Muscle histone deacetylase 4 upregulation in amyotrophic lateral sclerosis: potential role in reinnervation ability and disease progression

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disease and was negatively correlated with the extent of muscle reinnervation and functional outcome. Conversely, the proposed regulator of histone deacetylase 4, microRNA-206, was upregulated in both patient groups, but did not correlate with disease progression or reinnervation. We conclude that muscle expression of histone deacetylase 4 may be a key factor for muscle reinnervation and disease progression in patients with amyotrophic lateral sclerosis. Specific histone deacetylase 4 inhibitors may then constitute a therapeutic approach to enhancing motor performance and slowing disease progression in amyotrophic lateral sclerosis.

Keywords: ALS; motor neuron; neuromuscular junction; reinnervation; HDAC

Introduction

In amyotrophic lateral sclerosis (ALS), motor neuron loss results in rapidly progressive muscle paralysis, usually leading to death from respiratory failure in 3–5 years (Charcot and Joffroy, 1869; Nelson, 1995). Riluzole, the only drug currently available, does not improve muscle strength or function (Lacomblez et al., 1996). Although ALS is usually rapidly progressive, some patients with ALS can survive beyond 10 years (Zoccolella et al., 2008). Long-term ALS survivors, defined as patients living a tracheostomy-free life for >5 years after symptom onset, represented 14% of the total ALS population in a recent study (Mateen et al., 2010). Factors accounting for prolonged survival remain poorly understood. Early onset and predominance of upper motor neuron signs have been shown to be associated with long survival in some studies (Turner et al., 2003; Zoccolella et al., 2008), but not in others (Mateen et al., 2010). Genetic factors may also be involved and patients carrying mutations in the superoxide dismutase (SOD1) gene have been reported with very long survival (>40 years) (Weber et al., 2012).

In ALS, loss of functional motor neurons is initially followed by compensatory collateral reinnervation of denervated muscle fibres by the remaining motor neurons (Hansen and Ballantyne, 1978; McComas et al., 1993). Consequently, clinical muscle weakness results from the dynamic interaction between primary loss of lower motor neurons and secondary compensation by collateral reinnervation (Bromberg et al., 1993). Early in the course of the disease, motor neuron loss is compensated for by collateral reinnervation and strength is preserved. As the disease progresses, compensation fails leading to progressive muscle weakness. Compensatory reinnervation has also been demonstrated in mouse models of ALS (transgenic mice expressing human G93A or G85R mutation in the SOD1 gene). In these mouse models a selective loss of fast-fatigable neuromuscular synapses (on type Iib muscle fibres) is initially observed. Fast fatigue-resistant motor neurons innervating the same muscle compartment at first sprout to partially reinnervate neuromuscular junctions on type Iib muscle fibres, but over time fail to maintain these additional synapses. In contrast, slow-type synapses seem particularly resistant to the disease process (Frey et al., 2000; Pun et al., 2006). Factors that protect resistant motor units remain to be identified. Some of these factors may be external to motor neurons themselves, as motor neurons are subjected to the influence of various cell types, such as neighbouring microglia, astrocytes and muscle fibres they innervate (Schaefer et al., 2005). In a mouse model of ALS (G93A-SOD1 transgenic mice), a crucial role of muscle histone deacetylase 4 (HDAC4) and its regulator microRNA-206 (MIR206) was recently suggested in compensatory reinnervation and disease progression (Williams et al., 2009). MicroRNAs are small non-coding RNAs that negatively regulate gene expression at the post-transcriptional level (van Rooij and Olson, 2007). MIR206, a skeletal muscle-specific microRNA, was shown to promote reinnervation and slow disease progression in ALS mice. MIR206 was able to repress muscle expression of HDAC4 and upregulate the expression of the secreted factor fibroblast growth factor binding protein 1 (FGFBP1) (Williams et al., 2009), thus counteracting the negative effect of HDAC4 on reinnervation.

In this work, we sought to investigate whether the MIR206-HDAC4 pathway plays a role in muscle compensatory reinnervation in patients with ALS and underlies the diversity of disease progression. We studied reinnervation in muscle specimens from patients with ALS and showed that functional motor preservation and low progression rate were associated with a significantly greater compensatory reinnervation in muscle. We then analysed the expression of candidate genes involved in the reinnervation process and showed that upregulation of muscle HDAC4 could play a key role in muscle reinnervation and disease progression in patients with ALS.

Materials and methods

Patients and control subjects

Between March 2009 and April 2011, 11 patients with ALS (rapidly progressive ALS n = 6, long-term ALS survivors n = 5) referred from the Paris Motor Neuron Disease Centre were included in this study. The study was approved by the local ethics committee and all patients provided written informed consent consistent with institutional guidelines. All patients met the El Escorial World Federation of Neurology criteria for the diagnosis of definite or probable ALS (http://www.wfns.org) (Brooks et al., 2000). When enrolled in the study, all patients with ALS underwent extensive interview for family history of neurodegenerative disorders. None of the patients reported a family history of frontotemporal dementia. Only one patient (in the long-term survivors group) reported a family history of ALS, the remaining 10 patients being considered as apparently sporadic cases. With the patients’ written informed consent, genetic testing was performed for mutation in the SOD1, TARDBP and FUS genes. After 2011, genetic
testing was also performed when possible during patient follow-up for the recently identified GGGGCC hexanucleotide repeat expansion in the C9orf72 gene. All patients in the long-term survivors group had >5 years of disease progression without requiring respiratory support or gastrostomy feeding. All patients were prospectively followed up every 3 months after muscle biopsy. Functional impairment was assessed using the revised ALS Functional Rating Scale (ALSFRS-R). ALSFRS-R is a 12-item scale that rates the performance of activities of daily living and scores from 0 (total disability) to 48 (no disability). Muscle strength was measured by manual muscle testing according to the grading system of the Medical Research Council, ranging from 0 (no visible movement) to 5 (normal muscle strength). For normal control specimens, six histochemically normal biopsy specimens of deltoid muscle were obtained from patients who were investigated for suspicion of muscle disease but were considered free of any neuromuscular disorder after morphological and histochemical examinations.

Motor-point muscle biopsy procedure
All patients underwent a muscle biopsy. Muscle specimens were removed from deltoid muscle by open biopsy under local anaesthesia for eight patients. The neuromuscular junction region was determined by the small twitch provoked by the tip of the scalpel on the surface of the muscle fascicles. For three patients, muscle samples were taken from anconeus muscle, under regional anaesthesia, using the technique described by Maselli et al. (1991, 1993).

Confocal imaging study of neuromuscular junctions
Immediately after biopsy was performed, the presence of neuromuscular junctions on a longitudinal strip of the specimen was confirmed using the classic Koenne method revealing cholinesterase activity (Koelle and Friedenwald, 1949). After fixation with 4% paraformaldehyde, whole mounts of muscle specimens were stained for acetylcholine receptors with rhodamine-conjugated α-bungarotoxin (1/500, tetramethylrhodamine α-bungarotoxin, Molecular Probes, Invitrogen), and for neurofilaments. As the different neurofilaments may not be expressed at the same time during the reinnervation process and depending on the size of the growing axon (Donahue et al., 1988), immunostaining was performed for the three major neurofilament subunits for each patient, on separate muscle fragments. The following primary antibodies were used: 168 kDa neurofilament antibody (1/250, 2H3, Hybridoma Bank), 200 kDa neurofilament antibody (1/250, Chemicon) and 68 kDa neurofilament antibody (1/100, Abcam). Secondary antibodies were used Alexa Fluor® 488 anti-rabbit (1/200, Molecular Probes, Invitrogen) and Alexa Fluor® 488 anti-mouse (1/200, Molecular Probes, Invitrogen). The specimens were observed by confocal microscopy (Carl Zeiss LSM510). Neuromuscular junctions observed in each biopsy specimen were analysed and scored according to the relationship between the intrasynaptic axonal branches and the postsynaptic membrane. Neuromuscular junctions were classified into four categories (Huzé et al., 2009; Ben Ammar et al., 2013): normal, vacant, partially vacant and reinnervated neuromuscular junctions. Human normal neuromuscular junctions show a well-circumscribed postsynaptic apparatus that can be either ‘pretzel-shape’ or made of separated cups usually in close contact with each other. The appearance of the terminal arborization varies according to the staining procedure (Cöers, 1964). Under confocal microscope, after immunostaining for neurofilament, the axon terminal commonly ends as a fork made of thin branches. Vacant neuromuscular junctions (completely denervated neuromuscular junction) are characterized by fragmented and dispersed synaptic gutters with evanescent borders and absence of nerve terminal profiles. Partially vacant neuromuscular junctions are remodelled neuromuscular junctions in which the axon terminal innervates some but not all of its fragmented gutters. Reinnervated neuromuscular junctions were identified based on the careful observation of both the presynaptic compartment and the postsynaptic apparatus. The postsynaptic apparatus of a reinnervated neuromuscular junction is abnormal and usually consists of a flattened and fragmented synaptic gutter with separated cups and evanescent borders. Based on the morphology of the presynaptic compartment, we regrouped several patterns of reinnervation in the reinnervated neuromuscular junctions group: preterminal sprouting, terminal sprouting, reinnervation with a single axon terminal innervating several cups with branching or without branching (en passant innervation). Inentravered neofomed neuromuscular junctions (small newly formed synaptic cups contacted by a thin axon) were also classified in the reinnervated neuromuscular junctions group.

Reverse transcription quantitative real-time polymerase chain reaction analysis
MIR206 expression levels and messenger RNA levels of HDAC4, FGFBP1, α1 (CHRNA1) and γ (CHRNG) subunit of acetylcholine receptors were determined using reverse transcription quantitative real-time PCR. After tissue disruption using a FastPrep®-24 Instrument (MPBiomedicals), total RNA was isolated in 750 μl of TRI Reagent® (Sigma-Aldrich #T9424) according to the manufacturer’s instructions, except that a second isopropanol precipitation was performed. Reverse transcription-PCR with random hexamer primers was done on 1 μg of RNA samples using RevertAid™ H Minus Reverse Transcriptase (Fermentas, Thermo Fischer Scientific). Quantitative real-time PCR was performed twice in duplicate using the Rotor-Gene SYBR Green PCR Kit (Qiagen). Genes used for normalization were B2M, Hprt1, and ACTB. Sequences of the primers are listed in Supplementary Table 1.

Western blot determination of HDAC4 muscle content
For western blot determination of HDAC4 muscle content, tissues were homogenized by FastPrep® in 250 μl of cell suspension buffer (50 mM Tris pH 8, 150 mM NaCl, 2 mM EDTA, SigmaFAST™ Protease Inhibitor Cocktail Tablets EDTA-Free). Total protein content was extracted by addition of Triton (1% final), with 0.5-h incubation on ice and a brief sonication (3 x 10 s). Cell debris was pelleted at 1000g for 10 min and the supernatant was mixed with 83 μl of 4 x loading buffer (250 mM Tris pH 6.8, 8% SDS, 40% glycerol). Bromophenol blue (0.01% final) and DTT (0.1 M final) were added after protein determination, and the samples were boiled for 5 min. Protein lysates (25 μg) were resolved on 8% SDS-PAGE gels using standard procedures and blotted onto PVDF membranes. Antibodies against HDAC4 [Santa Cruz Biotechnology (N-18) #sc-5245] were used at 1:1000 dilution. HDAC4 protein expression was quantified and normalized to Coomassie staining using ImageJ software.
Myofibrillar ATPase staining and morphometric analysis

Myofibrillar activity staining was performed at pH 9.4 on cryostat sections from deltoid muscle specimens following the method proposed by Brooke and Kaiser (1969). The mean diameter of myofibres and the percentage of type I and type II fibres were assessed using sections stained for myofibrillar ATPase (pH 9.4). About 400 muscle fibres per specimen were analysed by measuring all muscle fibres in four consecutive, non-overlapping fields. Digital photographs were obtained with a Zeiss AxioCam HRc attached to a Zeiss Axioplan Bright Field Microscope and processed with the AxioVision 4.4 software (Zeiss). For each fibre type, atrophic fibres were defined as two standard deviations below the mean diameter measured in control subjects.

Statistical analysis

Statistical analysis was conducted with XLSTATS 2012 software (Addinsoft) and SAS software version 8.2 (SAS Institute). Mann-Whitney U-tests were used to compare continuous data and Fisher’s exact tests to analyse categorical data. Correlations were analysed using the non-parametric Spearman’s rank correlation test. We used a generalized linear mixed model for clustered multinomial outcomes to test for an association between innervation status and disease group. All statistics were two-tailed and the level of significance was set at $P = 0.05$.

Results

Demographic and clinical characteristics of patients with amyotrophic lateral sclerosis

The characteristics of patients and controls are summarized in Table 1 and Supplementary Table 2. Median disease duration at the time of the biopsy was 11.0 months (range 9–18) in the rapidly progressive ALS group and 100 months (range 72–193) in the long-term survivors group ($P = 0.004$). Long-term survivors were significantly younger at disease onset ($P = 0.022$). Other demographics were similar in the two groups. In the long-term survivor patient who reported a family history of ALS in a first-degree relative, we identified an exon 5 mutation on the $SOD1$ gene (c.418A>G, amino acid numbering according to the ALS online database: N139D). We calculated disease progression rate during follow-up as follows:

$$\text{Disease progression rate} = (\text{ALSFRS-R score at 'time of biopsy'} - \text{ALSFRS-R score at 'last follow-up'}) / \text{follow-up duration after biopsy}$$

The mean disease progression rate during follow-up was 3.018 units/month (range 0.584–9.130) in the rapidly progressive ALS group and $-0.001$ units/month (range $-0.180$–0.222) in the long-term ALS survivors group ($P = 0.004$). At the end of the study, four of six of the patients with rapidly progressive ALS were deceased [mean disease duration 28 (range 13–49) months], and one of six required permanent invasive ventilatory support with tracheostomy. The remaining patient required non-invasive respiratory support. Conversely, long-term survivors showed almost no functional decline during follow-up (Fig. 1) and none of them died.

Muscle reinnervation ability is higher in long-term amyotrophic lateral sclerosis survivors

Using high-resolution confocal microscopy, we classified all identified neuromuscular junctions in muscle biopsy specimens into four categories (Fig. 2, Supplementary Fig. 1 and Table 2): vacant, partially vacant, reinnervated and normal neuromuscular junctions. The innervation status was significantly different between the two ALS groups ($P = 0.001$). Vacant neuromuscular junctions were significantly more frequent in the rapidly progressive ALS group than in the long-term survivors group (29.2% and 9.2%, respectively; $P = 0.0004$). The proportion of partially vacant neuromuscular junctions was similar in the two groups. Reinnervated neuromuscular junctions were significantly more frequent in the long-term survivors group than in the rapidly progressive ALS group (55.6% and 36.7%, respectively; $P = 0.006$). Normal neuromuscular junctions were not observed. Disease progression rate correlated negatively with the proportion of reinnervated neuromuscular junctions and positively with the proportion of denervated neuromuscular junctions (Fig. 3).

HDAC4 muscle upregulation is associated with faster functional decline and lower muscle reinnervation ability

We used reverse transcription quantitative real-time PCR to determine relative transcript expression levels for $MIR206$, $HDAC4$ and $FGFBP1$ in muscle specimens (Fig. 4 and Supplementary Fig. 2). As molecular markers of denervation intensity, $CHRNT1$ and $CHRNG$ messenger RNA levels were also determined. $HDAC4$ messenger RNA was moderately upregulated in patients with ALS when compared with controls (mean fold-change ratio 1.25, not significant), but $HDAC4$ transcript upregulation was significantly higher in rapidly progressive patients with ALS (mean fold-change ratio 1.42 when compared with controls) than in long-term ALS survivors, who showed no $HDAC4$ induction (mean fold-change ratio 1.05) (Fig. 4A). Muscle $HDAC4$ transcript levels correlated positively with disease progression rate during follow-up (Fig. 4B). $CHRNA1$ and $CHRNG$ transcripts were significantly upregulated in patients with ALS and, although the difference was not statistically significant, reached higher levels in the rapidly progressive group (Fig. 4A). Muscle $HDAC4$ induction correlated positively with $CHRNA1$ and $CHRNG$ messenger RNA levels (not shown). $MIR206$ and $FGFBP1$ transcripts were significantly upregulated in patients with ALS and reached higher levels in the long-term survivors group, but the difference between the two groups was not statistically significant (Fig. 4A). The Spearman rank correlation test showed that $FGFBP1$ messenger RNA levels correlated positively with $MIR206$ transcript levels (Fig. 5), but not with $HDAC4$ transcript level nor with $HDAC4$ relative protein content.
The extent of reinnervation determined on muscle specimens correlated with HDAC4 protein level ($P = 0.008$, negative correlation, Fig. 4D), but not with MIR206 or FGFBP1 induction levels.

**Morphometric analysis**

Morphometric analysis (Supplementary Table 3) showed no difference between the two groups in mean fibre size and fibre atrophy. There was a trend for predominance of type 1 muscle fibres in patients with ALS.

**Discussion**

In this work, we show that low progression rate in patients with ALS is associated with greater compensatory reinnervation and have identified HDAC4 as a potential key deleterious factor for muscle reinnervation and disease progression in ALS.

The first major result of our study is the demonstration that functional motor preservation over time (i.e. long-term survival in ALS) is not associated with a high proportion of normal neuromuscular junctions (natively innervated by motor neurons spared by the disease process), but with greater compensatory reinnervation in muscle. We studied muscle reinnervation in five long-term ALS survivors and six patients with rapidly progressive ALS. Data from mouse models of ALS show that muscles are affected differently according to their fibre type composition and that the disease process preferentially affects fast-fatigable synapses, on type IIb muscle fibres (Frey et al., 2000; Pun et al., 2006). As deltoid and anconeus are ‘mixed’ muscles in normal subjects (Johnson et al., 1973; Saltin and Gollnick, 1983; Le Bozec and Maton, 1987), their vulnerability to disease can be considered as similar and we analysed data from these two muscles together. The characteristics of long-term ALS survivors were similar to those in previous reports on long survival in ALS, with younger age at onset (Turner et al., 2003; Zoccolella et al., 2008) and, although not statistically significant, less frequent bulbar presentation (Mateen et al., 2006).
et al., 2010). As shown by ALSFRS-R score and muscle testing values, patients of both groups were similarly and moderately disabled at the time of the biopsy, but patients with rapidly progressive ALS showed significantly less compensatory reinnervation in muscle. All patients were prospectively followed up after muscle biopsy and the two groups showed a strikingly different disease progression (3.018 units/month in the rapidly progressive ALS group and −0.001 units/month in the long-term ALS survivors group, the slightly negative mean disease progression rate in this group reflecting daily fluctuations in function usually based on tiredness or spasticity). It is noteworthy that we did not observe normal neuromuscular junctions in patients with ALS of either group. Thus, slow functional decline is correlated with the ability of long-term survivors to maintain a successful compensatory response to denervation. Identification of factors that promote compensatory reinnervation in these patients is of great importance as it may provide new targets for therapeutic intervention. We investigated whether this greater reinnervation is associated with differential expression of key muscle factors involved in the reinnervation process. In ALS mice, muscle Hdac4 and its regulator Mir206 were recently suggested to play a key role in compensatory reinnervation (Williams et al., 2009). HDAC4 is an important mediator of the action of neural activity on muscle gene expression and its muscle expression is dramatically induced in response

**Figure 2** Neuromuscular junctions observed in ALS patient muscle biopsy specimens on confocal microscopy. Whole-mount preparations were stained for acetylcholine receptor with α-bungarotoxin in red and axon terminals with neurofilament antibody in green. Scale bars = 5 μm. (A) Vacant neuromuscular junction, fragmented postsynaptic compartment with no axon profile, completely denervated neuromuscular junction. (B) Partially vacant neuromuscular junction (remodelled neuromuscular junction in which the axon terminal innervates some but not all of its fragmented gutters). (C) Reinnervated neuromuscular junction, the axon terminal innervates four isolated synaptic cups probably originating from the fragmentation of a single neuromuscular junction. (D) Normal neuromuscular junction (control patient), presence of a terminal axon ending as a fork made of thin branches, innervating a well-circumscribed postsynaptic apparatus.
Table 2  Classification of neuromuscular junctions observed by confocal microscope in muscle specimens of patients with ALS

<table>
<thead>
<tr>
<th></th>
<th>Patients with rapidly progressive ALS (n = 5)</th>
<th>Long-term ALS survivors (n = 4)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total neuromuscular junctions examined</td>
<td>120</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>Vacant n(%)</td>
<td>35 (29.2)</td>
<td>10 (9.2)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Partially vacant n(%)</td>
<td>41 (34.1)</td>
<td>38 (35.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Reinnervated n(%)</td>
<td>44 (36.7)</td>
<td>60 (55.6)</td>
<td>0.006</td>
</tr>
<tr>
<td>Normal n(%)</td>
<td>0 (0)</td>
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Innervation status could be determined for 9 of 11 patients with ALS (five in the rapidly progressive ALS group and four in the long-term survivors group). In two patients (one from each group) no neuromuscular junctions were present in the muscle fragment submitted to morphological analysis under the confocal microscope. All neuromuscular junctions observed in each biopsy specimen were analysed and classified into four categories: normal, vacant (fragmented and dispersed synaptic gutters with evanescent borders and absence of nerve terminal profiles, completely denervated neuromuscular junction), partially vacant (remodelled neuromuscular junctions in which the axon terminal innervates some but not all of its fragmented gutters), and reinnervated neuromuscular junctions (abnormally flattened and fragmented synaptic gutters made of separated cups with evanescent borders and one of the following patterns of reinnervation: preterminal sprouting, terminal sprouting, reinnervation with a single axon terminal innervating several cups with branching or without branching ‘en passant innervation’). Innervated newly formed neuromuscular junctions were also classified in the reinnervated neuromuscular junctions group (Fig. 2 and Supplementary Fig. 1).

*Alpha level is set at 0.017 (Bonferroni correction). NS = not significant.

Figure 3  Spearman rank test correlation between innervation status and disease progression rate during follow-up after biopsy. (A) Disease progression rate correlated negatively with the proportion of reinnervated neuromuscular junctions (Spearman rank correlation r = −0.672). (B) Disease progression rate correlated positively with the proportion of completely denervated (vacant) neuromuscular junctions (Spearman rank correlation r = 0.733). NMJ = neuromuscular junction.
Figure 4  HDAC4 muscle induction is greater in patients with rapidly progressive ALS, correlates with disease progression and negatively influences reinnervation. (A) Relative transcript levels of α1 (CHRNA1) and γ (CHRNγ) subunits of nicotinic acetylcholine receptor, MIR206, FGFBP1 and HDAC4 were quantified by reverse transcription quantitative real-time PCR. The y-axis represents relative transcript levels with control values set to 1. Genes used for normalization were β2-microglobulin, HPRT1 and ACTB. All the quantitative PCR primers used are listed in Supplementary Table 1. Data are expressed as mean fold-change ratios (±SEM) from two experiments. Mean values from the two experiments are presented for each individual in Supplementary Fig. 2. *P < 0.05 and **P < 0.01 by Mann-Whitney U-tests. NS = not significant. (B) Positive correlation between muscle HDAC4 messenger RNA relative expression and disease progression rate during follow-up after biopsy (Spearman rank correlation r = 0.700). Data shown are representative of two experiments with similar results. (C) Determination of HDAC4 muscle protein content. Top: Western blot analysis of the expression of HDAC4 protein in patients with rapidly progressive ALS (numbered from 1 to 6) and long-term ALS survivors (numbered from 7 to 11). Middle: Coomassie blue staining as loading control. Bottom: HDAC4 relative protein content was determined by the optical density of the HDAC4 bands normalized to the Coomassie staining (arbitrary units). These values were used for Fig. 4D. (D) Negative correlation between HDAC4 protein level and the proportion of reinnervated neuromuscular junctions (Spearman rank correlation r = −0.824).
be a potential new target for future treatments. Consistently, trichostatin A, one of the most potent HDAC inhibitors, has recently been shown to delay disease progression and to increase survival when administered to ALS mice after disease onset (Yoo and Ko, 2011). Interestingly, trichostatin A administration increased the number of fully innervated neuromuscular junctions and reduced muscle atrophy. Inhibition of muscle HDAC4 by specific inhibitors could then be a promising therapeautic approach to enhance motor performance and slow disease progression in patients with ALS.

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Supplementary material
Supplementary material is available at Brain online.

References


