Amyotrophic lateral sclerosis: delayed disease progression in mice by treatment with a cannabinoid

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Introduction

Amyotrophic lateral sclerosis (ALS) is the third most common neurodegenerative cause of adult death, after Alzheimer’s disease and Parkinson’s disease.1,2 ALS results in the degeneration of motor neurons in the cortex, brainstem and spinal cord.1,2 Most causes of ALS are presently unknown and several mechanisms of insult to motor neurons have been suggested.3–5 Two of the primary theories underlying motor neuron vulnerability are susceptibility to excitotoxicity and oxidative damage.4,5 Cannabinoid agonists have been reported to reduce both excitotoxic and oxidative cell damage.6,7 D9-tetrahydrocannabinol (Δ9-THC) was effective if administered either before or after onset of signs in the ALS mouse model (hSOD1G93A transgenic mice). Administration at the onset of tremors delayed motor impairment and prolonged survival in Δ9-THC treated mice when compared to vehicle controls. In addition, we present an improved method for the analysis of disease progression in the ALS mouse model. This logistic model provides an estimate of the age at which muscle endurance has declined by 50% with much greater accuracy than could be attained for any other measure of decline. In vitro, Δ9-THC was extremely effective at reducing oxidative damage in spinal cord cultures. Additionally, Δ9-THC is anti-excitotoxic in vitro. These cellular mechanisms may underlie the presumed neuroprotective effect in ALS. As Δ9-THC is well tolerated, it and other cannabinoids may prove to be novel therapeutic targets for the treatment of ALS. (ALS 2004; 5: 33-39)

Keywords: amyotrophic lateral sclerosis – Δ9-THC – cannabinoid – anti-oxidant – anti-excitotoxicity – neuroprotection
bred with background matched B6SJL wild type females (Jackson Laboratories, Bar Harbor, ME). The total DNA was isolated from tail clips of the progeny by proteinase K digestion and subsequent phenol-chloroform extraction. The progeny were genotyped using primers specific to exon 4 of the human SOD1 gene within the transgenic construct and segregated and used for subsequent studies. Transgenic mice were housed in micro-isolator cages in a barrier facility and were seronegative for mouse hepatitis virus, Sendai virus and other common viral and bacterial pathogens. The mice were observed twice a week during the first 60 days of age and subsequently monitored every day for general health and signs of illness. Body weight was taken once a week and every day after the onset of disease.

Treatment protocols
Male hSOD1<sup>G93A</sup> mice were injected intraperitoneally with vehicle (18:1:1 ratio of normal saline: emulphor: ethanol) or 5 or 10 mg/kg body weight Δ⁹-THC (Research Triangle Park, NIDA/NIH) dissolved in the same vehicle, daily from day 60 of their age. Alternatively, mice were administered 20 mg/kg Δ⁹-THC or vehicle beginning on day 75 when tremors were first observed. Mice were given ad libitum access to food and water, including moistened food on the floor after the onset of disease.

Evaluation of motor function
Motor neuron function in mice was evaluated using a rotarod (Accuscan Instruments, Columbus, OH). Mice were trained on the rotarod for 10 minutes at speeds of 5 and 10 rpm beginning at 40 days of age. After the treatment started the mice were evaluated on the rotarod at 10 rpm on a weekly basis. The time they remained on the rotarod was registered automatically. If the mouse remained on the rod for 10 minutes, the test was completed and scored as 10 minutes. Mice were tested on the rotarod at 5 rpm after failing at 10 rpm in order to follow the decrement in motor function.

Clinical end points
The clinical condition of mice was monitored twice a week after entry into each protocol and observed daily after entering into the treatment schedule. The earliest clinical signs observed were tremors and shaking of their limbs when mice were suspended briefly in the air by their tails. Progression of disease was measured by the decrement in the ability of mice to remain on the rotarod. To determine ‘mortality’ as an independent measure and humanely, mice were euthanized when they could not right themselves within 3 seconds after being placed on their sides.

Oxidative damage in mixed spinal cord cultures
Mixed spinal cord cultures were prepared from 13-day-old mouse embryos using methods previously described. At week 1 in culture. To induce oxidative damage, cultures were treated for 5 hours in the presence of 200 μM tert-butyl hydroperoxide (TBH). The compounds evaluated were added at the same time as TBH. Δ⁹-THC (0.5 μM) and SR14716A (1 μM) were diluted from 1 mM stock solutions in ethanol; the final concentration of ethanol was kept constant in all treatments (0.1%). After the 5-hour treatment, cell viability was quantified by a combination of lactate dehydrogenase (LDH) activity and visually confirmed by propidium iodide fluorescence.

Statistical analysis
A logistic response curve was fitted to the endurance time for each mouse using a nonlinear mixed effects model. The model is described by the equation:

\[
\text{Time} = 10(1 + \exp((\text{Age} - A)/B))^{-1},
\]

where Time is rod endurance time (range 0–10 mins), Age is mouse age in days, and A and B are constants representing day at which endurance is reduced to 50% (A) and rate of decline (1/B). The fitting program allows A and B to vary from mouse to mouse and can be used to test whether A and B differ by drug dose, delay in treatment and rpm of the rod. First we fitted the model allowing both A and B to vary from mouse to mouse and can be used to test whether A and B differ by drug dose, delay in treatment and rpm of the rod. First we fitted the model allowing both A and B to vary from mouse to mouse, but neither A nor B were dependent on dose, delay or rpm. Next, we fitted a sequence of models starting with A and B each a linear function of dose and rpm, (A=0.01+A1 Dose +A2 delay +A3 rpm, and B=B0 +B1 Dose +B2 delay +B3 rpm). The coefficients for A and B and their standard errors were calculated by the program and Wald statistics were used to test whether each of the coefficients (A0, A1,...,B3) differed significantly from zero. A and B terms with nonsignificant (P>0.05) coefficients were removed and the model was refitted. This process was repeated until all remaining terms were taken into account. Summary fit curves were prepared for all mice in each treatment group. Survival data were summarized by Kaplan-Meier curves. For survival, a Cox proportional hazard model was fitted to the data and the effect of dose was tested by the likelihood ratio test and Wald statistics. First we tested for a difference among all 3 doses (log-rank test P=0.003). Then we tested pairwise differences finding that each dose effect on survival was significantly different from vehicle (0) dose (P=0.004 for 10 mg and P=0.01 for 20 mg) but that there was no difference between 10 and 20 (P=0.56). All calculations were carried out in S-Plus version 6. Mortality results are expressed as means of individual animals with s.e.m. per group. Results of in vitro experiments are expressed as means of cultures±s.e.m. per group. These data were assessed using an unpaired t-test with GraphPad Prism software (GraphPad, San Diego, CA).

Results
Δ⁹-THC delays disease progression and improves survival
The primary aim of this study was to evaluate the effectiveness of a cannabinoid in the treatment of a mouse model of ALS. A second aim was to improve the analysis of disease progression in the mouse model of ALS by using a more
powerful statistical model. hSOD1<sup>G93A</sup> mice were administered Δ<sup>9</sup>-THC (5 and 10 mg/kg body weight) or vehicle beginning at 60 days of age, i.e., prior to onset of motor dysfunction. The earliest clinical signs of disease observed were tremors and shaking of their limbs when mice were suspended briefly in the air by their tails. These signs were never seen in non-transgenic littermates, but were always seen in hSOD1<sup>G93A</sup> mice after 75 days. In a subsequent set of experiments, mice were administered 20 mg/kg Δ<sup>9</sup>-THC beginning on day 75 when tremors were first observed, i.e., after onset of disease signs. Mice were evaluated on a rotarod to follow disease progression. No changes in motor behavior were observed in the Δ<sup>9</sup>-THC treated animals at any of the doses given, confirming earlier reports. Furthermore, no significant difference in weights was observed between the treatment groups.

In order to assess the effect of Δ<sup>9</sup>-THC on disease progression, a logistic response curve was fitted to the endurance time for each mouse using a nonlinear mixed effects model. For these experiments, each mouse was assessed on the rotarod at 5 and 10 rpm. Figure 1A shows the observed data and fitted endurance curves at 10 rpm for each mouse. The results from the nonlinear mixed effects model showed that both dose and rpm (but not delay in treatment initiation) significantly affected age at which endurance declined to 50% (i.e., 5 minutes, abbreviated as A50% henceforth). These results are summarized in Table 1 and shown graphically in Figure 1B. We found that A50% increased 3.3 days (±1.1 day) per 10 mg/kg of THC (2-sided P=0.003). In other words, disease progression as assessed by rotarod performance was delayed 3.3 days in the 10 mg/kg group and 6.6 days in the 20 mg/kg group as compared to the vehicle treated animals. This represents a 3% increase in motor performance endurance in the 10 mg/kg group and a 6% increase in the 20 mg/kg group.

We were able to assess animals later in the disease by testing them at the slower rotarod speed (5 rpm). In the data analysis, we found that A50% increased 6.5 days (±0.4 day) when changing from 10 rpm to 5 rpm. However, compared with the rate of 10 rpm, the rate at 5 rpm was increased by a factor of 2. This rate of decline can also be inferred by expressing it as number of days to decline from 9 minutes to 1 minute endurance, based on the fitting equation, i.e., at 10 rpm, it took 17.1 (±1.76) days to decline from 9 to 1 minute compared with 8.55 (±0.88) days at 5 rpm. These results are summarized in Table 2 and shown graphically in Figure 1B.

In these experiments, the treatment effect was to slow the progression of disease (Figure 1b). Furthermore, treatment with Δ<sup>9</sup>-THC improved survival. Treatment with 10 mg/kg Δ<sup>9</sup>-THC extended mean survival from 125.9±1.6 days (vehicle, n=15) to 131.8±2.4 days (Δ<sup>9</sup>-THC, n=8, P=0.004) (Figure 2a, c). This represents a 4.9 day (4.6%) increase in survival in the 10 mg/kg Δ<sup>9</sup>-THC-treated group. At a (delayed) dose of 20 mg/kg the increase in mean survival was 6.4 days (5.1%) (P=0.01, Figure 2a,c).

Δ<sup>9</sup>-THC is anti-oxidant and anti-excitotoxic in vitro

Δ<sup>9</sup>-THC is known to be effective in attenuating in vitro excitotoxic and oxidative cell damage. Both of these mechanisms have been implicated in the progression of ALS. We had previously reported that Δ<sup>9</sup>-THC was as effective as the anti-excitotoxic compound NBQX, an AMPA/Kainate receptor antagonist, in protecting spinal cord neurons against direct excitotoxin (kainate) exposure. To evaluate the possibility that Δ<sup>9</sup>-THC may also have antioxidant properties in spinal
Table 1
Summary of results

<table>
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<td>20</td>
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<td>8.55</td>
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</tr>
<tr>
<td>10</td>
<td>20</td>
<td>106.6</td>
<td>17.10</td>
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Table 2
Predictions based on model

Figure 2
Δ⁹-THC extends survival in hSOD1^{G93A} mice. Cumulative survival in hSOD1^{G93A} mice treated with 10 mg/kg Δ⁹-THC, begun at 60 days of age (a) or 20 mg/kg Δ⁹-THC, started at 75 days of age. Vehicle controls were treated at 60 days and 75 days of age, respectively; no difference was seen in the lifespan between the two vehicle treatment paradigms, so the data were pooled for analysis. (b). Mortality of hSOD1^{G93A} mice treated with Δ⁹-THC or vehicle (c). Survival was significantly increased in the 10 and 20 mg/kg Δ⁹-THC treated groups compared to vehicle controls (*, P=0.004, P=0.01, respectively, error bars represent s.e.m.).
cord cultures, we determined whether Δ²-THC could protect against oxidative damage produced by direct application of the oxidant tert-butyl hydroperoxide (TBH).\textsuperscript{23} Δ²-THC was extremely effective at reducing oxidative damage produced by TBH in mixed spinal cord cultures. Exposure to 200 μM TBH for 5 hours resulted in 74 (±14) % cytotoxicity, which was reduced to 28 (±8) % in the presence of 0.5 μM Δ²-THC (n=4, P<0.001) (Figure 3). These quantitative data were visually confirmed by measuring propidium iodide uptake in parallel cultures (data not shown): both neurons and glia were affected by TBH as previously published.\textsuperscript{23} To test if this profound antioxidant effect was CB\textsubscript{1} receptor mediated, 1 μM SR141716A was used to block the receptor (Figure 3). There was no difference in the level of protection, suggesting the antioxidant effect was not CB\textsubscript{1} receptor mediated.

**Discussion**

Our study is innovative in at least two characteristics. First, treatment at 20 mg/kg began after first appearance of disease signs. This design more realistically mimics treatment in humans where it is not possible to treat prior to symptom onset. Secondly, the use of a nonlinear mixed effects model for data analysis increased statistical power to detect a large range of effects and to test separately for delayed disease onset and rate of disease progression. Use of the model eliminates the complication of defining an age at onset based on observed symptoms. The use of a logistic model provides an estimate of the age at which muscle endurance has declined by 50% with much greater accuracy than could be attained for any other measure of decline. This is because statistically maximum precision is attained at the median of a logistic regression. Use of the logistic model also allowed us to test whether the dose effect at 20 mg/kg was diminished by delaying treatment until the first appearance of disease signs. The results showed that endurance and survival could be increase in the hSODG\textsuperscript{G93A} mice even when Δ²-THC was administered after the onset of disease.

The mechanisms for neuroprotection by Δ²-THC appear to be multifaceted. In this study, the antioxidant neuroprotective effect of Δ²-THC was not CB\textsubscript{1} receptor mediated because the CB\textsubscript{1} receptor antagonist SR141716A did not diminish the antioxidant effect. CB\textsubscript{2} receptors were detected in neurons and glia in the spinal cord cultures by immunocytochemistry and Western blot analysis, thus the lack of effect of receptor antagonist is not due to the absence of receptors in the cultures.\textsuperscript{10} CB\textsubscript{2} receptor expression has not been demonstrated in neurons or astrocytes though we can readily detect CB\textsubscript{2} receptor expression in cells from the immune system.\textsuperscript{28} Other cannabinoid receptor subtypes have been proposed, however they have not been identified. Thus while it is possible that other cannabinoid-type receptors could be involved with the antioxidant effect, there are no reagents to test this possibility. Previous investigations into the potential neuroprotective effects of cannabinoids have focused on models of cerebral ischemia\textsuperscript{9,29}, multiple sclerosis\textsuperscript{11} and epilepsy.\textsuperscript{26} Our results are in line with the in vitro models of cerebral ischemia in which the predominant neuroprotective effect of cannabinoids is as an antioxidant but not CB\textsubscript{1} receptor mediated.\textsuperscript{7,29} In addition, cannabinoids were equally effective as neuroprotective antioxidants in cultured neurons from CB\textsubscript{2} receptor knockout mice or control wild-type littersmates.\textsuperscript{30} Furthermore, cannabidiol and (+)-11-OH-Δ⁴-THC (HU-211) which have no CB\textsubscript{1} receptor activity are also effective antioxidants.\textsuperscript{7} Δ⁴-THC, cannabidiol and HU-211 were found to possess greater antioxidant properties than either ascorbate or α-tocopherol, similar to that of BHT.\textsuperscript{7}

The antioxidant properties of Δ²-THC may also contribute to the attenuation of excitotoxicity in primary neuronal cultures.\textsuperscript{7,31} We previously found that Δ²-THC protected against kainate-mediated toxicity in mixed (neuronal and glial) spinal cord cultures.\textsuperscript{10} The amount of cytotoxicity produced by kainate was about half of that produced by the oxidative stress paradigm described in this report, but is consistent with a neuronal toxicity (excitotoxicity) as opposed to a combined neural and glial toxicity (oxidative stress).\textsuperscript{23} That Δ⁴-THC protects glia as well as neurons may contribute towards its effectiveness in the hSODG\textsuperscript{G93A} mice. Astrocytic cell damage is believed to contribute to the disease process by suppressing the activity of EAAT2 glutamate transporters that are necessary for recovering synaptic glutamate and/or preventing repetitive motor neuron firing.\textsuperscript{3}

A potential therapeutic application of Δ²-THC for the symptoms of ALS includes the relief of spasticity.\textsuperscript{6,32–34} Similar results were found in a mouse model of multiple sclerosis in which cannabinoids quantitatively ameliorate both tremor and spasticity.\textsuperscript{11} Recently, increased levels of the endogenous ligands anandamide, 2-arachidonylethanolamide were found in the brain and spinal cord, areas associated with the induced nerve damage.\textsuperscript{12} The endogenous ligands were also anti-spastic; in addition, inhibitors of re-uptake and hydrolysis were shown to significantly attenuate spasticity.\textsuperscript{12}

In order for a pharmacological treatment to be clinically effective for ALS, it must be able to protect the remaining motor neurons (as up to 50% are lost at the time of clinical diagnosis).\textsuperscript{35} Other treatments to date have focused on antioxidants, neurotrophic, immunosuppressive and neuroprotective...
agents, most of which have been shown to be neuroprotective in cellular and animal models of ALS. Nevertheless, over 25 large clinical studies based on these strategies have shown no benefit, apart from riluzole. As a result, riluzole is the only drug approved and marketed for the treatment of ALS. However, candidate compounds are usually tested in the mouse model by administration prior to the onset of disease signs, which may explain their failure in clinical trials.

An important additional consideration is that ALS is a chronic disease, therefore long-term toxicity of treatment drugs becomes an important issue. Δ9-THC is well tolerated and already in clinical usage for nausea associated with cancer chemotherapy and appetite stimulation with the AIDS wasting syndrome. In a pilot study of the safety and tolerability of Δ9-THC in ALS patients, symptomatic benefits were seen in insomnia, appetite and spasticity. Δ9-THC is effective in a pre-clinical model of ALS, suggesting it could be evaluated for its effectiveness in the human disease.

Conclusion

The data presented here indicate that Δ9-THC delays progression of disease and increases survival time in hSODG93A mice even when administered after onset of signs. Our finding that Δ9-THC is both anti-excitotoxic and antioxidant in vitro suggests it may act additively towards its therapeutic effect in the hSODG93A mice.

Acknowledgements

The authors would like to thank Robert G. Miller, Billy R. Martin and George Kunos for critical reading of the manuscript, and the ALS and Neuromuscular Research Foundation and NS041639 for support.

References