± 2.14 ms) was still significantly faster than vehicle-treated TA (58.61 ± 5.15 ms; P = 0.026). Similar improvements were observed in the contractile characteristics of antibody-treated EDL muscles at both 90 and 120 days, so both TTP and 1/2RT were significantly faster than in vehicle-treated EDL: at 90 days in antibody-treated EDL, TTP: 25.58 ± 0.41 ms; 1/2RT: 49.87 ± 1.23 ms; in vehicle EDL, TTP: 30.13 ± 1.13 ms; 1/2RT: 61.53 ± 3.50 ms (P = 0.006 and 0.013, respectively); at 120 days in antibody-treated EDL, TTP: 31.24 ± 1.60 ms; 1/2RT: 62.56 ± 2.89 ms; in vehicle-treated EDL, TTP: 35.85 ± 1.51 ms; 1/2RT: 72.90 ± 5.08 ms.

The long-term effects of an anti-Nogo-A antibody on disease progression in SOD1G93A mice

Since treatment of SOD1G93A mice with an anti-Nogo-A antibody was found to significantly improve the neuromuscular phenotype of 90 day old mice, we next examined whether these beneficial effects were maintained in the long term, at 120 days of age, a time when vehicle-treated SOD1G93A mice were reaching end stage, by assessing muscle innervation, muscle force and motor unit survival.

Analysis of muscle innervation revealed that in EDL muscle of 120 day old WT mice, only 8.53 ± 0.43 and 8.94 ± 1.58% of endplates were denervated in vehicle and Nogo-A antibody-treated mice, respectively, which is not significantly different (P = 0.83) and is similar to the levels observed at 90 days of age (Fig. 7A). In contrast, by 120 days, denervation of EDL muscles in SOD1G93A mice were reaching end stage, by assessing muscle innervation, muscle force and motor unit survival.

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Figure 7. The effects of an anti-Nogo-A antibody on disease phenotype in 120 day old SOD1<sup>G93A</sup> mice. The long-term effects of treatment of SOD1<sup>G93A</sup> mice with the anti-Nogo-A antibody were established at 120 days of age by comparing WT and SOD1<sup>G93A</sup> mice treated with either vehicle or anti-Nogo-A antibody. The bar charts show the number of denervated endplates expressed as a percentage of the total number of endplates counted in (A) EDL and (B) soleus muscles of WT and SOD1<sup>G93A</sup> mice. The bar charts show the mean maximum tetanic force of (C) TA and (D) EDL muscles of vehicle and antibody-treated WT mice and SOD1<sup>G93A</sup> mice. (E) The mean number of motor units in the EDL muscle of WT mice and SOD1<sup>G93A</sup> mice is summarized. (F) The photomicrographs show examples of cross-sections of TA and EDL muscles of WT and SOD1<sup>G93A</sup> mice at 120 days of age, stained for SDH. (*P < 0.05) n = min 5 mice in each group, muscles from both legs were analysed. Error bars = SEM. Scale bar = 70 μm.
Nogo-A inhibition on muscle innervation were still apparent and even at this very late stage of disease only 28.18 ± 0.86% of endplates were denervated, which is significantly less than that in vehicle-treated mice. The exact Wilcoxon two-sample test shows Nogo-A significantly reduces denervation in EDL muscles from SOD1G93A animals by 120 days of age (P = 0.028).

As observed in EDL muscles, as disease progressed the level of denervation of soleus muscles of vehicle-treated SOD1G93A mice increased, and by 120 days, 36.40 ± 1.57% of endplates were denervated compared with only 7.19 ± 0.70% in vehicle-treated WT mice (P < 0.0001; Fig. 7B). Although there was an improvement in the level of innervation of soleus muscles in SOD1G93A mice treated with the anti-Nogo-A antibody, with 27.43 ± 3.41% of fibres denervated, this was not significantly different from that observed in vehicle-treated mice (P = 0.057; see Fig. 7B).

Despite the improvement in innervation observed in 120 day old SOD1G93A mice treated with the antibody, and in contrast to 90 days, this improved innervation was not reflected in an increase in muscle force. Thus, the maximum tetanic force produced by TA in anti-Nogo-A-treated SOD1G93A mice at 120 days was 20.56 ± 3.20 g, which is not significantly different from that in vehicle-treated SOD1G93A mice, which produced 18.06 ± 2.75 g (Fig. 7C) and represents a decrease in force of 80% and 82%, respectively, with WT mice. Similarly, the maximum tetanic force of EDL muscles in 120 day SOD1G93A mice was decreased compared with WT mice, by 80% to 10.45 ± 1.31 g in anti-Nogo-treated mice and by 82% to 11.15 ± 0.98 g in vehicle-treated mice. Thus, the reduction in force in antibody- and vehicle-treated SOD1G93A mice is not significantly different (Fig. 7D). Likewise, there was no significant improvement in motor unit survival observed in 120 day old SOD1G93A mice treated with the anti-Nogo antibody, where only 15 ± 0.7 motor units survived compared with 13 ± 1 motor units in vehicle-treated SOD1G93A mice (P = 0.248, Fig. 7E).

Finally, muscle histochemistry revealed that in contrast to 90 days of age, treatment with the anti-Nogo-A antibody did not prevent the transformation in fast-twitch TA muscle fibres from primarily glycolytic to oxidative fibres that is a characteristic feature of fast-twitch muscles in SOD1G93A mice. Thus, as can be seen in Figure 7F, in contrast to WT TA muscle, in SOD1G93A mice treated with either the antibody or vehicle, the majority of TA muscle fibres stain very darkly for SDH, with no obvious difference in the pattern of staining between the vehicle- and antibody-treated muscles, indicating a shift in the phenotype of these muscle fibres.

The changes in disease phenotype in TA muscles of antibody-treated SOD1 mice observed in this study was reflected in the muscle weight (see Fig. 8A). Thus, there was a significant reduction in TA weight in vehicle-treated SOD1G93A mice at 90 days, and this loss was ameliorated in antibody-treated SOD1G93A mice at this stage. However, by 120 days, the beneficial effects of the antibody are no longer apparent, and the reduction in TA weight in antibody- and vehicle-treated mice is the same. The improvement in TA weight observed at 90 days was not however reflected in a significant improvement in body weight of antibody-treated mice (see Fig. 8B) and at both 90 and 120 days of age, vehicle- and antibody-treated SOD1G93A mice had a similar body weight.

**DISCUSSION**

Our results show that compared with vehicle-treated SOD1G93A mice, early symptomatic treatment with anti-Nogo-A antibody from 70 days of age significantly improves muscle innervation, muscle function and phenotype and increases motor neuron survival in symptomatic mice SOD1G93A mice at 90 days of age. However, this improvement in muscle function and motor neuron survival is not maintained and by 120 days most of the beneficial functional effects of anti-Nogo-A treatment are no longer observed in SOD1G93A mice.

In this study, we were able to confirm the previously reported increases in Nogo-A mRNA and protein in muscle fibres of SOD1G93A mice. By staining sections from gastrocnemius muscle, we were able to show an increased expression of Nogo-A protein levels in muscle fibres in atrophic muscle fibres. Co-staining of muscle sections with an anti-idiotype antibody directed against the anti-Nogo-A antibody, showed that the drug was reaching the muscle with immunostaining observed between the muscle fibres. Quantification of the immunostaining showed that, although there was a trend towards lower Nogo-A protein levels in anti-Nogo-A-treated animals at the 90 day time point, this was not statistically significant and this modest effect was lost by the 120 day time point. Similarly, we were able to confirm that increases in expression of Nogo-A RNA were paralleled by increases in markers of muscle denervation/neuromuscular dysfunction such as the alpha subunit of the acetylcholine receptor (CHRNA1) and muscle-specific receptor.
tyrosine kinase (MuSK). Treatment with anti-Nogo-A reduced the accumulation of mRNAs for Nogo-A and CHRNA1/MuSK at the 90 day time point, although the effect was lost at the 120 day time point. These results are consistent with those previously reported for Nogo-A ablation in the SOD1<sup>G86R</sup> model (29).

Effects on Nogo-A protein levels were modest, but there was a trend towards lower expression in the anti-Nogo-A-treated SOD1<sup>G93A</sup> at the 90 day time point, but again this was lost at Day 120.

Treatment with anti-Nogo-A antibody results in an increase in muscle force in both TA and EDL muscles of 90 day old SOD1<sup>G93A</sup> mice. This improvement in muscle force is reflected in an increase in motor unit survival in EDL muscles of 90 day old anti-Nogo-A-treated SOD1<sup>G93A</sup> mice compared with their vehicle-treated SOD1<sup>G93A</sup> littermates. Moreover, this improvement in motor unit survival is, as would be expected, reflected in an increase in motor neuron survival in the ventral horn as assessed morphologically in 90 day old anti-Nogo-treated SOD1<sup>G93A</sup> mice compared with their vehicle-treated SOD1<sup>G93A</sup> littermates. Phenotypic changes that are known to take place in the fast-twitch muscles TA and EDL in SOD1<sup>G93A</sup> mice during disease progression occur to a much lesser extent in anti-Nogo-treated SOD1<sup>G93A</sup> mice at 90 days of age. Thus, histochemical analysis of the oxidative capacity of muscle fibres reveal that in TA muscles of vehicle-treated SOD1<sup>G93A</sup> mice, there is a remarkable increase in the number muscle fibres that stain darkly for the oxidative enzyme SDH, whereas in anti-Nogo-treated SOD1<sup>G93A</sup> mice, there appears to be only a slight increase in the proportion of darkly stained fibres compared with TA muscles of WT mice. Analysis of the contractile characteristics of TA and EDL muscles confirms that treatment of SOD1 mice with the anti-Nogo-A antibody prevents the change in the phenotype of these fast-twitch muscles to slowly contracting muscles that occurs in vehicle-treated SOD1 mice.

Treatment of SOD1<sup>G93A</sup> mice with anti-Nogo-A exerts a beneficial effect by maintaining muscle innervation of EDL muscles, as observed at 90 days of age. The improvement in innervation of EDL muscles in 90 day old SOD1<sup>G93A</sup> mice treated with anti-Nogo compared with vehicle-treated mice is even greater at later stages of disease. Thus, at 90 days, ~10% more endplates are innervated in anti-Nogo-A treated than saline-treated SOD1<sup>G93A</sup> mice and this increases to 19% by 120 days of age. This increase in EDL innervation is likely to be due to a sprouting effect. It has been shown that compensatory, regenerative sprouting occurs when muscle innervation is lost during disease progression in SOD1<sup>G93A</sup> mice (12). In addition, pharmacological inhibition of Nogo A promotes terminal axon sprouting (29). Our results suggest that sprouting is increased in anti-Nogo-A-treated SOD1<sup>G93A</sup> mice compared with vehicle-treated mice. Indeed, even although no difference in motor neuron and motor unit survival could be observed at 120 days between experimental SOD1<sup>G93A</sup> groups, the number of innervated endplates was greater in EDL muscle of anti-Nogo-treated mice.

In the soleus muscle, an improvement in muscle innervation in anti-Nogo-A-treated SOD1<sup>G93A</sup> mice was observed at 120 days of age, although it did not reach statistical significance. Different types of muscles have been shown to exhibit a differential vulnerability to ALS pathology (11,30). Thus, EDL and TA are fast-twitch, fatigable muscles, which have been shown to be more vulnerable to ALS pathology compared with slow-twitch, fatigue resistant muscles such as the soleus, which is considered to be largely resistant to SOD1-induced toxicity, particularly during the earlier stages of disease progression (30). Previous studies in our lab and elsewhere have shown that fast-twitch muscles such as EDL start to show signs of denervation as early as 45–50 days (11,12,30), whereas slow-twitch soleus only shows signs of significant denervation in SOD1<sup>G93A</sup> animals from 90 days of age (12). Considering the relatively low level of denervation in soleus muscle at 90 days, it is therefore not surprising that in the present study, the beneficial effects of anti-Nogo-A on muscle innervation in soleus muscles only become significant at a later stage of disease progression (120 days).

These results suggest that anti-Nogo-A antibody treatment exerts a beneficial effect early in disease progression, perhaps at the time of active muscle fibre denervation, increasing compensatory nerve terminal sprouting and reinervation of denervated endplates. However, the improvement in muscle phenotype observed in 90 day old animals is not maintained in the long term, so that no significant difference in muscle force and motor unit survival were observed when SOD1<sup>G93A</sup> mice were assessed at the end stage of disease.

It is possible that the loss of beneficial effects of the antibody in 120 day old mice may be directly related to the specific mouse model that was used in this study. Although the high copy SOD1<sup>G93A</sup> mouse is currently the standard model which is used for pre-clinical testing in ALS (26,27,31–33), the duration of disease, i.e. time from symptom onset to end stage, is relatively rapid, most likely due to high expression levels of the mutant SOD1 protein in these mice. In the high copy SOD1<sup>G93A</sup> mice, the expression of mutant SOD1 protein is ~20 times higher than normal, resulting in a rapid, aggressive disease phenotype. In ALS patients, such rapid denervation of muscles is unlikely to occur. It is therefore possible that the full potential of anti-Nogo-A antibody treatment may only be determined in a slower progressing model of ALS such as the low copy SOD1<sup>G93A</sup> mouse (34) (see the Jackson Laboratory website for details). In addition, our recent discovery of the genetic components involved in ALS has significantly improved in the last few years with the identification of several genes causing familial forms of the disease, namely TARDBP, FUS, OPTN and VCP (35–40), as well as the recent discovery of a hexanucleotide repeat expansion in the C9ORF72 gene which accounts for >30–40% of familial cases in Europe and up to 10% of apparent sporadic cases (2,41). Therefore, these newly discovered gene defects are likely to lead to the creation of different ALS disease models (for review42). Development and characterization of these models will be extremely useful not only to understand the disease pathology but also to test targeted therapeutic strategies and carry out pre-clinical testing.

In conclusion, targeting Nogo-A may be an effective therapeutic strategy for the treatment of ALS during the earlier stages of disease, when significant benefits on muscle innervation and muscle function were observed in this study. Several studies have suggested an involvement of Nogo-A, a protein known to inhibit neurite outgrowth and nerve regeneration in the CNS, in ALS pathology. Our results are consistent with previous reports of accumulation of Nogo-A in muscle fibres in SOD1 transgenic mouse models (29), that Nogo-A...
overexpression promotes denervation and induces NMJ instability (20) and that Nogo-A deletion in SOD1G93A mice prolongs lifespan, reduces muscle denervation, increases motor neuron survival and reduces the number of ubiquitinated inclusions (21). Indeed, it has been shown that levels of Nogo-A expression increase with severity of symptoms (19,43) and Nogo-A expression is upregulated in atrophic slow muscle fibres in ALS patients. Interestingly, pharmacological intervention in vivo using neutralizing antibodies has shown that anti-Nogo-A treatment after spinal cord lesion improves functional recovery and promotes axonal sprouting in rats (44,45).

Taken together with the results of the present study, these data strongly suggest that Nogo-A represents a valid therapeutic target to treat ALS since treatment with an anti-Nogo-A antibody clearly improves the neuromuscular phenotype early in disease progression in SOD1G93A mice, an ALS model with rapid disease progression.

**MATERIALS AND METHODS**

**Breeding and maintenance of transgenic SOD1G93A mice**

A colony of SOD1G93A mice (TgN[SOD1-G93A]1Gur; Jackson Laboratories, Bar Harbour) were bred and maintained in Biological Services, UCL Institute of Neurology by breeding male heterozygous carriers with female (C57BL/6 × SJL) F1 hybrids. In this colony, male SOD1G93A mice have an average lifespan of 123 days and females an average lifespan of 130 days. All studies were carried out according to the guidance issued by the Medical Research Council and under licence from the UK Government (Animals (Scientific procedures) Act 1986), following ethical approval from the Institute of Neurology.

Transgenic SOD1G93A mice were identified by genotyping for mutations in the human SOD1 transgene using DNA extracted from ear snips (46). For each animal, the genotype was also confirmed at the end of the study, on completion of the acute experiment. In view of the known effects of gender on disease progression in SOD1G93A mice, only female animals were examined in this study.

**Experimental groups**

A murine monoclonal antibody directed against Nogo-A (GSK577548) was generated by GSK and supplied as a stock of 6.3 mg/ml in Dulbeccos phosphate buffered saline. The effect of treatment with anti-Nogo-A on disease progression in SOD1G93A mice was examined at two stages of disease, a late symptomatic stage, at 90 days, and near to end-stage disease, at 120 days. SOD1G93A mice and their WT littermates were treated weekly (i.p.) with either the anti-Nogo-A antibody (GSK577548) or vehicle (PBS) from 70 days of age. Two cohorts of animals were established (i) to look at markers of disease progression and (ii) to examine muscle physiology.

The first cohort of animals contained 54 female mice assigned into three experimental groups consisting of SOD1G93A mice and their WT littermates:

- **Group 1**: WT vehicle: WT mice treated with vehicle, PBS (n = 20)
- **Group 2**: SOD1G93A-anti-NOGO-A: SOD1G93A mice treated with GSK577548 at a dose of 30 mg/kg (n = 20)
- **Group 3**: SOD1G93A vehicle: SOD1G93A mice treated with vehicle, PBS (n = 22)

Approximately half of the animals in each group (Group 1: 6 WT, Group 2: 9 SOD1G93A, Group 3: 10 SOD1G93A) were treated until 90 days of age, and received three injections. The remaining animals (Group 1: 6 WT, Group 2: 11 SOD1G93A, Group 3: 12 SOD1G93A) were treated until 120 days of age, and received seven injections. 

**RNA analysis**

The animals were anaesthetized (4.5% chloral hydrate solution, 1 ml/100 g body weight, i.p.; Sigma–Aldrich, Poole, UK) and the gastrocnemius muscle from the right leg was dissected, weighed and immersed in RNALater (according to the manufacturer’s instructions) and kept overnight at 4°C. The next day, the tissue was stored at −80°C. Samples were homogenized in Trizol using a polytron homogenizer and transferred to 96-well deep-well plates in a pre-determined randomized order. RNA was isolated using a Qiagen BioRobot the standard RNeasy tissue protocol. Following RNA isolation sample quality was assessed using Agilent Bioanalyser and transferred to 96-well deep-well plates in a pre-determined randomized order. RNA was isolated using a Qiagen BioRobot the standard RNeasy tissue protocol. RNA yields ranged between 3.5 and 26.7 μg (mean = 11.9 μg).

RNA samples were converted to cDNA using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). cDNA conversion was performed in a single batch, triplicate cDNA conversions for each RNA along with reverse-
transcriptase minus controls for each sample. Real-time PCR results were generated using the 5′ nuclelease assay (TaqMan) and the ABI 7900HT Sequence Detection System (Applied Biosystems). Each reaction included cDNA from 20 ng of RNA, 900 nM of each primer and 100 nM of probe and Universal PCR Master Mix (Applied Biosystems). PCR parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min. Where possible, a linear regression line calculated from the standard curves of serially diluted genomic DNA allowed relative transcript levels in RNA-derived cDNA samples to be calculated from the fluorescent signal in each run. Primers were purchased from Sigma Genosys and FAM-TAMRA probes purchased from Biosearch.

Data were normalized using a covariate for RNA loading calculated as the ratio of the housekeeper abundance of ACTB and PPIB (GAPDH was not used to normalize as it showed significant time and genotype interactions) in the sample to the average of that housekeepers in all grouped samples. This value was then used to adjust the copy number of each gene of interest to correct for inconsistencies in RNA loading. Datapoints were marked invalid if they had statistically inconsistent behaviour with the other housekeepers in those samples with similar tissue types. Mixed model analysis of variance was fitted to the TaqMan data with the addition of a covariate to correct for RNA loading (log(copy no.) = covariate + genotype x time point x treatment (three-way interaction)). Each TaqMan assay was analysed independently using this model.

**Laser scanning cytometry**

Frozen sections of hindlimb muscle containing the gastrocnemius, plantarus and soleus were cut at 5 μm and mounted on positively charged slides. Sections were air dried overnight then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 10 min at room temperature. Fixed sections were transferred to Bond Wash Buffer in preparation for IHC staining.

Custom IHC protocols were optimized on the Lecia Bond Max IHC/ISH stainer to detect Nogo-A expression and GSK577548. Briefly, for detection of NOGO-A, sections were blocked, stained with Sheep anti Human Nogo A 2 μg/ml (R&D Systems, AF3515) and detected with biotinylated rabbit anti Sheep IgG (Vector Labs) and visualized with streptavidin AP Label (Biocare Medical)/AP Red chromagen (Lecia Microsystems). For detection of GSK577548, sections were blocked, stained with biotinylated anti-Id9G12/3B3 and visualized with Streptavidin HRP (Biocare Medical)/DAB chromagen (Biocare Medical)/DAB enhancer (Leica Microsystems). Completed sections were air dried and mounted in Cytoseal 60.

Quantitative assessment was performed using the iCyte Laser Scanning Cytometer (CompuCyte). Slides were loaded into sample carriers and processed according to workspace specifications. A 20× mosaic scan was performed with 20 μm steps followed by a high-resolution field scan at 40× with a 0.5 μm step. The 488 argon laser was used for excitation of the red chromagen. Phantom countering was used to detect intensity and integral values of the Nogo A staining. In addition, primary contours were used to measure the area of section and Nogo A positive fibres.

**Analysis of muscle innervation**

The extent of innervation of the soleus and EDL muscles of anti-Nogo-A and vehicle-treated WT and SOD1G93A mice was established at 90 and 120 days of age. In order to accurately determine the extent of endplate innervation, presynaptic nerve terminals and endplates were simultaneously visualized using a modified version of the combined silver-cholinesterase stain (47). This technique uses potassium ferricyanide to stain AChE at the endplate region and silver impregnation to label presynaptic nerve terminals.

At 90 or 120 days of age, WT and SOD1G93A mice were terminally anaesthetized (4.5% chloral hydrate solution, 1 ml/100 g body weight, i.p.; Sigma–Aldrich). The soleus and EDL muscles were carefully dissected with tendons intact, slightly stretched, pinned on silgard-covered Petri-dish and fixed in a 4% formaldehyde fixative solution (40% formaldehyde, 10% CaCl2, 5% MgCl2, 1%CdCl2·2.5H2O) in veronal-acetate buffer for 6 h. Muscles were then transferred to a 10% sucrose solution overnight and kept at 4°C. Longitudinal sections (40 μm) were cut on a freezing microtome and held in distilled water for 10 min prior to processing. Free-floating sections were incubated in an acetylcholine iodide solution (0.04% acetylcholine iodide, 56% 0.1 M sodium hydrogen maleate, 4% 100 m M sodium citrate, 8.7% 30 mM copper sulphate, 8.7% distilled water, 8.7% 5 mM potassium ferricyanide and 13% sucrose) for 20 min on ice. The sections were then rinsed in distilled water for 30 s and immersed in potassium ferricyanide solution (0.25% potassium ferricyanide) for 10 min. Sections were then rinsed three times and immersed in absolute ethanol for 1 h, followed by two washes in distilled water for 15 min each. The sections were then incubated in a silver solution (0.1% CaCO3, 0.05% CuSO4·5H2O and 10% AgNO3) for 40 min at 37°C. The sections were then briefly washed in distilled H2O and immersed in a reducer solution (1% C6H4(OH)2, 10% Na2SO3) and monitored under a dissecting microscope for appropriate development of the silver stain, which usually takes ~2–3 min. When nerve terminals became labelled with silver, the sections were quickly removed from the reducer solution and placed in a large volume of distilled H2O. The sections were then mounted on double gelatinised slides. The extent of denervation of each muscle was calculated by expressing the number of denervated endplates as a percentage of the total number of endplates counted per muscle. For each muscle, a minimum of 200 endplates was assessed.

**In vivo assessment of muscle force and motor unit number**

The maximum force of the TA and EDL muscles of each animal was assessed at either 90 or 120 days of age. The animals were anaesthetized (4.5% chloral hydrate solution, 1 ml/100 g body weight, i.p.; Sigma – Aldrich) and prepared for in vivo isometric tension recordings of muscle contraction (26,31). The distal tendons of the TA and EDL muscles were exposed, dissected free from surrounding tissue and cut. The sciatic nerve was exposed and sectioned, and all of its branches were cut apart from the deep peroneal nerve, which innervates the TA and EDL muscles. The hindlimbs of the animals were rigidly secured to the table with stainless steel pins, and the distal tendons of the TA and EDL muscles were attached to an
isometric force transducer (Dynamometer UFI Devices, Welwyn Garden City, UK) via silk thread. Once attached, the length of each muscle was adjusted to obtain maximal twitch tension. Both muscles and nerves were kept moist throughout the experiment with saline, and the experiments were performed at room temperature. Isometric contractions were elicited by stimulating the nerve to TA and EDL using square-wave pulses of 0.02-ms duration and supramaximal intensity via platinum electrodes. Contractions were elicited by trains of stimuli at a frequency of 20, 40 and 80 Hz. Maximum twitch and tetanic tensions were determined.

The number of motor units innervating the EDL muscles in each animal was determined by stimulating the motor nerve with stimuli of increasing intensity, resulting in stepwise increments in twitch tension because of successive recruitment of motor axons. The number of stepwise increments was counted to give an estimate of the number of functional motor units present in each EDL muscle.

Muscle histochemistry and immunohistochemistry

At the end of the acute physiological experiment, the TA muscles were dissected, weighed and snap frozen in melting isopentane cooled in liquid nitrogen. The muscles were then stored in a −80°C freezer until being processed for histochemical analysis. Serial cross-sections (12 μm) were cut on a cryostat and stained for SDH activity to determine the oxidative capacity of the muscle fibres, as described previously (31).

Morphological analysis of motor neuron survival

Following removal of the hindlimb muscles, the animals were terminally anaesthetised and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (4% PFA). The lumbar region of the spinal cord was removed, post-fixed in 4% PFA for 6 h and cryoprotected in 30% sucrose for a minimum of 8 h before processing.

Serial cross-sections (20 μm) of lumbar spinal cord were cut on a cryostat and stained with galloycyanin, a Nissl stain. The number of Nissl-stained motor neurons in the sciatic motor pool was determined in every third section (number of Nissl-stained motor neurons in the sciatic motor pool) using a Nikon E995 digital camera and the images downloaded (×10, ×20 and ×40 magnification). Images were captured using a Nikon E995 digital camera and the images downloaded into Adobe Photoshop CS. To optimize image contrast, levels adjustment operations were performed, but no other image manipulations were made.

Statistical analysis

Statistical analysis of muscle innervation was performed by Clinical Pharmacology & Biometrics, GSK using the SAS/STAT® module of the SAS® System, Version 9.2. The ANOVA model including genotype, treatment and the interaction between genotype and treatment was used to analyse the innervation data at 90 and 120 days of age. Pairwise comparisons were made between groups. In case any significance difference was detected, further investigation on the time point the difference existed was performed if the data were longitudinal. In this exploratory study, no adjustment was made for multiple testing. For analysis of muscle force and motor unit survival, statistical significance among the groups was assessed using a Mann–Whitney U-test. In these experiments, significance was set at \( P < 0.05 \).

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Conflict of Interest statement. This study was undertaken as part of a Research Collaboration Agreement between UCL (L.G.) and GSK. D.K., A.T., S.B., M.C. and A.P. are employees of and hold stock in GlaxoSmithKline.

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