Extramotor involvement in ALS: PET studies with the GABA<sub>A</sub> ligand [¹¹C]flumazenil

C. M. Lloyd, M. P. Richardson, D. J. Brooks, A. Al-Chalabi and P. N. Leigh

1Department of Neurology, Guy's, King's and St Thomas' School of Medicine and the Institute of Psychiatry, London
2Medical Research Council Cyclotron Unit, Hammersmith Hospital and 3Institute of Neurology, London, UK

Correspondence to: P. N. Leigh, Department of Neurology, Institute of Psychiatry, De Crespigny Park, London SE5 8AF, UK
E-mail: n.leigh@iop.kcl.ac.uk

Summary
We used the benzodiazepine GABA<sub>A</sub> marker [¹¹C]flumazenil to study cerebral dysfunction in amyotrophic lateral sclerosis (ALS) with PET. Seventeen non-demented patients with clinically definite or probable ALS were scanned and statistical parametric maps were derived to localize changes in regional flumazenil volumes of distribution (FMZVD), which correlate closely with receptor density (B<sub>max</sub>), and the results were compared with those of 17 controls. The ALS group showed statistically significant decreases in relative FMZVD in the prefrontal cortex (areas 9 and 10 bilaterally), parietal cortex (area 7 bilaterally), visual association cortex (area 18 bilaterally) and left motor/premotor cortex (including area 4) (P < 0.001). Relative reductions in FMZVD were also seen in the left ventrolateral and dorsolateral prefrontal cortex (areas 45, 46 and 47), Broca's area and the right temporal (area 21) and right visual association cortex (area 19). These observations suggest that cerebral dysfunction in ALS involves motor/premotor and extramotor areas, particularly the prefrontal regions.

Keywords: amyotrophic lateral sclerosis; PET; [¹¹C]flumazenil; extramotor cortex

Abbreviations: ALS = amyotrophic lateral sclerosis; FMZVD = flumazenil volumes of distribution; FDG = [¹⁸F]2-fluoro-2-deoxyglucose; rCBF = regional cerebral blood flow

Introduction
Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder of uncertain aetiology that predominantly affects the corticospinal tracts, the brainstem and the anterior horn cells of the spinal cord (Charcot and Marie, 1885; Holmes, 1909; Lawyer and Netsky, 1953; Smith, 1960; Brownell et al., 1970).

Recent pathological, neuropsychological and functional imaging studies, however, have challenged the view that ALS is a disorder restricted to the motor system. Dysfunction of the extramotor cortex, and in particular the prefrontal cortex, has now been identified (Ludolph et al., 1992; Kew et al., 1993a, b; Abrahams et al., 1996, 1997).

Although the cardinal pathological features of ALS include loss of the anterior horn cells in the spinal cord and cells in the lower motor cranial nerve nuclei, and degeneration of the corticospinal tracts (Charcot and Marie, 1885; Holmes, 1909; Lawyer and Netsky, 1953; Smith, 1960; Brownell et al., 1970), other extramotor systems are involved to varying degrees. For example, Smith found degenerating projections from the sensory cortex, parietal and temporal lobes and the cingulate gyrus in addition to the primary motor areas (Smith, 1960). In patients with ALS and dementia (Hudson, 1981), there is often marked neuronal loss in the frontotemporal cortex (Morita et al., 1987; Neary et al., 1990; Jackson et al., 1996).

Dementia occurring in association with ALS has the characteristics of dementia of the frontal type, with predominant changes in personality and social conduct and the inability to use strategies and planning to accomplish tasks (Neary et al., 1990). Recently, more widespread cognitive impairments have been detected by formal neuropsychological testing in patients who are not clinically demented. In particular, tests of frontal lobe function (fluency, sorting and planning) are impaired, suggesting that cognitive impairment in ALS may represent a continuum with frank dementia at one extreme (Gallassi et al., 1985, 1989; David and Gillham, 1986; Ludolph et al., 1992; Hartikainen et al., 1993; Kew et al., 1993b; Abrahams et al., 1996, 1997, 2000; Chari et al., 1996; Massman et al., 1996).

Previous PET studies have also suggested that cortical involvement in ALS is not confined to the motor cortex. Studies using [¹⁸F]2-fluoro-2-deoxyglucose (FDG) have demonstrated reductions in glucose metabolism throughout the cerebral hemispheres, although this was most marked in
impaired verbal PET activation studies have shown that ALS patients with 
et al.
prefrontal and premotor cortex (Abrahams 
with PET using the benzodiazepine GABA A marker 
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ALS have also shown marked reductions in regional
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M = male; F = female; D = definite; P = probable; + = present; - = absent.

the sensorimotor cortex in patients with classical ALS (Dalakas et al., 1987; Hatazawa et al., 1988). PET studies in ALS have also shown marked reductions in regional cerebral blood flow (rCBF) at rest in the primary sensorimotor cortex and the adjacent premotor and parietal areas (Kew et al., 1993a, b). Recent combined neuropsychological and PET activation studies have shown that ALS patients with impaired verbal fluency show reduced activation of the prefrontal and premotor cortex (Abrahams et al., 1995).

In this study we sought to extend these observations with PET using the benzodiazepine GABA A marker [11C]flumazenil. Because GABA A receptors are widely distributed in the cerebral cortex and are located both on pyramidal cells and interneurons, [11C]flumazenil PET provides a sensitive means of detecting extramotor as well as motor dysfunction in ALS.

**Subjects and methods**

**Subjects**

Seventeen non-demented patients with typical ALS were selected from the MND (Motor Neurone Disease) Care and Research Centre at King’s College Hospital, London. All the patients underwent a full medical history and examination, and on clinical and electrophysiological grounds fitted the El Escorial criteria for probable or definite ALS (Lambert and Mulder, 1957; Brooks, 1994). None of the patients had clinical evidence of cognitive impairment. Patients were not receiving riluzole or insulin-like growth factor. The patients were able to travel and to lie supine without moving for 2.5 h whilst in the PET scanner. In addition, 17 age-matched normal volunteers with no history of neurological disease or family history of ALS were scanned.

Of the 17 patients studied, 15 were male and two were female, with an age range of 24–72 years (mean 52.8 years). The patients had had ALS for 5–48 months (mean 18 months). The full clinical details are given in Table 1. One patient (Patient 10) had a 2 year history of maturity-onset diabetes, and another (Patient 7) had a 6 month history of polycythaemia rubra vera, treated with venesection. The 17 normal volunteers had an age range of 25–70 years (mean 49.0 years) and included 13 males and four females. All ALS subjects were judged to be cognitively intact and none of them had detailed neuropsychological test data. No statistically significant differences were found between the ALS subjects and controls (data not shown).

The PET study was approved by the ethics committees of the Hammersmith Hospital and the Institute of Psychiatry. Permission to administer [11C]flumazenil was obtained from the Administration of Radioactive Substances Advisory Committee (ARSAC), Department of Health, UK. Written informed consent was obtained from all the subjects according to the Declaration of Helsinki.

**PET scanning protocol**

The subjects were asked to abstain from alcohol for 48 h before the scan. None of the patients or normal subjects had been taking benzodiazepine drugs at the time of PET and all had been drug-free for at least 1 week before the study. A 21-gauge cannula was inserted into the radial artery after infiltration with 0.5% bupivacaine, to allow collection of blood samples. A 21-gauge cannula was also inserted into a vein in order to administer the radioactive labelled ligand. Subjects were positioned in the PET scanner with their head held in an individually constructed head-mould and positioned with the glabella–inion line parallel to the detector rings so that the transaxial planes were parallel to the intercommissural (AC–PC) line. The subject was observed throughout the scan by video monitoring to ensure the head did not move. Scans were performed with the subject in an awake resting state.

The scans were performed with an ECAT-953B PET scanner (CTI/Siemens, Knoxville, Tenn., USA) at the Medical Research Council Cyclotron Unit, Hammersmith Hospital.
London; the characteristics of this model of scanner have been described previously (Spinks et al., 1992). The data were acquired in 3D mode with the septa retracted to improve sensitivity by a factor of 6.4 (Bailey, 1992). Dual-energy window scatter correction was employed in reconstruction to produce images with a resolution of $4.8 \times 4.8 \times 5.2$ mm. Scans were displayed as 31 contiguous transaxial slices with pixel dimensions $2.09 \times 2.09 \times 3.43$ mm.

A short transmission scan was performed using three retractable rotating germanium-68 rods as sources to ensure the correct positioning of the head in the scanner. This was followed by a 19 min transmission scan to correct for attenuation of $\gamma$-radiation by skull and brain tissue. $[^{11}C]$Flumazenil tracer (10 mCi) of high specific activity, prepared by a modification of the method of Maziere (Maziere et al., 1984) was injected intravenously. A dynamic series of scans consisting of 20 time frames was acquired over a period of 90 min. Arterial blood radioactivity was measured continuously using an on-line bismuth germanate (BGO) detector and to derive the metabolite corrected input function, as described previously (Luthra et al., 1991). Spectral analysis was employed to minimize anatomical uncertainties, we co-registered to the standard stereotaxic space of Talairach and Tournoux (Talairach and Tournoux, 1988).

Data analysis
The scan data were analysed on a Sun SPARC classic workstation (Sun Microsystems, Mountain View, Calif., USA) using Imagentool software (CTI), Analyse version 7.0 (Biodynamics Research Unit, Mayo Foundation, Rochester, Minn., USA) (Robb and Hanson, 1990), MATLAB (Math Works Incorporated, Natick, Mass., USA) and Statistical Parametric Mapping (SPM) software (Wellcome Department of Cognitive Neurology, Institute of Neurology, London, UK).

Production of parametric maps
The 20 frames of the dynamic acquisition were initially co-aligned to one another by an automated least squares technique using software from our unit designed for this purpose in order to significantly reduce any movement artefact (Friston et al., 1995a). Spectral analysis was employed to produce voxel-by-voxel parametric images of flumazenil volumes of distribution (FMZVD) consisting of 31 contiguous slices with voxel dimensions $2.09 \times 2.09 \times 3.43$ mm (Cunningham and Jones, 1993). In the case of $[^{11}C]$Flumazenil, which has minimal non-specific binding, the volumes of distribution closely correlate with receptor availability ($B_{\text{max}}$) (Koepe et al., 1991).

Statistical parametric mapping
Statistical parametric maps are statistical processes that are used to localize regionally specific effects in imaging data (Friston et al., 1995b). SPM95 was used. After they had been realigned, all FMZVD images were transformed into the standard stereotaxic space of Talairach and Tournoux (Friston et al., 1995a). The procedure involves linear and quadratic 3D transformations followed by 2D non-linear matching using a set of smooth basis functions that allow normalization at a finer anatomical scale. The parameters were estimated using standard least squares after linearizing the problem. The images were then smoothed using a Gaussian filter ($10 \times 10 \times 6$ mm) both to minimize the effects of individual variations in gyral anatomy and to increase the signal-to-noise ratio (Friston et al., 1990). These procedures generated a normalized set of 26 planes with an interplane distance of 4 mm corresponding directly to the atlas of Talairach and Tournoux (Talairach and Tournoux, 1988).

The effect of variance in global FMZVD on focal values was removed by the use of analysis of covariance (ANCOVA) and by normalizing all global FMZVD values to an arbitrary value (Friston et al., 1990; Richardson et al., 1996, 1998). In this procedure, the mean of all voxels above a threshold value (volume of distribution) is calculated and used as the global mean in the ANCOVA. Because global normalization was undertaken, all differences between ALS subjects and controls are referred to as relative changes in FMZVD.

This process segments out CSF, white matter and extracranial regions so that the voxel values are adjusted relative to mean grey matter VD. In addition, this process excludes regions of markedly low cortical FMZVD such that even large regions of reduced relative FMZVD do not bias the global mean so as to artefactually increase areas after ANCOVA (Frey et al., 1996; Richardson et al., 1996, 1998). Differences in mean patient and control relative FMZVD values at each voxel were then compared by Student’s $t$-test. This resulted in a set of voxel $t$-values for each contrast (i.e. patient versus control) and generated a map of the $t$-statistic (a ‘$t$-map’).

Subsequently, these $t$-maps were transformed into a normal distribution to produce a statistical parametric map (SPM) of $Z$ scores. The SPM was thresholded at a $Z$ threshold of 3.09 (or $P = 0.001$). The resulting foci were then characterized in terms of spatial extent ($\kappa$) and peak height ($\mu$). The significance of relative FMZVD changes in each region was estimated by the use of distributional approximations from the theory of Gaussian fields. This looks at the probability that the observed cluster of voxels in a region of altered FMZVD could have occurred together by chance [$P(n_{\text{max}} > \kappa)$], or that the peak height observed could have occurred by chance [$P(n_{\text{max}} > \mu)$] over the entire volume subject to the analysis (i.e. a corrected $P$-value) (Friston et al., 1994). The threshold chosen for $P(n_{\text{max}} > \kappa)$ and $P(n_{\text{max}} > \mu)$ was $P < 0.05$.

In order to minimize anatomical uncertainties, we co-registered MRI data from eight normal controls with the PET ($[^{11}C]$Flumazenil) scans from these individuals, using SPM95. The PET images were then transformed to the standard template and co-registered MRI images were then transformed by the same parameters. Hence, MRI scans co-registered with the same individuals’ PET scans in the standard...
(approximately Talairach) space were produced. A ‘mean MRI image’ was then produced by averaging these eight MRI scans, voxel by voxel. Significant regions of relative change in flumazenil binding (FMZVD) were then superimposed on this mean MRI image.

Results
The PET data were analysed by considering the entire group of ALS patients in comparison with the controls. There was no evidence of significant focal increases in relative FMZVD in patients compared with the controls. However, widespread significant focal reductions in relative FMZVD were found (Fig. 1 and Table 2). At a threshold of $P < 0.001$ (corrected) there were areas of relatively reduced FMZVD which involved the left ventrolateral and dorsolateral prefrontal cortices (Brodmann areas 45, 46, 47 and 10), the right dorsomedial prefrontal cortex (areas 9 and 10) and the left dorsal prefrontal cortex (area 9), but only a small part of the motor and premotor cortex (areas 4 and 6). There was also a relative reduction of FMZVD in Brodmann area 44 on the left (Broca’s area), in area 21 on the right, and bilaterally in the superior parietal and visual association areas. When subgroups of patients with and without pseudobulbar features (defined as in Abrahams et al., 1997) were separately compared with controls, similar focal reductions in relative FMZVD were found in the ALS groups but there were no significant differences between patients with and without pseudobulbar features (data not shown). As predicted, a less conservative statistical threshold ($P < 0.05$) revealed more extensive reductions in relative FMZVD (Fig. 1B).

Discussion
Our [$^{11}$C]flumazenil PET study has shown decreases in relative FMZVD in the motor/premotor cortex in ALS, but perhaps more striking are the decreases in [$^{11}$C]flumazenil uptake in the prefrontal and other association areas of the cortex. These changes are unlikely to be an artefact of the SPM method used (Richardson et al., 1996, 1998; Koepp et al., 1998) and probably reflect involvement of these areas in the degenerative process.
Although the patients selected for this study were a little younger (mean age 53 years) than the average age for sporadic ALS patients, this probably reflects the pattern of referrals to a specialized research centre. The ratio of men to women in this study (7.5 : 1) was also higher than that seen in most epidemiological studies (Chancellor and Warlow, 1993; Dean et al., 1994). We feel it unlikely, however, that these factors would have influenced the general validity of our observations.

Flumazenil is an antagonist at the benzodiazepine subunit of the GABA<sub>A</sub> receptor. Reduced binding of [11C]flumazenil may reflect loss of cortical pyramidal and interneurones, which are thought to bear GABA<sub>A</sub> receptors, or it may reflect the downregulation of postsynaptic GABA<sub>A</sub> receptor expression in intact neurones. In the absence of precise knowledge about the location of GABA<sub>A</sub> receptors in the human brain, it is difficult to know which of these factors are most relevant; indeed, the observed reductions in FMZVD in ALS could reflect all the above mechanisms. However, as the prefrontal cortex has few pyramidal cells, the loss of these in isolation is unlikely to explain our findings. Additionally, changes in FMZVD in the primary motor cortex were less striking than those in the premotor and prefrontal areas, suggesting that the decreases are more likely to reflect loss of interneuronal function.

Histopathological studies examining the involvement of the extramotor cortex in ALS are limited. Smith demonstrated degenerating nerve fibres extending anteriorly from the precentral gyrus (motor cortex) into the prefrontal cortex, the postcentral gyrus (sensory cortex) and the parietal cortex adjacent to this area (Smith, 1960). In addition, degenerating nerve fibres were noted in the upper cingulate, temporal lobe and in the corpus callosum. In keeping with these findings, Kiernan and Hudson, using MRI, found evidence of loss of white matter underlying the anterior frontal lobes in ALS subjects (Kiernan and Hudson, 1994), and Kato and colleagues identified progressive atrophy affecting particularly the frontal and temporal lobes in ALS patients who were studied with serial CT and MRI scans (Kato et al., 1993).

In addition, several studies have shown pathological involvement of the thalamus and basal ganglia, although we found no evidence of decreased relative FMZVD in subcortical areas (Brownell et al., 1970; Hayashi and Kato, 1989). The majority of pathological studies have been limited to the motor and premotor areas that are known to be affected in ALS and have not examined the prefrontal or occipital areas systematically.

In classical ALS, cell loss targets the pyramidal neurones in layer V of the cortex and there is little evidence to support the loss of interneurones as an explanation for our observed reductions in FMZVD. However, in studies of ALS with dementia, marked cell loss has been reported in layers II and III of the cortex, the layers in which interneurones are most numerous. It has been suggested that the characteristic appearance of status spongiosus is due to loss of the dendrites and spines on cortical cells (Horoupian et al., 1984). This is not, however, proven and it could reflect loss of interneurones.

Post-mortem studies of GABA and GABAergic markers have been inconclusive in ALS; Yoshino and colleagues reported normal tissue GABA levels (Yoshino et al., 1979), whereas Perry and colleagues showed significantly reduced levels in the cerebellum, caudate nucleus, brainstem and spinal cord and a trend towards reduction in the frontal and occipital lobes (Perry et al., 1987). These neurochemical studies did not identify which cells were involved in the pathological process.

Parvalbumin and calbindin D28K are intracellular calcium-binding proteins and are particularly associated with GABAergic interneurones, acting as neuronal markers for these cells. It is postulated that they play a role in intracellular calcium buffering, preventing hyperpolarization of cells and possibly protecting them from excitotoxic damage (Ince et al., 1993). Ince and colleagues did not find decreased parvalbumin

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in the motor cortex of ALS patients (Ince et al., 1993), but reductions in calbindin D28K have been found in patients with frontotemporal dementia associated with ALS (Ferrer et al., 1993); and reductions in both calbindin D28K and parvalbumin were found in dementia of the frontal type associated with hereditary spastic paraparesis (Ferrer et al., 1995). These pathological studies would support our interpretation that reduced FMZVD in ALS reflects neuronal loss, and more specifically the loss of GABAergic interneurones. However, as none of these pathological immunocytochemical studies has examined the prefrontal cortex in non-demented ALS patients, we cannot be sure whether our prefrontal findings can be interpreted as reflecting loss of GABAergic (or other) interneurones in ALS patients.

It is interesting that we did not find more extensive changes in relative FMZVD in the primary motor cortex. This observation may, however, be in keeping with our hypothesis that changes in relative FMZVD are largely attributable to damage to or loss of interneurones. Corticospinal tract degeneration in ALS may be predominantly a dying back process (Cavanagh, 1979; Chou, 1995) and loss of large pyramidal neurones or giant Betz cells in the primary motor cortex is not always evident (Davison, 1941; Brownell et al., 1970; Chou, 1995). It has long been known that the postcentral as well as the precentral cortex may show neuronal loss in ALS (Charcot and Marie, 1885; Holmes, 1909). In the monkey, corticospinal neurones are found in the precentral regions as well as in the primary motor cortex (Dum and Strick, 1991). Our findings are in keeping with cell loss in this extensive area of origin of the corticospinal tracts, but also indicate the involvement of non-motor areas (e.g. the dorsolateral and dorsomedial prefrontal areas).

The finding of reductions in cortical \([^{11}C]\)flumazenil binding are broadly in keeping with previous metabolic and blood flow studies using PET and single photon emission computed tomography (SPECT). Global resting blood flow has been reported to be reduced throughout the brain, particularly in the frontal cortex, in SPECT studies in ALS (Waldemar et al., 1992). PET studies using FDG and region-of-interest analysis have shown extensive decreases in resting metabolism throughout the frontal, temporal, parietal and occipital cortex, although changes were most marked in the motor cortex in patients with typical ALS (Dalakas et al., 1987; Hatazawa et al., 1988; Ludolph et al., 1992). Although Hatazawa and colleagues found widespread reduction in the cerebral metabolic rate for glucose (CMRG) in ALS subjects compared with controls (Hatazawa et al., 1988), this was not confirmed by a later FDG study (Hoffmann et al., 1992). Tanaka and colleagues noted reductions in blood flow and oxygen metabolism in the anterior frontal lobes in non-demented ALS patients, although this did not reach significance (Tanaka et al., 1993). Taking into account advances in data analysis (including the use of SPM analysis, which reduces the bias inherent in region-of-interest analysis), and the use of Talairach coordinates to relate changes in tracer or ligand distribution to standard stereotaxic space, the changes we have found in relative FMZVD are likely to reflect relatively localized changes in brain pathology in ALS. The use of SPM analysis in this study allowed us to detect the locations of regions of focal reductions in relative FMZVD without having to make prior assumptions as to their whereabouts. This is because SPM compares binding across the entire brain volume in an exploratory fashion, unlike region-of-interest analysis, in which locations have to be defined a priori.

In keeping with these changes in relative FMZVD, previous PET activation studies from our unit, using a joystick movement paradigm, have also shown extensive cortical involvement extending beyond the primary motor cortex in non-demented patients with classical ALS. Regions of reduced resting rCBF included the primary sensorimotor and lateral premotor cortex, the supplementary motor area, the anterior cingulate cortex, the paracentral lobule and the parietal cortex (Kew et al., 1993b). These areas were subsequently shown to be disinhibited during the performance of paced joystick movements in freely selected directions whereas prefrontal areas were underactive. PET activation studies using a Verbal Fluency Index (VFI) have demonstrated extensive prefrontal and premotor underactivity in ALS (Abrahams et al., 1996). These findings are all consistent with our finding of reduced prefrontal relative FMZVD in non-demented patients with ALS. It would appear that \([^{11}C]\)flumazenil binding is a sensitive method for detecting prefrontal dysfunction in ALS, as it reveals abnormalities in prefrontal areas at rest in non-demented patients, whereas rCBF studies require the use of activation paradigms.

In summary, \([^{11}C]\)flumazenil PET demonstrates widespread cortical dysfunction in clinically non-demented patients with ALS. We cannot be certain which GABA\(_A\)-bearing cells are dysfunctional, but it is likely that corticospinal neurones, other pyramidal neurones and interneurones are all involved. A recent study using an unbiased stereological approach to neuronal counting found no evidence of neuronal loss in the motor cortex and extramotor cortex in ALS (Gredal et al., 2000). However, more detailed and extensive analyses using this technique (Hyman et al., 1998) are required before we can conclude that there is no significant neuronal loss in the motor or extramotor cortex in ALS.

Whatever the cellular basis for the changes observed, this study provides further evidence to support the concept that, although motor neurones are most vulnerable, ALS is a multi-system degeneration.

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