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Differential expression of the NMDA NR2B receptor subunit in motoneuron populations susceptible and resistant to amyotrophic lateral sclerosis

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Abstract

We have compared the expression pattern of NMDA receptor subunits (NR1 and NR2A-D) and NR1 splice variants (NR1-1a/1b,-2a/2b,-3a/3b,-4a/4b) in motor neuron populations from adult Wistar rats that are vulnerable (hypoglossal, XII) or resistant (oculomotor, III) to death in amyotrophic lateral sclerosis (ALS). The major finding was higher levels of expression of the NR2B subunit in the hypoglossal nucleus. Quantitative real-time PCR showed that NR1 was expressed at a greater level than any of the NR2 subunits (>15 fold greater, $P \leq 0.05$, $n = 11$ animals), while conventional RT-PCR showed no difference in NR1 splice variant expression (with all variants except NR1-3 detected in both nuclei; $n = 6$ animals). Within III, the NR2B subunit was expressed 1.7 to 2.6-fold lower than the other NR2 subunits ($P \leq 0.05$), while in XII all NR2 subunits were expressed at equal levels. When comparing levels between the 2 nuclei, mRNA for the NR2B subunit was expressed 2.1-fold higher in XII compared to III ($P \leq 0.05$), while their was no difference in mRNA expression for the other subunits. Immunohistochemical analysis confirmed greater NR2B protein levels within individual hypoglossal neurons compared to oculomotor neurons (1.8-fold greater, $P \leq 0.05$, $n = 5$ animals). Lower expression of the NMDA NR2B subunit may constitute one factor conferring protection to oculomotor neurons in ALS.

Keywords: Glutamate receptors; Oculomotor; Hypoglossal; Quantitative real-time PCR; Immunohistochemistry; NR2B

Amyotrophic lateral sclerosis (ALS) is a common neurodegenerative disease of adult onset characterized by progressive loss of most cranial and spinal motor neurons (MNs). A striking characteristic of ALS is differential vulnerability of MN populations, with cranial motor nuclei V, VII, and XII, and most spinal cord MNs being most susceptible to MN death in ALS, whereas MN in cranial motor nuclei III, IV, and VI are spared until the end stages of the disease [8]. The great majority of cases of ALS are sporadic [19] and the basis for differential vulnerability of MNs remains unknown.

Current evidence suggests that multiple factors contribute to MN injury in ALS. Three key hypotheses include genetic factors (such as mutations in the copper/zinc superoxide dismutase gene) [18], oxidative stress (such as superoxide radical accumulation), and glutamatergic excitotoxicity [19]. Evidence suggests that excessive activation of glutamate receptors and disruption of calcium homeostasis may contribute significantly [13]. Comparison of resistant and vulnerable MNs in control animals has established that resistant MNs have a greater calcium-buffering capacity than vulnerable MNs [12]. Several potential sources for glutamate-evoked increases in intracellular Ca^{2+} into MNs exist, including the activation of ionotropic Ca^{2+}-permeable *N*-methyl-D-aspartate (NMDA) receptors. Moreover, differences in the expression of glutamate receptors in resistant and susceptible MN pools may regulate the level of Ca^{2+} influx in response to excessive glutamate. For example, vulnerable MN populations have been shown to express high levels of the metabotropic glutamate receptor mGluR1a [10].

NMDA receptors are critical for neuronal survival, however, their excessive activation results in excitotoxicity and cell death. The NMDA receptors are multimeric complexes composed of an obligatory NR1 subunit (of which there are eight splice variants)

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and combinations of the NR2A, B, C and D subunits. Both the specific type of NR1 splice variant and the different composition of NR2 subunits confer unique functional and pharmacological characteristics, including significant effects on Ca^{2+} permeability and opening duration [2,6]. Differential expression of NMDA receptor subunits between resistant and vulnerable MN pools in normal animals has not been previously addressed. The aim of this study was to compare the relative expression of the NR1 and NR2 subunits within two cranial motor nuclei that differ in their vulnerability to ALS (hypoglossal-susceptible and oculomotor-resistant MN pools), using mRNA analysis and immunohistochemical techniques.

The Animal Ethics Committee of Murdoch University approved all protocols. Experiments were performed on adult Wistar rats of either sex (10–14 weeks old). For reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR studies, rats were briefly anaesthetized with carbon dioxide, decapitated and the brain immediately frozen. Results from six animals were pooled for the analysis of the NR2 subunits. Results from a second group of five animals were pooled for the NR1 subunit real-time and splice variant analysis. For immunohistochemistry, rats ($n=5$) were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (100 mg/kg) and perfused fixed as described by us previously [7].

For total RNA extraction, coronal sections (200 μm) were cut on a cryostat, followed by bilateral tissue punches taken from sections containing the hypoglossal nucleus (10 punches pooled in total from five slices containing XII), extending −14.30 mm to −13.24 mm Bregma, and the oculomotor nucleus (six punches in total pooled from three sections) extending −7.04 mm to −6.4 mm Bregma [16]. Tissue punches were taken using blunt 18 and 23-gauge needles, respectively. Total RNA was extracted using an RNAgents RNA Isolation Kit (Promega, USA) according to the manufacturer's instructions. RT was performed as described by us previously [7] using Superscript III (Invitrogen, USA). Reactions containing no reverse transcriptase or RNA functioned as negative controls.

Three different primer pairs were used to detect the specific NR1 splice variants [7]. The NR1a/b primer set detected both the NR1-a and NR1-b splice variants (604 and 667 bp, respectively). The NR1-1/2 primer set detected both the NR1-1 and NR1-2 splice variants (345 and 234 bp, respectively) and the NR1-3/4 primer set was designed to detect both the NR1-3 and NR1-4 splice variants (586 and 475 bp, respectively). PCR was performed as single reactions and products were resolved by electrophoresis on a 2% agarose gel. Negative controls were included as described above.

The NR1 (all isoforms) and NR2 subunit genes were quantitatively assessed in a multiplexed fluorescence-based real-time PCR reaction using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primers, probes and reaction conditions for the NMDA subunits and the endogenous control (neuronal specific enolase; NSE) are as described previously [7]. Cycle threshold (Ct) values were normalised to NSE to give a ΔCt value. PCR was performed with Taqman Universal PCR Master Mix (Applied Biosystems).

ΔCt values are reported as mean ± standard error (S.E.). Duplicate and triplicate reactions from individual animals were averaged. These values were then used to carry out statistical comparisons between and within regions ($n=5$ for NR1 results and $n=6$ for NR2 results). Fold variation was then determined using the $2^{-(\Delta\Delta Ct)}$ method according to previously published protocols [7]. Range values were determined using the formula $2^{-(\Delta\Delta Ct \pm SE\Delta Ct)}$. Individual genes from III were used as a reference for the expression level of each gene when comparing the two regions. NR2 subunit expression within each motor pool was analysed relative to NR1 expression due to preliminary data indicating NR1 did not vary between the two nuclei.

Statistical analysis was performed using the SPSS Statistical Package (SPSS, IL, USA). For comparison of genes within each nucleus, a one-way ANOVA was used followed by a Tukey HSD post hoc analysis. To compare individual genes between nuclei, a paired two-tailed Student's *t*-test was used with the assumption of equal variance. Significance was set at 0.05.

Fifty-micrometer thick coronal sections containing the hypoglossal and oculomotor nuclei were cut on a Vibratome within the regions described above. Sections were split into three equal consecutive series of seven sections each for the hypoglossal and four sections each for the oculomotor. From three animals, two series were used for cell counting purposes and the third was used for semi-quantitative analysis. From a further two animals, one series was used for semi-quantitative analysis (total $n=5$ for semi-quantitative analysis).

For cell counting, fluorescence immunohistochemistry was performed using the methodology described by us previously [7]. Sections were treated with either rabbit anti-NR1 antibody (detecting all splice variants; 1:100; Chemicon, Temecula, CA) or a rabbit anti-NR2B antibody (1:200; Chemicon). NR1 and NR2B expression was detected using a species-specific secondary antibody (donkey anti-rabbit FITC; 1:500, Jackson ImmunoResearch, USA). Control sections were processed in parallel without the addition of primary antibody.

For semi-quantitative analysis, immunoperoxidase immunohistochemistry was performed using the same NR2B primary antibody. Endogenous peroxidase activity was blocked by 0.3% H_2O_2 and non-specific binding sites were blocked with DAKO Biotin Blocking Kit (using the manufacturers instructions), followed by a 4-day incubation at 4 °C with anti-NR2B antibody diluted in IHC Select Immunoperoxidase Blocking reagent (Chemicon). Antibody binding was visualised using a biotin-streptavidin peroxidase system (DAKO LSAB) with diaminobenzidine (DAB) as the primary substrate (Pierce). Control sections were processed in parallel either in the absence of primary antibody or with rabbit serum to control for non-specific staining.

Fluorescent sections were examined using a Nikon Bio-Rad confocal dual laser-scanning TCS 4D system microscope (Nikon, Germany). For cell counts, composite figures of the hypoglossal and oculomotor nuclei were assembled using PhotoShop software (Adobe Systems, Mountain View, CA). Cells were counted from the right side of each section and only cell profiles that included a nucleus were included. The ratio of NR1 to NR2B immunoreactive cells was then calculated for each