Evidence for neuronal localisation of enteroviral sequences in motor neurone disease/amyotrophic lateral sclerosis by *in situ* hybridization

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Sequences resembling those of human enterovirus type B sequences have been associated with motor neurone disease/amyotrophic lateral sclerosis. In a previous study we detected enteroviral sequences in spinal cord/brain stem from cases of motor neurone disease/amyotrophic lateral sclerosis, but not controls. Adjacent tissue sections to two of those strongly positive for these sequences by reverse-transcriptase polymerase chain reaction were analyzed by *in situ* hybridization with digoxigenin-labelled virus-specific antisense riboprobes. In one case, a female aged 83 showing 12 month rapid progressive disease, signal was specifically localized to cells identifiable as motor neurones of the anterior horn. In another case, a male aged 63 with a 60-month history of progressive muscle weakness, dysarthia, dyspnoea and increased tendon reflexes, signal was located to neurones in the gracile/cuneate nuclei of the brain stem tissue block that had been analyzed. This case showed loss of neurones in the anterior horn of the spinal cord by histopathologic examination which would account for clinical signs of motor neurone disease/amyotrophic lateral sclerosis. Dysfunction of the gracile/cuneate nuclei might have been masked by the paralytic disease. These structures are adjacent to the hypoglossal nuclei, and suggest either localised dissemination from hypoglossal nuclei or a possible route of dissemination of infection through the brainstem to the hypoglossal nuclei. These findings provide further evidence for the possible involvement of enteroviruses in motor neurone disease/amyotrophic lateral sclerosis.

Key words: enterovirus; echovirus; in situ hybridization; motor neurone disease; amyotrophic lateral sclerosis.

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Two independent studies using reverse transcriptase-polymerase chain reaction (RT-PCR), capable of detecting a wide range of enteroviruses, including poliovirus, identified a 73% or >80% incidence of enterovirus nucleic acid sequences in amyotrophic lateral sclerosis (ALS) cases vs a 0% or 4% incidence in controls, respectively (Woodall et al., 1994; Berger et al., 2000). Further recent studies have also shown similar sequences in Japanese ALS cases (Giraud et al., 2001). Viral sequences were similar from all three studies and were related to human coxsackie B/echoviruses, but not human coxsackievirus A, poliovirus or other picornaviruses infecting humans and other mammals (Woodall et al., 1994; Berger et al., 2000). Two other studies found no enterovirus sequences in ALS cases and controls (Swanson et al., 1995; Walker et al., 2001), and another, enterovirus sequences in ALS, other neurological diseases and controls (Muir et al., 1996).

Poliovirus specifically targets lower motor neurones in infectious poliomyeltis; in addition, clinical histories and histopathological comparisons with poliomyeltis suggest that enteroviruses may be implicated in ALS (Poskanzer et al., 1969; Roos et al., 1980). Polioviruses, extreme examples of acute lytic infective agents of the CNS, although not detected in ALS spinal cord, do exhibit some characteristics that illustrate how this group of viruses as a whole may be capable of acting as aetiologic agents in ALS. Poliovirus can enter the CNS through damaged motor end plates and track along nerves from the periphery to motor neurones, then spread within the motor system (Gromeier et al., 1998; Ohka et al., 1998; Ponuraj et al., 1998; Ponuraj et al., 2001 Strebel et al., 1995). Attenuated polioviruses might persist in motor neurones, but may not directly lyse them and induce only mild inflammation (Ponuraj et al., 2001) and poliovirus can be adapted to persist in mice motor neurones for more than 12 months (Destombes et al., 1997). RT-in situ-PCR located

these sequences in the cytoplasm of neurone-like cells of the spinal cord (Berger et al., 2000). Two virus-sequence positive ALS cases repeatedly showed a very strong RT-PCR signal on RNA isolated from sequential wax sections in one study (Woodall et al., 1994). Adjacent wax sections to those analyzed by RT-PCR were therefore further analyzed by in situ hybridization, a technique which gives a higher resolution than RT-in situ-PCR, and here we present evidence for localization of enterovirus (EV) sequences to neurone-like cells in the spinal cord or lower medulla of these cases. A previous study had given an indication of the presence of enteroviruses in MND/ALS spinal cord by in situ hybridization (Brahic et al., 1985), but in this study we were able to enhance sensitivity by using highly viral sequence specific probes.

Materials and Methods

Samples

Cases used in this study were obtained from routine *post-mortem* material at two different sources in the West of Scotland between 1986 and 1988. Diagnosis of ALS was confirmed histopathologically in both cases (Woodall et al., 1994). Case #1, at post mortem, was a female aged 83 showing a 12 month rapid progressive history of difficulty in swallowing and dysarthia, with limb fasciculation/weakness and loss of neurones in the anterior horn of the spinal cord and the hypoglossal nuclei. Case #2 was a male aged 63 with a 60 month history of progressive muscle weakness, dysarthia, dyspnoea and increased tendon reflexes. Loss of neurones was recorded in the anterior horn of the spinal cord. The hypoglossal nuclei were not investigated. The control case was a 76 year old female who died from pneumonia and was autopsied 26 hrs after death.

Appropriate ethical approval had been obtained for this study.

In situ hybridization

Sections (8.0 μm) from ALS cases #1 and 2 and a control spinal cord were dewaxed in xylene, rehydrated, then pretreated with 0.2N HCl for 10 minutes, 0.3%v/v triton X-100 for 10 min, 10.0 mg/mL of proteinase K at 40°C for 15 minutes and post-fixed in 4%w/v paraformaldehyde for 5 minutes. Sections were washed extensively after each of these procedures in PBS. One section was further treated with 20 mg/mL RNase A and 400 u RNase T1 for 20 minutes at 37°C, extensively washed, post-fixed in 4% w/v paraformaldehyde for 1 minute and again extensively washed. Sections were prehybridized in 2x SSPE/50% formamide at 37°C for 1 hour. RT-PCR products were amplified from the viral 5' untranslated region of coxsackievirus B3, or from abelson cellular mRNA as previously described (Woodall et al 1994) and cloned directly into a transcription vector (pCR-Script, Stratagene). Clone orientation was determined by sequencing analysis. Plasmids were double purified and either cleaved with Not-1 or EcoR1 (Roche), Qiagen tip purified and digoxigenin labelled sense and antisense riboprobes synthesized from either T7 or T3 RNA polymerases (20 u) for 2 hours (Roche). The reaction was terminated and the riboprobe/vector mix alkali treated at 60°C for 20 minutes, neutralized with sodium acetate buffer, pH 6.0, then precipitated with the addition of 1 μL of 20 mg/mL glycogen (Roche) and 2 volumes of ethanol. Riboprobes were dissolved in 100 μL of RNase free water and 5.0 μL checked by gel electrophoresis. A 6.0 μL volume of riboprobe was added/100 μL hybridization mix/section (hybridization mix was 1mM Tris-HCl, pH 7.0, 1x Denhardts solution, 2x SSPE, 50% v/v formamide, 0.5% SDS, 0.5% dextran sulphate, 0.25 mg/mL salmon sperm DNA). Separate adjacent sections were either probed with antisense riboprobes (detection of the + strand viral genome) or sense probes (negative control, but also will hybridize to the viral replicative intermediate). RNase pre-treated sections were probed with the anti-sense riboprobe. Riboprobes in hybridization mix, and prehybridise-mix treated sections, were incubated at 85°C for 2 minutes in parallel, then the riboprobe/hybridization mix applied to the sections and hybridized at 50°C overnight. Sections were washed in 2x SSPE, 0.1 x SSPE for 10 minutes, 0.1 x SSPE for 30 minutes at 50°C then 0.1 x SSPE for 20 minutes. Standard colour development was performed — the digoxigenin antiserum was diluted 1/2000 in 10% normal rat serum (Roche), and staining developed for 5 hours at 4°C in the dark. Sections were then washed in PBS, briefly counter-stained with eosin, washed in sterile distilled water, then mounted in Crystalmount (Biomeda, UK). Positively stained cells appeared as a deep purple against a light pink background. Negatively stained cells appeared pink, occasionally with a brown coloration. Differentially treated adjacent sections were matched for individual cells, or if this was not possible as a result of tissue damage or

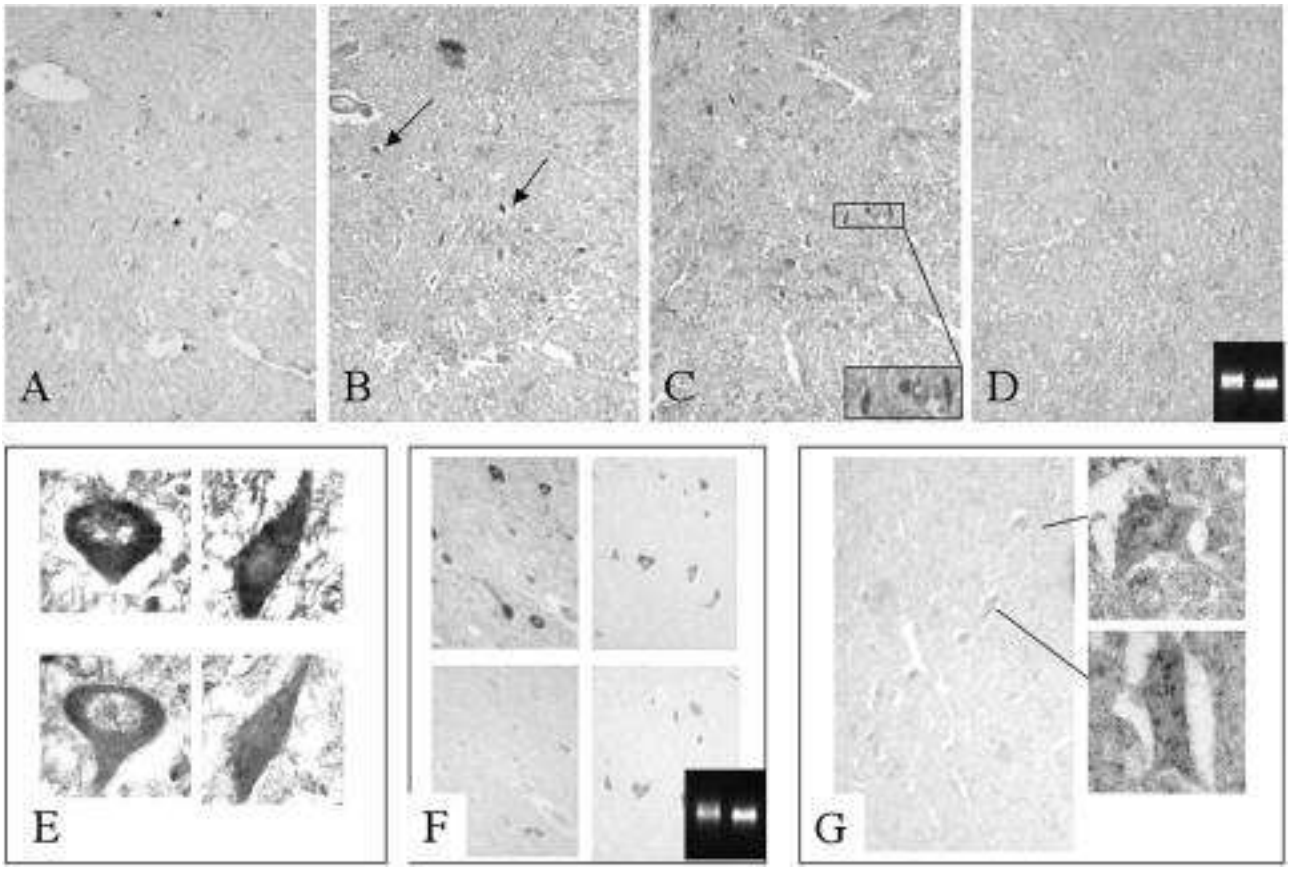

Figure 1. Cervical ventral horn of case 1. A. Right ventral horn from section stained with the coxsackievirus B sense (negative control) riboprobe (x40). B, Adjacent section showing matching neurones in the right ventral horn staining for the antisense coxsackivirus riboprobe (some of which are arrowed, x40). C. Left ventral horn showing staining of neurones with the coxsackivirus antisense riboprobe (x40). C inset. Enlargement (x200) showing stained ventral horn neurones. D. An adjacent section showing the left ventral horn staining for the antisense coxsackievirus riboprobe following treatment of the section with RNase. D (inset). Ethidium bromide stained gel showing enteroviral antisense (left) and sense (right) riboprobes. E. Matched ventral horn neurones stained with the coxsackievirus anti-sense probe (upper) and the sense riboprobe (lower). x1000 (oil immersion). F. Ventral horn neurones stained with proto-oncogene abelson anti-sense (left, upper) and sense probes (left, lower). Control spinal cord ventral horn neurons, antisense (right, upper) and sense (right, lower) staining for abelson (x200). F inset. Ethidium bromide stained gel showing abelson antisense(left) and sense (right) riboprobes. G. Ventral horn from a control spinal cord stained with the antisense riboprobe (x200). Selected neurones are shown x1000.

separation of sections, for the same region. Experimental and control photomicrographs, up to x400 magnification were taken under exactly the same manual exposure conditions using a Olympus BH-2 microscope with an attached Olympus DP50 CCD camera. Images were digitized using AnalySIS 3.1 software (Soft imaging system, Gmbh). Photomicrographs at x1000 were taken with an oil immersion x100 objective onto photographic slide film, then subsequently scanned into Adobe Photoshop 4.0 for Apple Macintosh.

Results

Staining of the cervical spinal cord of case #1 is shown in Figure 1. The ventral horns of this case were considerably denuded of neurones, however remaining isolated neurones stained positive for the antisense viral riboprobe. Signal was located in neurones of both ventral horns (Figure 1 B,C) and was not observed in any other regions of the section. On an adjacent section the same ventral horn neurones were not stained by the sense (negative control) riboprobe (Figure 1 A) and the antisense probe did not detect sequences in the anterior horn of a control spinal cord (Figure 1 G). Signal was not observed with the antisense riboprobe if a further adjacent section was RNase pre-treated (Figure 1 D). Examples of paired neurones stained with antisense or sense probes are shown (Figure 1 E). Signal for the mRNA of the cellular Abelson protein

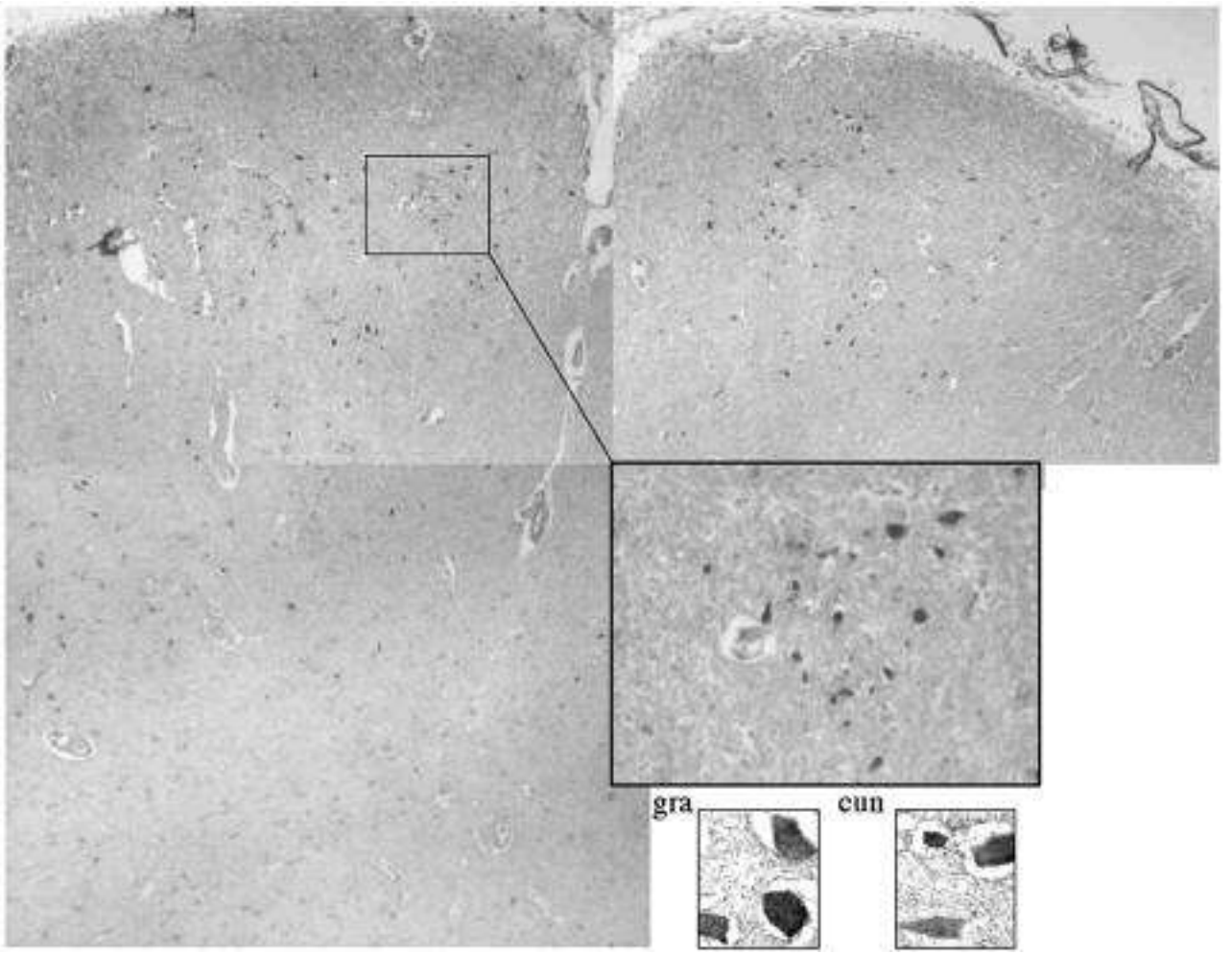

Figure 2. Case #2. Composite image showing the distribution of signal in the gracile and cuneate nuclei in a section from the lower medulla (antisense coxsackievirus riboprobe x40). A selected cluster of stained neurones are shown x200. Stained (and unstained) neurones from the gracile (gra) and cuneate (cun) nuclei (x1000, oil immersion).

kinase was observed in neurones but not in other cell types, with the antisense, but not the sense riboprobe (Figure 1 F). Case #2 showed a distinct pattern of staining in both the left and right gracile and cuneate nuclei, where many but not all neurones were stained (Figure 2). The staining pattern within these regions showed evidence of clustering, but there was not obvious perivascular staining. Staining was not observed in the gracile/cuneate nuclei of adjacent sections with the sense (negative control) riboprobe (Figure 3A) or RNase pre-treated sections stained with the anti-sense riboprobe (Figure 3B). Closer examination revealed stained neurone-like cells with a granular appearance in the gracile and cuneate nuclei (Figure 2). This staining was not observed with the sense riboprobe (Figure 3A, inset). Neurones showed faint labelling when stained with an abelson antisense, but not sense probe (Figure 3C).

These data indicate that staining in both MND/ALS cases was virus-specific and localized to neurone-like cells. Ablation of signal from the anti-sense probe after RNase treatment, shows that RNA is specifically stained. Comparison of staining with sense and anti-sense probes, which were applied to adjacent sections in equal concentrations (see Figure 1D inset) shows that staining is specific to viral genome (+ sense) RNA. Not all neurones were labelled. The above evidence also suggests that this was not artefactual staining of neurones.

Discussion

The use of probes, the specificity of which was previously determined by sequencing RT-PCR products from adjacent sections, allows the highly sensi-

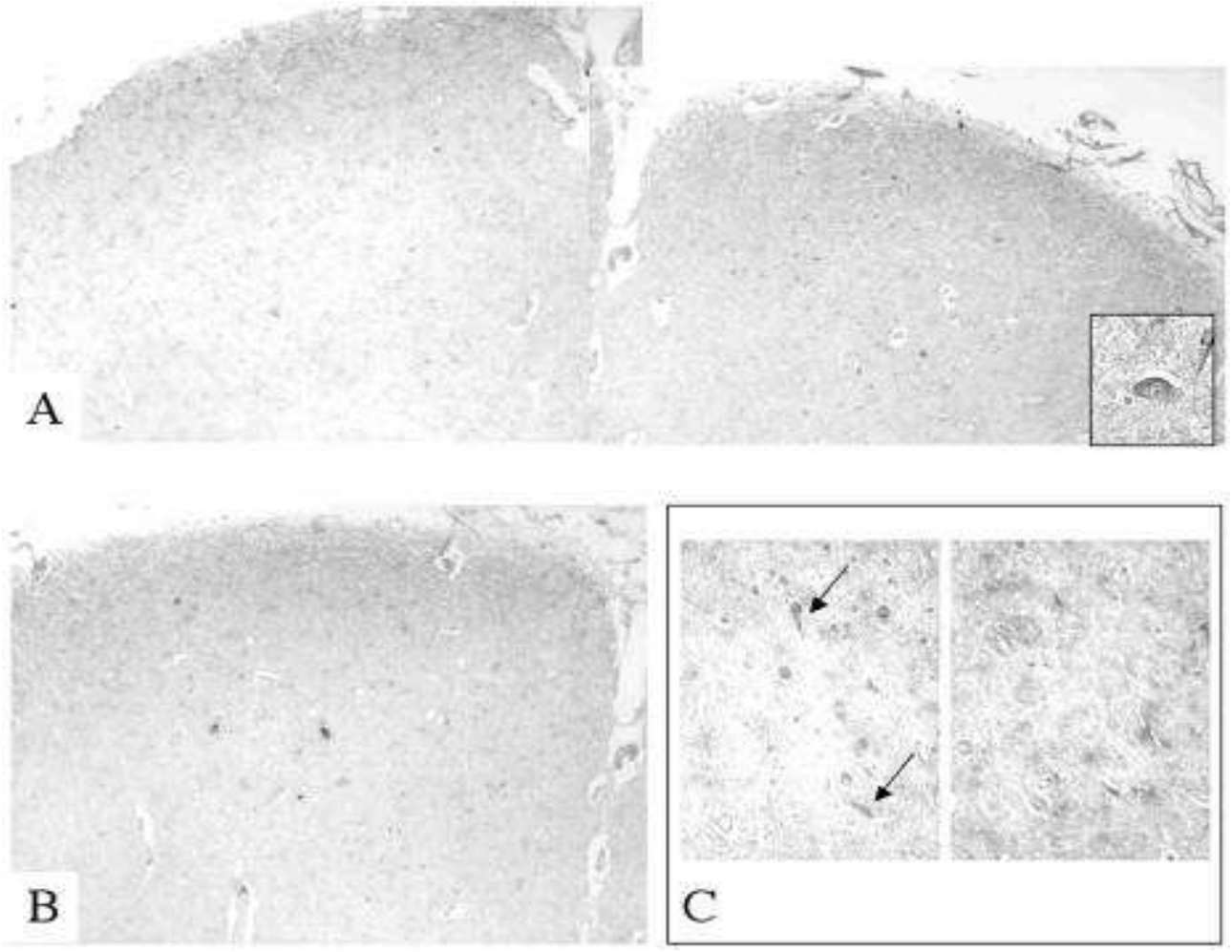

Figure 3. A. Case #2. Composite image showing gracile/cuneate nuclei probed with the coxsackievirus B sense (negative control) riboprobe on sections adjacent to those probed with the antisense riboprobe in Figure 2 (x40). Inset. Neurone from this section (x1000, oil immersion). B. An adjacent section showing the right gracile and cuneate nuclei probed with the antisense coxsackievirus B riboprobe following treatment with RNase (x40). C. Neurones probed with the proto-oncogene abelson antisense (left) and sense (right) riboprobes (x200). Neurones are arrowed.

tive staining with digoxigenin-labelled riboprobes. Staining of cervical spinal neurones in case #1 identified as motor neurones from their morphology and anatomical location in the ventral horn, is consistent with a role of enteroviruses in ALS but this is not evidence for an etiologic role. Examination of positively stained neurones indicates a cytoplasmic staining and a punctate appearance that is consistent with replication of enteroviruses (Bolten et al., 1998; Klinger et al., 2001). Many neurones in the gracile/cuneate nuclei of the lower medulla are stained for virus in case #2. The gracile and cuneate nuclei are responsible for two point discrimination, vibration sense and conscious proprioception. Therefore, dysfunction of these neurones per se would not be expected to be responsible for the clinical signs of ALS. However, case #2, in addition had shown loss of ventral horn motor neurones by routine histopathological examination, indicative of ALS, but the hypoglossal nuclei had not been investigated. It is possible that loss of gracile/cuneate nuclei function is a secondary disease that could be masked by paralytic disease, especially if clinical manifestation occurred later in MND/ALS disease progression. The progression of this case was relatively slow (60 months), and this might have permitted wider dissemination of the virus into other systems from the motor system. Infection of the cuneate/gracile nuclei could originate from a persistent viral infection within the CNS by direct cell to cell contact similar to a mechanism observed in other picornaviruses (Wada et al., 1993). Notably, the gracile and cuneate nuclei lie adjacent to the hypoglossal nuclei in the brain stem, providing a possible opportunity for localized dissemination of virus from the motor system. Alternatively, this may

be a widely disseminated acute infection, spread into the CNS by viraemia and is unrelated to MND/ALS. The lack of an observed inflammatory response, or obvious perivascular clustering of infected cells however, argues against this possibility, but does not exclude it. Further blocks from different levels of the spinal cord from case #2 are available to us for further studies.

These viruses are incapable of latency, and therefore must be replicating in the cells they infect. They are committed to producing viral gene products that have potential to effect changes in cellular function and survival (Barco et al., 2000; Goldstaub et al., 2000; Huber et al.,1999; Iordanov et al., 2000; Teterina et al., 1997). This would support the hypothesis that the presence of these viruses indicates a contributory role in the pathogenesis of MND/ALS. Cases of MND/ALS, however, may be susceptible to opportunistic infection by enteroviruses. Motor end plate damage in infected atrophic striated muscle, for example, is known to stimulate entry into motor axons (Andrew et al., 1984; Gromeier et al., 1998; Sato et al., 1978), and therefore axonal withdrawal from muscle during motor neurone degeneration might provide an opportunity for enterovirus infection of the CNS.

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