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EXPRESS COMMUNICATION

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Autoradiography with [^{3}H]PK11195 of spinal tract degeneration in amyotrophic lateral sclerosis

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Abstract The diagnostic hallmarks of amyotrophic lateral sclerosis (ALS) are degeneration of upper and lower motor neurons and of corticospinal tracts. Here, we demonstrate the suitability of the gliosis marker [^{3}H]PK11195 for quantitative evaluation of tract degeneration in ALS in vitro. Binding of [^{3}H]PK11195 was increased in lateral and ventral white matter of ALS spinal cords but not in the anterior horn, in spite of a dramatic loss in muscarinic binding sites and a high level of oxidatively modified protein. Labeling of activated microglia with [^{11}C]PK11195 may also allow tract degeneration in ALS to be visualized in vivo.

Keywords Amyotrophic lateral sclerosis · Peripheral benzodiazepine receptor · Activated microglia

Introduction

The diagnostic hallmarks of amyotrophic lateral sclerosis (ALS) are degeneration of motor neurons in spinal cord and brain stem, and of cerebral motor neurons giving rise to lateral and ventral corticospinal tracts. The most common (sporadic) form of this neurodegenerative disease does not usually occur before 50 years of age, but then rapidly progresses to severe motor disability and death. No effective treatment is available.

The density of binding sites for the gliosis marker [^{3}H]PK11195 (a peripheral-type benzodiazepine receptor ligand unrelated to the GABA receptor complex) is low in the CNS under normal conditions; increased binding has been demonstrated in the brain of experimental animals after seizure-induced [3] and after ischemic [3, 5, 13] brain damage, and post mortem in brain tissue from patients suffering from Alzheimer's disease [6], multiple sclerosis (MS) [2, 14], stroke and astrocytoma [2]. In human imaging studies in vivo, [^{11}C]PK11195 has been used to visualize glioblastoma [10] and acute white matter lesions in MS [14]. Using spinal cord from four sporadic and one juvenile case, we assessed for the first time the autoradiographic distribution of [^{3}H]PK11195 binding sites in the spinal cord of patients dying at the end stage of ALS. For comparison, we also visualized the autoradiographic distribution of muscarinic binding sites using [^{3}H]QNB (a sensitive marker for motor neurons [4]), the distribution of material immunoreactive for HLA-DR (a microglia marker) and, as an indicator for oxidative stress, determined the content of oxidatively modified protein in microdissected specimens [12].

Patients and methods

Spinal cords (cervical and thoracic levels) were collected from five patients who died with ALS (four sporadic cases, age 57–66 years; one juvenile case: age 24 years), and from seven controls who died of non-neurological diseases (age 50–78 years). The tissue was removed 7–20 h after death and immediately frozen on dry ice. For histology, 4-μm-thick sections were routinely stained with H&E, Luxol-fast blue (for myelin), and Kanzler (for fibrillary gliosis) stains. Immunocytochemistry was performed on three ALS cases (two sporadic and one juvenile case) and three controls using a monoclonal antibody against HLA-DR (Dako, 1:100) to visualize the distribution of microglia. As a secondary system we used Dako's optimized staining system. All neurochemical parameters obtained from the juvenile case fell within the range of the sporadic cases; therefore, data obtained from all five ALS patients were pooled. For determination of protein carbonyl content, spinal cord was dissected according to the method of Malessa et al. [8] and samples were treated according to the method of Shaw et al.

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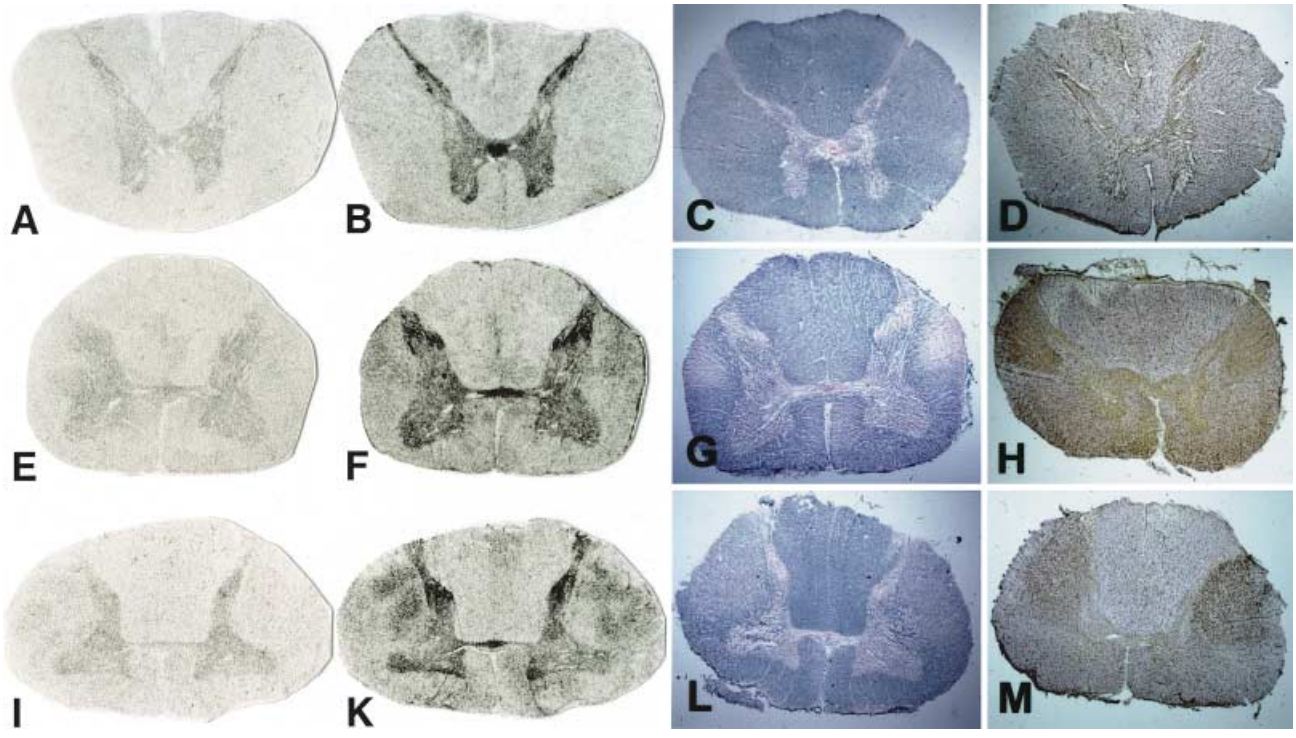

Fig. 1 Transverse sections of the spinal cord from a control subject (**A**–**D**), a sporadic (**E**–**H**) and a juvenile (**I**–**M**) ALS patient. The *first two columns* (**A**,**E**,**I** and **B**,**F**,**K**) represent quantitative slice autoradiography of [^{3}H]PK11195 binding. The *first column* (**A**,**E**,**I**) denotes unspecific binding in the presence of an excess of unlabeled ligand, the *second column* (**B**,**F**,**K**) total binding of [^{3}H]PK11195. *Column three* (**C**,**G**,**L**) demonstrates severe loss of myelin in the corticospinal tracts of ALS spinal cords (**G**,**L**) compared to normal control (**C**); Luxol-fast blue staining. *Column four* (**D**,**H**,**M**) shows accumulation of microglia in the corticospinal tracts of ALS spinal cords (**H**,**M**) compared with normal control (**D**); anti-HLA-DR staining. **A**–**M** ×3.6

[12] using reaction with 2,4-dinitrophenylhydrazine. The spectrum was read for each sample between 340 and 400 nm and the carbonyl content was calculated relative to reagent blank using a molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$. For autoradiographic studies, 16-μm sections were cut in a cryostat, mounted on coated glass slides, and incubated with the muscarinic radioligand [^{3}H]QNB as described [4]. Peripheral-type benzodiazepine binding sites were labeled by incubating slide-mounted sections in 50 mM TRIS acetate buffer, pH 7.0, containing 3 nM [^{3}H]PK11195 (85.5 Ci/mmol, NEN, Boston, Mass.) for 2 h on ice; slices were washed three times for 1 min in cold buffer, dipped in distilled water and rapidly dried in a stream of air. Dried sections were processed for autoradiography and densitometry as described [4]. Statistical significance, as given in the tables, was calculated using the *t*-test for unpaired samples.

Results

Histology confirmed the classical findings of a motor neuron disease with loss of motor neurons and astrogliosis in the anterior horn and degeneration of corticospinal tracts (Fig. 1G, L); findings which were absent in controls (Fig. 1C). Kanzler stain revealed focal astrocytic gliosis in the lateral and ventral white matter (not shown). Immunocytochemistry for HLA-DR demonstrated accumulation of microglia in the corticospinal tracts of ALS patients (Fig. 1H, M) but not in controls (Fig. 1D).

[^{3}H]PK11195 binding was hardly detectable in the white matter of control spinal cords (Fig. 1B). However, in the ALS material the density of these sites was increased in patches, comprising large parts of the lateral and the ventral white matter, with no labeling of the dorsal white matter (Fig. 1F, K). In the gray matter, high levels of [^{3}H]PK11195 binding were detected in both ALS and control tissue, with the highest values in the substantia gelatinosa (posterior horn). The density of [^{3}H]PK11195 binding sites in these gray areas was not significantly al-

Table 1 Specific binding of [^{3}H]PK11195 (3 nM) as determined by quantitative slice autoradiography with tissues taken from the cervical and the thoracic level of ALS and control spinal cords; means ± SD (fmol/mg tissue) (*ALS* amyotrophic lateral sclerosis)

	Control (*n*=7)	ALS (*n*=5)	Significance
Cervical			
Anterior horn	207±139	251±112	n.s.
Posterior horn	286±125	289±114	n.s.
Lateral white	12± 16	180±157	$P<0.05$
Ventral white	11± 9	139± 78	$P<0.05$
Dorsal white	31± 22	29± 14	n.s.
Thoracic			
Anterior horn	147± 35	258±142	n.s.
Posterior horn	217±126	279±109	n.s.
Lateral white	11± 7	208±164	$P<0.01$
Ventral white	7± 6	141±130	$P<0.01$
Dorsal white	20± 21	24± 19	n.s.

Table 2 Specific binding of [^{3}H]QNB (1 nM) as determined by quantitative slice autoradiography with tissues taken from the cervical and the thoracic level of ALS and control spinal cords; means ± SD (fmol/mg tissue)

	Control (n=7)	ALS (n=5)	Significance
Cervical			
Anterior horn	50.9± 6.8	25.2± 5.9	P<0.01
Posterior horn	38.5±10.2	51.3±11.8	n.s.
Thoracic			
Anterior horn	52.7±11.2	23.1± 5.6	P<0.01
Posterior horn	41.6±12.2	35.1±10.5	n.s.

Table 3 DNP-reactive material ("protein carbonyl content") of tissues taken from the thoracic level of ALS and control spinal cords; means ± SD (nmol DNP/mg protein) (*DNP* dinitrophenylhydrazine)

	Control (n=5)	ALS (n=4)	Significance
Thoracic			
Anterior horn	1.5±0.2	3.7±1.2	P<0.01
Posterior horn	1.3±0.5	2.1±0.5	n.s.
Lateral white	1.1±0.6	2.7±0.8	P<0.05
Dorsal white	1.1±0.3	1.3±0.2	n.s.

tered by the disease process (Table 1). In contrast, a dramatic loss in muscarinic binding sites was observed in the anterior horn of ALS spinal cord (more than 50% at both the cervical and the thoracic level Table 2). In addition, microdissected tissue from ALS spinal cords consisting predominantly of anterior horn gray matter contained three times more material reactive for dinitrophenylhydrazine than equivalent material from control spinal cord (Table 3), indicative of a high level of oxidatively modified protein in pathological tissue.

Discussion

It has been repeatedly demonstrated that the appearance of specific binding sites in CNS tissue for the peripheral-type benzodiazepine ligand [^{3}H]PK11195 reflects immigration of activated microglia in response to injury and neuronal degeneration [1, 5, 13]. Even subtle injuries resulting in retrograde or anterograde degeneration of neuronal pathways can be visualized by [^{3}H]PK11195 autoradiography [1]. Our results, obtained both with [^{3}H]PK11195 and with the immunological microglia marker HLA-DR, suggest that the appearance of activated microglia accompanies the degeneration of the motor pathways descending in the spinal cord, a characteristic pathological feature of ALS. To our surprise, the increase in [^{3}H]PK11195 binding did not reach significance in the anterior horn of ALS spinal cords, although other parameters indicated oxidative damage and loss of more than 50% of motor neurons in that area. Others have found increased MAO-B in ALS ventral horn and have interpreted this finding as reactive gliosis [7]. While peripheral-type benzodiazepine binding sites are predominately situated in organs outside the blood-brain barrier, they are also found in the outer mitochondrial membrane of neurons, without any relationship to activated microglia [11]. If a certain degree of microglia immigration also occurred in the ventral horn of ALS spinal cords, it may have been unrecognizable against this considerable background of neuronal [^{3}H]PK11195 binding, given the relatively low number of cases studied. Alternatively, degeneration of motor neurons in ALS may proceed via some type of apoptotic cell death program [9] involving oxidative stress, but to a lesser degree the immigration of activated microglia.

In conclusion, we demonstrated the suitability of a high-affinity radioligand for quantitative evaluation of tract degeneration in ALS in vitro; although [^{11}C]PK11195 has already been used as a gliosis marker in the human brain in vivo [2, 10, 14], its application as a diagnostic tool to the living spinal cord awaits further refinements in the resolution of current tomographic techniques.

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