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Altered sensitivity to excitotoxic cell death and glutamate receptor expression between two commonly studied mouse strains

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Abstract

Alterations in glutamatergic synapse function have been implicated in the pathogenesis of many different neurological disorders including ischemia, epilepsy, Parkinson's disease, Alzheimer's disease, and Huntington's disease. While studying glutamate receptor function in juvenile Batten disease on the C57BL/6J and 129S6/S_vE_v mouse backgrounds, we noticed differences unlikely to be due to mutation difference alone. We report here that primary cerebellar granule cell cultures from C57BL/6J mice are more sensitive to NMDA-mediated cell death. Moreover, sensitivity to AMPA-mediated excitotoxicity is more variable and is dependent upon the treatment conditions and age of the cultures. Glutamate receptor surface expression levels examined *in vitro* by *in situ* ELISA and *in vivo* by Western blot in surface cross-linked cerebellar samples indicated that these differences in sensitivity are likely due to strain-dependent differences in cell surface receptor expression levels. We propose that differences in glutamate receptor expression and in excitotoxic vulnerability should be taken into consideration in the context of characterizing disease models on the C57BL/6J and 129S6/S_vE_v mouse backgrounds.

Keywords

129S6/S_vE_v; C57BL/6J; strain differences; glutamate receptor; AMPA; NMDA

Introduction

A large number of studies have investigated the anatomical and behavioral differences between the 129 and C57BL/6J mouse strains and substrains (Balogh et al. 1999; Bothe et al. 2004; Cook et al. 2002; Crabbe et al. 1999; Fujii et al. 1997; Hefner et al. 2008; Montkowski et al. 1997; Paulus et al. 1999; Thomsen and Caine 2006; van Bogaert et al. 2006). C57BL/6J mice, as compared to 129 mice, have much higher learning capacity as

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well as better motor coordination (Balogh et al. 1999; Kelly et al. 1998; Koike et al. 2006; Pollak et al. 2005; Tarantino et al. 2000; Thomsen and Caine 2006) suggesting differences in neurotransmitter receptor function between the two mouse strains. In fact, a comparison of four different mouse strains has demonstrated significant differences regarding sensitivity to kainate type glutamate receptor-induced cell death in the hippocampus (Schauwecker and Steward 1997).

Neuronal relay of information is mediated by inhibitory and excitatory synaptic transmission. In the mammalian central nervous system, glutamate is the main excitatory neurotransmitter (Michaelis 1998). Two classes of receptors make possible glutamate-mediated neurotransmission: the G-protein coupled metabotropic glutamate receptors and a group of ionotropic receptors. Ionotropic glutamate receptors are glutamate-gated ion channels and mediate fast excitatory neurotransmission. This group of receptors is made up of three classes based on the agonists originally determined to activate them: kainite receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, and *N*-methyl-D-aspartate (NMDA) receptors (Kew and Kemp 2005). NMDA, AMPA, and kainite receptors are heterotetramers, and the channel properties of the receptors can vary based on subunit composition (Kew and Kemp 2005).

We were originally drawn to investigate the differences between mouse strains by our research on juvenile Batten disease. Juvenile Batten disease is a debilitating lysosomal storage disorder characterized by progressive neurodegeneration. Our lab maintains colonies of two different mouse models of this disease: *Cln3*$^{\Delta ex1–6}$ on 129S6/S_vE_v and *Cln3*$^{\Delta ex7–8}$ on C57BL/6J. The *Cln3*$^{\Delta ex1–6}$ mouse model of juvenile Batten disease displays a deficit in motor coordination (Kovacs et al. 2006). As excitatory glutamatergic transmission of cerebellar granule cells mediates cerebellar coordination of motor function (Hashimoto et al. 1999; Jensen et al. 1999), we investigated the sensitivity of primary neuronal cultures to glutamate-mediated excitotoxicity and found that *Cln3*$^{\Delta ex1–6}$ cerebellar granule cells were significantly more sensitive to AMPA receptor-mediated cell death when compared to their WT 129S6/S_vE_v counterparts (Kovacs et al. 2006). When we tried to reproduce these results in granule cell cultures from *Cln3*$^{\Delta ex7/8}$ mice on a C57BL/6J background, the selective difference between the mutant and WT cultures disappeared (unpublished data). This result suggested to us that fundamental differences in the glutamatergic systems of these two mouse strains may affect the phenotype of CLN3 deficiency. To confirm these suspicions, we compared the sensitivity of WT 129S6/S_vE_v and C57BL/6J neuronal cultures to glutamate receptor-mediated toxicity. These toxicity studies are widely used as a method of assessing glutamate receptor function since the expression level, subunit composition, and other receptor properties determine the extent of neuronal death..

Significant differences were found in the sensitivity to NMDA- and AMPA-type glutamate receptor-mediated cell death between the two genotypes studied, suggesting that there is an inherent difference in glutamate receptor function. We also measured the surface expression of AMPA and NMDA receptor subunits *in vivo* in cerebellar samples and found marked differences. This significant difference in glutamate receptor expression and function between C57BL/6J and 129S6/S_vE_v mice should be taken into consideration when generating mouse models of human neurodegenerative diseases in which dysregulated glutamate receptor function may contribute to neuronal death and neurological dysfunction.

Materials and methods

Chemicals

Neurobasal medium, B-27 neuronal serum replacement, glutamine, and Penicillin-Streptomycin liquid were purchased from Gibco BRL, Invitrogen Corporation (Grand

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Island, NY). DPBS was prepared according to the Gibco recipe. N-methyl-D-aspartate (NMDA), MK-801, (RS)-AMPA, Cyclothiazide, and CPW-399 used here were products of Tocris Cookson (Bristol, UK). Clear, polystyrene 48 and 96 well plates were obtained from Corning (Corning, NY) for cell culture and viability assay readout, respectively. Isopropanol was acquired from JT Baker/Mallinckrodt Baker (Phillipsburg, NJ). Hydrogen peroxide was from VWR (West Chester, PA). The membrane-impermeable cross-linking agent, BS^3, and the o-phenylenediamine were purchased from Pierce (Rockford, IL). All other chemicals, unless stated otherwise, were procured from Sigma (St. Louis, MO).

Animals

Mice used in this study were wild type $129S6/S_vE_v$ or C57BL/6J obtained from our in-house breeding colony. All experiments were carried out according to the Animal Welfare Act, NIH policies, and the guidelines developed by the University of Rochester Institutional Animal Care and Use Committee.

Cell cultures

Cerebellar granule cell cultures were prepared from seven-day-old pups as previously described (Kovacs et al. 2006) with some modifications. Briefly, cerebella were dissected and the meninges and blood vessels were removed. Tissue was then minced with a tissue chopper (McIlwain Tissue chopper, Brinkmann) making two perpendicular passes through the cerebella at 275 μm intervals. After incubation in 0.25% trypsin for 15 minutes at 30 °C, tissue was mechanically dissociated in a solution containing 0.05% soybean trypsin inhibitor and 0.01% DNase before being plated in 48 well culture dishes precoated with poly-L-lysine. Plating density was 1.5×10^5 cells per well in Neurobasal medium supplemented to include 2% B-27 supplement, 25 mM KCl, 0.5 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Maintaining cultures in serum-free medium eliminates the need to add a mitotic inhibitor. In primary neuronal cultures maintained in Neurobasal/B-27, the rate of GFAP-positive cells is 1–2% (Kovacs et al. 2001). Cultures were grown in a humidified environment of 5% CO_2/95% air maintained at 37 °C. Twenty-four hours after plating, medium on the cultures was changed completely. Every three days for the duration of *in vitro* growth, half of the culture medium was removed and replaced.

Agonist treatments

In rich culture medium, such as DMEM and Neurobasal, the sensitivity of cerebellar granule cells to glutamate receptor-mediated toxicity develops rather slowly (Didier et al. 1997). Therefore, cultures were treated after two as well as three weeks of *in vitro* development. At the chosen *in vitro* age, culture medium was removed and replaced with fresh medium containing the desired concentration of the glutamate receptor agonist. Cells were treated for 24-hour stretches: agonists were diluted in Neurobasal medium containing B-27 to the desired concentrations chosen to avoid maximum toxicity and enable us to detect even small differences in sensitivity. This solution was then used to replace the culture medium at the desired *in vitro* age. Twenty-four hours after this medium change, viability was assessed by MTT. To investigate the properties of glutamate receptors in the absence of insulin and antioxidants, two-hour treatments were done in the absence of B-27 following a 30-minute pre-incubation in medium lacking the serum replacement. To avoid confounding our results with trophic factor deprivation-induced cell death, we had to limit our B-27-lacking treatments to two hours. At the cessation of each treatment, the agonist-containing medium was removed and replaced with Neurobasal medium containing B-27. Twenty-four hours after agonist removal, viability was assessed by MTT.

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Cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability assay—After agonist treatments, cultures were inspected under the microscope to assess the approximate levels of cell death (both shrinkage due to apoptosis and disintegration by osmotic lysis); the extent of excitotoxic death was then quantified using an MTT viability assay. We found that the results of the MTT assays performed correlated well with our microscopic analysis of relative cell death. MTT assays were performed in 48 well culture plates as previously described (Kovacs et al. 2006). The assay was initiated by removing the culture medium and replacing it with B-27-lacking Neurobasal medium (composition described above) containing 0.3 mg/ml MTT. Cells were incubated for one hour at 37 °C before the MTT was aspirated and the plates were allowed to dry. 150 μL of isopropanol was added to each well to lyse cells and dissolve the formazan crystals. Aliquots (100 μL) were transferred to a 96 well microplate. Absorbance values were determined by reading at 562 nm and subtracting background at 690 nm. Cell viability was expressed as a percentage of untreated control. MTT is taken into cells by endocytosis and converted into a purple insoluble product by intracellular dehydrogenases (Liu et al. 1997). Although this conversion depends on enzymatic activity and may depend on redox state, it correlates well with cell viability(Aras et al. 2008).

Receptor surface expression assays

In Situ ELISA in cerebellar granule cell cultures—To quantify the surface and total expression of the GluR1 AMPA receptor subunit, in situ ELISA was applied as described by (Pickard et al. 2000), with some modifications. Granule cell cultures were grown in 48 well culture plates for two or three weeks, washed twice with Ca^{2+}- and Mg^{2+}-containing Hank's balanced salt solution, and then fixed in 4% paraformaldehyde for 10 min at room temperature (RT). After washing three times with Dulbecco's PBS (DPBS), cells were either left non-permeabilized (to detect surface GluR1) or were incubated with 0.1% Triton X-100 (in DPBS) for 5 min at RT to permeabilize the cell membrane (to detect total GluR1). Endogenous peroxidase activity was quenched by incubating the cells with 3% H_2O_2 in DPBS for 5 min. After washing the cells with DPBS, the unspecific binding sites were blocked with DPBS containing 5% goat serum (GS), 1% bovine serum albumin, and 0.05% Na-azide (DPBS-5%GS-1%BSA-azide) for 1 h at RT. Then cells were incubated with a rabbit anti-GluR1 antibody raised against the N-terminal, extracellular part of GluR1 (Calbiochem, Cat. No. PC246) (1:20, diluted in DPBS-5%GS-1%BSA-azide) for 1 h at 37 °C. This anti-GluR1 antibody has been effectively used to detect the surface expression of GluR1 in rat and mouse neuronal cultures (Gao and Wolf 2007; Lee et al. 2004; Nakamoto et al. 2007). At the end of the 1-h incubation with the anti-GluR1 antibody, cells were washed four times with DPBS, 5 min each, then incubated with a donkey anti-rabbit IgG coupled to horse radish peroxidase (GE Health; 1:200, diluted in DPBS-5%GS-1%BSA) for 1.5 h at RT. After washing five times with DPBS (5 min each), freshly made *o*-phenylenediamine (OPD) solution (0.4 mg/ml in 0.1 M citrate buffer, pH 5.0) containing 0.06% H_2O_2 was added to the cells, and the reaction was stopped by adding 2 M H_2SO_4 when the negative controls (no primary antibody) began to become yellow. Aliquots were transferred to a 96 well plate and the absorbance was measured at 492 nm in a microplate reader. GluR1-specific absorbance values were normalized to cell numbers determined in sister culture wells by the MTT assay immediately before fixing the cultures with paraformaldehyde.

Surface crosslinking in acute cerebellar slices—Using surface cross-linking in acutely isolated brain slices, the cell surface receptors (the receptor subunits forming the receptor and adjacent proteins) are covalently cross-linked resulting in high-molecular-weight aggregates, whereas intracellular receptors are not modified (Boudreau and Wolf

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2005; Conrad et al. 2008). Consequently, the surface and intracellular receptors can be separated and distinguished by molecular weight using SDS-PAGE and Western blotting.

Surface cross-linking was carried out as described by Boudreau & Wolf (2005), with some modification. One-month-old (P31) C57BL/6J and 129S6/S_vE_v male mice were euthanized with CO_2 and decapitated. The brain was rapidly extracted, and using a brain matrix a 2-mm horizontal slice was cut from the cerebellum containing the middle part of the cerebellum. The slice was put into ice-cold artificial cerebrospinal fluid (ACSF; 20 mM HEPES, 147 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM dextrose) and kept on ice. The cerebellum was then isolated from the slice, and the dissected tissue pieces were cut into 400-μm slices using a McIllwain tissue chopper. Slices were transferred to microtubes containing 500 μl of ice-cold ACSF. The cross-linking reaction was started by adding 500 μl of 4 mM BS^3 to the microtubes (BS^3 was dissolved in ice-cold ACSF immediately before adding it to the microtubes). The microtubes were incubated with gentle agitation at +4 °C for 30 min. The cross-linking reaction was quenched by adding 1 M glycine to the tubes (final concentration: 100 mM), and incubating them with gentle agitation at +4 °C for 10 min. Then the tissue pieces were pelleted (200 g, 2 min, +4 °C), resuspended in ice-cold lysis buffer containing protease and phosphatase inhibitors [25 mM HEPES, pH 7.4, 500 mM NaCl, 2 mM EDTA 20 mM NaF, 1 mM sodium orthovanadate, 0.1% NP-40 substitute, 1X protease inhibitor cocktail, and 1X phosphatase inhibitor cocktail (Sigma, St. Louis, MO)], and homogenized by sonication. Total protein concentration of the lysates was determined by the Pierce 660 nm protein assay (Pierce, Rockford, IL). The samples were aliquotted and stored at −80 °C until further analysis,

For Western blotting, samples (30 or 50 μg total protein) were loaded and electrophoresed on a 5% (for NMDA receptor subunits) or a 6% (for AMPA receptor subunits) Tris-HCl gel under reducing conditions; proteins were then transferred onto nitrocellulose membranes. Membranes were rinsed twice with ultrapure water and blocked with 5% nonfat dry milk in Tris-buffered salt solution containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature. Membranes were then incubated with antibodies to either AMPA receptor subunits [rabbit anti-GluR1 (Cat. No. AB1504), 1:1000; rabbit anti-GluR2 (Cat. No. AB1768), 1:2000; rabbit anti-GluR4 (Cat. No. 06-308), 1:1000; all from Millipore (Temecula, CA)] or NMDA receptor subunits [goat anti-NR2A (Cat. No. sc-1468), 1:1000; goat anti-NR2B (Cat. No. sc-1469), 1:500; both from Santa Cruz Biotech. (Santa Cruz, CA)] in TBS-T containing 5% nonfat dry milk at 4 °C for 65–68 h. In the case when β–tubulin was also detected, the membrane was horizontally cut immediately after the protein transfer, and the membrane piece containing the proteins with molecular weight below 70 kDa was incubated with a rabbit anti-β–tubulin antibody (1:20000; Sigma, St. Louis, MO). Membranes were rinsed twice with ultrapure water, washed with TBS-T (first for 10 min then three times for 5 min), and incubated with horse radish peroxidase-conjugated anti-rabbit IgG (1:5000; GE Healthcare, Piscataway, NY) or anti-goat IgG (1:5000; Santa Cruz Biotech, Santa Cruz, CA) for 1.5 h at room temperature. Membranes were then rinsed twice with ultrapure water and washed with TBS-T (first for 10 min then four times for 5 min). After rinsing twice with ultrapure water, membranes were incubated in Amersham ECL Plus chemiluminescence detection reagent (GE Healthcare, Piscataway, NY) for 5 min, and exposed to Kodak chemiluminescence BioMax film. Densities of surface and intracellular bands in each lane were determined using the ImageJ program (NIH). Care was taken to avoid measuring the density of saturated bands; for the visibility of some bands, figures were made using images with longer exposure times. Band intensities in each lane were normalized to total protein (determined by staining membranes with Ponceau S).

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Statistics

Two-way ANOVA with Bonferroni's post test for pairwise multiple comparison and unpaired *t*-test were performed using GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA)

Results

To compare glutamatergic function of C57BL/6J and 129S6/SvEv neurons, we measured the vulnerability of cultured cerebellar granule cells to glutamate receptor overactivation. This receptor overactivation can be achieved by acute, shorter (0.5–2 hr) treatments or chronic (24 hr) exposure to receptor agonists. The short-term treatments require significantly higher agonist concentrations to induce toxicity. Glutamate receptor-mediated cell death is widely used as a measure of glutamate receptor function because the expression level, the subunit composition, and the kinetic and ion permeability properties of glutamate receptors determine the extent of neuronal death. The extent of excitotoxic death was quantified using the MTT cell viability assay after microscopic inspection of the cultures was done to assess approximate levels of cell death. MTT is taken into cells by endocytosis and converted into a purple insoluble product by intracellular dehydrogenases (Liu et al. 1997). Although this conversion depends on enzymatic activity and may depend on redox state, it correlates well with cell viability (Aras et al. 2008).

C57BL/6J cerebellar granule cell cultures are more sensitive to NMDA receptor-mediated cell death than their 129S6/S_vE_v counterparts

Overactivation of NMDA receptors with 1 mM or 5 mM NMDA for two hours in the absence of insulin and anti-oxidant-containing B-27 neuronal serum replacement induced noticeable death in two-week-old cerebellar granule cell cultures isolated from both genotypes as measured by a MTT viability assay (Fig. 1A). NMDA at 5 mM caused significantly more death in the C57BL/6J cultures ($p < 0.05$). Exposure to NMDA at lower concentrations over the course of 24 hours in the presence of insulin and anti-oxidants also induced cell death in cultures of the same age (Fig. 1B). At both 100 μM and 300 μM, the difference in the extent of cell death between the strains was significant ($p < 0.05$ and $p < 0.001$ respectively), but the higher concentration had a much more dramatic effect.

Significant differences in sensitivity were also seen in three-week-old cultures. Both 100 μM and 300 μM NMDA treatments induced significantly more receptor-mediated death in C57BL/6J cultures ($p < 0.01$) when cells were exposed to the agonist for two hours in the absence of insulin and anti-oxidants and then allowed to recover for 24 hours (Fig. 1C). Continuous exposure of 21-day-old cultures to NMDA at 30 μM or 100 μM concentrations for 24 hours in the presence of insulin and anti-oxidants also induced significantly different responses from the two genotypes (Fig. 1D; $p < 0.01$ and $p < 0.001$ respectively).

Differential sensitivity to AMPA-mediated cell death is determined by *in vitro* age of cultures and treatment conditions

No differences in AMPA-mediated cell death between C57BL/6J and 129S6/S_vE_v granule neurons were seen when cells were exposed to 0.5 mM or 1 mM AMPA in the absence of insulin and anti-oxidants for two hours after two weeks of culture (Fig. 2A) in the presence of 100 μM cyclothiazide (CTZ) added to prevent the rapid desensitization of AMPA receptors. Twenty-four-hour exposure to 100 μM or 500 μM AMPA and 50 μM CTZ in the presence of insulin and anti-oxidants at the same *in vitro* developmental time point induced significantly more cell death in 129S6/S_vE_v neuronal cultures than in cultures from C57BL/6J mice (Fig. 2B; $p < 0.001$).

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After three weeks in culture, comparative sensitivity to AMPA-mediated cell death had switched. In response to both 2-hour and 24-hour treatments, C57BL/6J granule cells were found to be significantly more sensitive to AMPA-mediated cell death than their 129S6/S_vE_v counterparts (Fig 2C, 2D; $p < 0.001$ and $p < 0.05$ respectively). CTZ is selective for AMPA receptors formed by the flip splice variants of the receptor subunits, and the developmental expression of the flip variants may be different in C57BL/6J and 129S6/S_vE_v cultures.

CTZ treatment alone did not have significantly different effects on the two genotypes. Exposure to 100μM CTZ for two hours in the absence of insulin and anti-oxidants induced an equivalent decrease in viability in cultures from both genotypes after two or three weeks of *in vitro* development (Fig. 2A, 2C). Treatment of two-week-old cultures with 50 μM CTZ for 24 hours in the presence of insulin and anti-oxidants or treatment of three-week-old cultures with 10 μM CTZ under equivalent conditions also failed to affect 129S6/S_vE_v and C57BL/6J cultures differently (Fig. 2B, 2D).

Cerebellar granule cell cultures from C57BL/6J mice are significantly more sensitive to cell death induced by exposure to the AMPA receptor agonist, CPW-399

CPW-399 is an AMPA receptor agonist that induces a slow or non-desensitizing response (Campiani et al. 2001). Exposure of granule neurons to 500 μM or 2.5 mM concentrations of this compound in the absence of insulin and anti-oxidants for two hours after two weeks of *in vitro* development induced significantly more cell death in C57BL/6J cultures (Fig. 3A; $p < 0.001$). There was no difference between the genotypes at 100 μM, the lowest concentration tried. Twenty-four-hour treatment of cultures of the same age also showed the C57BL/6J neurons to be more sensitive, this time with all concentrations used (Fig. 3B; $p < 0.01$ for 10 μM and $p < 0.001$ for 30 μM and 100 μM).

The same pattern of differential sensitivity was seen in response to treatments both with and without insulin and anti-oxidants after three weeks of *in vitro* development. There was no difference in response to a two-hour, insulin and anti-oxidant lacking treatment of the lowest concentration, 30 μM, but the two higher concentrations used, 100 μM and 300 μM, showed the C57BL/6J neurons to be significantly more sensitive to CPW-399-induced cell death (Fig. 3C; $p < 0.001$). Exposure of three-week-old neuronal cultures to 3 μM, 10 μM, or 30 μM CPW-399 for 24 hours in the presence of insulin and anti-oxidants induced significantly more death in C57BL/6J cultures than in 129S6/S_vE_v cultures (Fig. 3D; $p < 0.001$).

Cultured C57BL/6J cerebellar granule cells express higher levels of GluR1 on their cell surface

Function of AMPA receptors is regulated, in part, by surface expression. To investigate whether an increase in receptor surface expression is the driving force behind the differential sensitivity to AMPA receptor agonists, we performed an *in situ* ELISA to compare surface levels of the GluR1 subunit to total subunit expression. After two weeks of *in vitro* development, C57BL/6J granule neurons displayed significantly higher total GluR1 levels ($p = 0.0003$) as well as higher surface expression (Fig. 4A; $p = 0.0091$) although both genotypes maintained very similar ratios of surface to total receptor levels (Fig. 4B). Three-week-old cultures followed the same pattern: C57BL/6J cells expressed significantly more of the receptor subunit (Fig. 4C; $p < 0.0001$) but the surface to total ratio was unchanged between C57BL/6J and 129S6/S_vE_v neurons (Fig. 4D). Comparison of the two time points also showed that the difference between the two mouse strains increased with time in culture.

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There are marked differences between C57BL/6J and 129S6/S_vE_v mice in AMPA and NMDA receptor subunit expression *in vivo*

Our results from cerebellar granule cell cultures indicated that AMPA and NMDA receptor function is significantly enhanced in C57BL/6J neurons as compared to 129S6/S_vE_v neurons, at least *in vitro*. To examine if glutamate receptor function of C57BL/6J neurons is also increased *in vivo*, we measured the surface and intracellular expression levels of AMPA and NMDA receptor subunits in the cerebellum of one-month-old (P31) C57BL/6J and 129S6/S_vE_v male mice. (We chose the age of one month because it roughly corresponds to the absolute age of the cerebellar granule cell cultures prepared from seven-day-old pups and maintained for three weeks in vitro.) Using surface cross-linking in acutely isolated brain slices, the cell surface receptors (the receptor subunits forming the receptor and adjacent proteins) are covalently cross-linked resulting in high-molecular-weight aggregates, whereas intracellular receptors are not modified (Boudreau and Wolf 2005; Conrad et al. 2008). Consequently, the surface and intracellular receptors can be separated and distinguished by molecular weight using SDS-PAGE and Western blotting (Fig. 5A). Previous studies have shown that the membrane-impermeable cross-linking agent, BS^3, does not cross-link intracellular proteins (Boudreau and Wolf 2005; Conrad et al. 2008). Our results with the intracellular protein, β-tubulin (see Fig. 5A) confirm these previous findings. We normalized the expression levels of the GluR1 glutamate receptor subunit to the β-tubulin and total protein levels and obtained identical results (data not shown). As there was no significant difference between the two methods, all further blots were normalized to total protein levels as determined by Ponceau S staining. The difference in surface and total (surface + intracellular) expression levels of the GluR1 AMPA receptor subunit between the C57BL/6J and 129S6/S_vE_v mice did not reach statistical significance, but the intracellular pool of GluR1 was almost two-fold larger in the C57BL/6J cerebellum (Fig. 5). Conversely, there was no difference in the intracellular expression levels of the GluR2 and GluR4 AMPA receptor subunits between the two strains, but the surface and total (surface + intracellular)expression levels of GluR2 and GluR4 in the C57BL/6J mice were approximately two-fold greater than those for the 129S6/S_vE_v mice (Fig. 6 and 7).

The cerebellar expression levels of the NR2A NMDA receptor subunit were identical in both mouse strains (Fig. 8). The surface expression of the NR2B NMDA receptor subunit, however, was markedly (nearly three times) higher in the cerebellum of C57BL/6J mice as compared to 129S6/S_vE_v mice (Fig. 9).

Discussion

The 129 mouse strain encompasses a large number of genetically (Simpson et al. 1997) and behaviorally (Balogh et al. 1999; Cook et al. 2002; Montkowski et al. 1997) distinct substrains. This strain, however, has anatomical, genetic and behavioral abnormalities (Balogh et al. 1999; Koike et al. 2006; Paulus et al. 1999) that make the phenotypic (particularly neurobehavioral) characterization of the genetically modified mice difficult. Therefore, in many studies the knockout or transgenic mice are back-crossed with C57BL/6J mice to exchange the genetic background. Several reports have indicated, however, that the C57BL/6J background can also modify (even suppress) the effect of a gene deletion (Bilovocky et al. 2003; Duysen and Lockridge 2006; Mahajan et al. 2004; Tang et al. 2003; Yang et al. 2005).

It has been well established that there are significant differences between the C57BL/6J and 129 mouse strains. These discrepancies have been documented at the neurobehavioral (Balogh et al. 1999; Bothe et al. 2004; Crabbe et al. 1999; Paulus et al. 1999; Thomsen and Caine 2006; van Bogaert et al. 2006), genetic (Bothe et al. 2004; Koike et al. 2006; Simpson et al. 1997), and morphological levels (Balogh et al. 1999; Fujii et al. 1997). These

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functional differences between the C57BL/6J and 129 strains are of particular interest due to the frequency with which these mice are used in the creation of knock-outs and animal models of disease.

Several studies have investigated the effect of genetic background on the disruption of particular genes of interest (Bilovocky et al. 2003; Duysen and Lockridge 2006; Kelly et al. 1998; Lariviere et al. 2001; Lloret et al. 2006; Magara et al. 1999; Mahajan et al. 2004; Nguyen et al. 1997; Tang et al. 2003; Yang et al. 2005). In some cases, the strain context determined viability of knock-outs (Bilovocky et al. 2003; Nguyen et al. 1997). Others linked genetic background to fertility of the knock-out (Mahajan et al. 2004) or the severity of the resultant phenotype (Yang et al. 2005). The well described corpus callosum defect seen in 129 mice was shown to confound the phenotype seen in a β-amyloid precursor protein knock-out (Magara et al. 1999). It has also been postulated that the same corpus callosum defect extends the lifespan of an acetylcholine esterase knock-out by decreasing the severity of seizures that result from the induced genetic mutation (Duysen and Lockridge 2006). Our own experimental results suggest that the differences between these two genetic backgrounds also affect the phenotypic readout of *Cln3* mutations (unpublished data).

To explore the mechanisms driving the differences between C57BL/6J and 129S6/S_vE_v mice, we investigated the functional properties of NMDA and AMPA receptors in cerebellar granule neuron cultures from the two strains and found significant differences in response to agonist-mediated excitotoxic cell death. To further investigate these differences, we next examined surface expression levels of receptor subunits.

In response to NMDA exposure, cultured C57BL/6J cerebellar granule neurons were found to be more sensitive to agonist-stimulated excitotoxicity. This finding is complemented by our *in vivo* surface expression measurements. NR2B surface expression was almost three times higher in the cerebellum of C57BL/6J mice as compared to 129S6/S_vE_v mice. As neurotoxicity has been linked to NR2B function in cortical neuron cultures and *in vivo* (Heng et al. 2009; Mizuta et al. 1998), it is possible that this differential NR2B expression level drives the increased sensitivity of cerebellar granule cell cultures to NMDA.

Sensitivity of cultured granule cells to AMPA-mediated cell death was dependent upon treatment conditions employed as well as age of the cultures. There are a few possible explanations for this variability in AMPA sensitivity over time in culture. First, AMPA receptor expression and subunit composition change as granule cells develop *in vitro* (Hack et al. 1995). It is possible that this developmental process is differentially regulated in cultures isolated from the two strains. Second, CTZ displays a preference for AMPA receptors containing flip splice variants of the receptor subunits (Johansen et al. 1995; Partin et al. 1996; Partin et al. 1994). It has been shown that mRNA splicing is developmentally regulated in cultured granule cells (Longone et al. 1998; Mosbacher et al. 1994) so it is possible that the switch in relative sensitivity that occurs when cells mature between two and three weeks in culture could be due to a change in the regulation of mRNA splicing. To investigate the functional properties of AMPA receptors in the absence of CTZ without inducing receptor desensitization, we also treated cultures with the non-desensitizing AMPA receptor agonist, CPW-399, and found C57BL/6J neurons to be more susceptible to the induced excitotoxicity at both *in vitro* ages under both treatment conditions. We were able to quantify surface expression levels of AMPA receptor subunits both *in vitro* and *in vivo* using *in situ* ELISA and surface cross-linking in acutely isolated brain slices, respectively. Our *in vitro* results demonstrating significantly higher surface expression of the GluR1 AMPA receptor subunit in C57BL/6J cerebellar granule cell cultures offer a possible explanation for their increased sensitivity to AMPA receptor-mediated toxicity, particularly that induced by CPW-399. CPW-399 preferentially activates GluR1- and GluR2-containing AMPA

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receptors, showing a 20-fold selectivity for these receptors over those containing GluR3 and GluR4 (Campiani et al. 2001). The fact that there is a difference between the strains in terms of neuronal sensitivity to AMPA treatment after two weeks in culture in the presence, but not the absence, of insulin- and anti-oxidant-containing B-27 suggests that C57BL/6J granule neurons can more efficiently endocytose glutamate receptors and thereby modulate receptor function. Endocytosis of AMPA receptors has been shown to be an insulin-dependent process (Beattie et al. 2000); removal of insulin eliminates the ability of neurons to efficiently endocytose their receptors and thus removes the difference between the two populations. It is also possible that the difference between the two-hour and 24-hour treatments is due to differential regulation of cell death pathways activated by more prolonged agonist exposure.

Our *in vivo* data show significantly higher surface and total expression levels of the GluR2 and GluR4 AMPA receptor subunits in the cerebellum of C57BL/6J mice that, in conjunction with the *in situ* ELISA data for GluR1 offer a plausible explanation for the increased sensitivity of C57BL/6J neurons to AMPA receptor-mediated excitotoxicity. In contrast to the *in situ* ELISA data in cerebellar granule cell cultures, our *in vivo* surface expression assay showed a considerably higher intracellular expression of GluR1 in the cerebellum of C57BL/6J mice. This could be due to the fact that the in situ ELISA was done in highly purified granule cell cultures, and the *in vivo* surface expression assay used cerebellar slices that contained all the cell types of the cerebellum including astrocytes and oligodendrocytes.

Dysregulation of Glutamatergic neurotransmission and glutamate receptor-mediated cell death have been implicated in a number of different neurological diseases including ischemia, epilepsy, Parkinson's disease, Alzhiemer's disease, and Huntington's disease (Gardoni and Di Luca 2006). A considerable amount of research has been done specifically investigating alterations in NMDA receptor function seen in animal models of the latter (Estrada Sanchez et al. 2008). Recent evidence had also implicated altered AMPA receptor function in the pathogenesis of Juvenile Neuronal Ceriod Lipofuscinosis (juvenile Batten disease) (Kovacs and Pearce 2008; Kovacs et al. 2006).

As C57BL/6J and 129S6/S_vE_v mouse strains are so commonly used in the creation of disease model animals, we propose that genetic background should be carefully considered while characterizing these models. Despite the fact that C57BL/6J is generally considered a better model of human physiology, the 129S6/S_vE_v substrain would be a better choice for studying neurodegenerative diseases where enhanced glutamate receptor function may be involved in the pathophysiology as our results in cerebellar granule cell cultures indicate that glutamate receptor function is already highly enhanced in C57BL/6J neurons as compared to 129S6/S_vE_v ones.

Acknowledgments

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
CTZ	cyclothiazide
NMDA	N-methyl-D-aspartate

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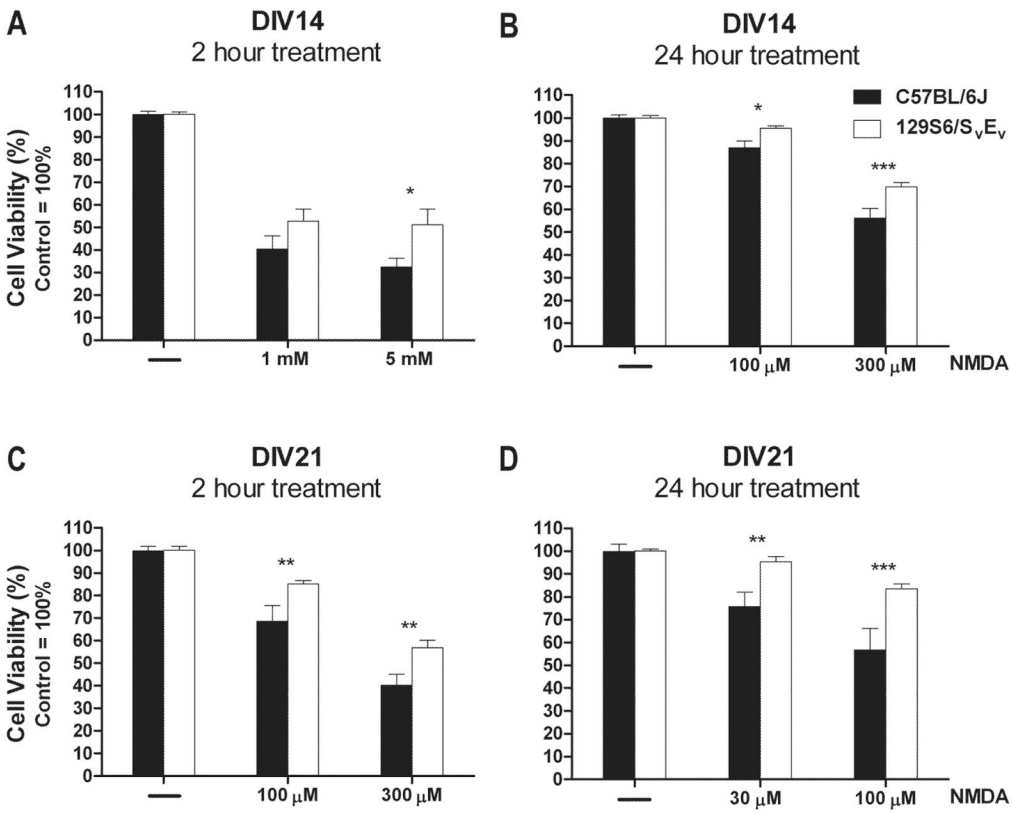

Figure 1. Cerebellar granule cells from C57BL/6J mice are more sensitive to NMDA-mediated cell death

NMDA-mediated cell death was investigated in primary cultures of cerebellar granule cells from wild type C57BL/6J (filled bars) and 129S6/S_vE_v (open bars) mice. Cultures were prepared from seven-day-old pups and treated with NMDA at the indicated concentrations after (A, B) two or (C, D) three weeks of *in vitro* development. Twenty-four hours after the cessation of the two-hour treatment or the commencement of the 24-hour treatment, cell viability was assessed via MTT assay. Columns and bars represent mean ± S.E.M. of four separate experiments with four different culture preparations (n=9–20). Statistical significance was determined by two-way ANOVA with Bonferroni's post test for multiple comparisons: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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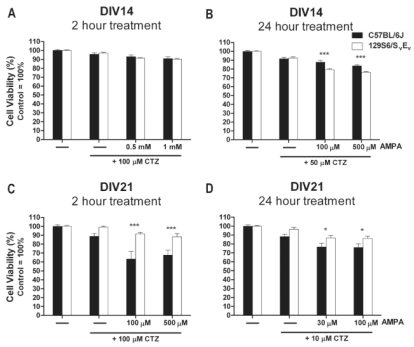

Figure 2. Sensitivity of granule cell cultures to AMPA-mediated cell death is determined by treatment duration and length of time in culture

AMPA-mediated cell death was investigated in primary cultures of cerebellar granule cells from wild type C57BL/6J (filled bars) and 129S6/S_vE_v (open bars) mice. Cultures were prepared from seven-day-old pups and treated with AMPA at the indicated concentrations after (A, B) two or (C, D) three weeks of *in vitro* development. To prevent transactivation of NMDA receptors, treatments were done in the presence of 50 μM MK-801. Cyclothiazide was added at the indicated concentrations to prevent desensitization of AMPA receptors. Twenty-four hours after the cessation of the two-hour treatment or the commencement of the 24-hour treatment, cell viability was assessed via MTT assay. Columns and bars represent mean ± S.E.M. of four separate experiments with four different culture preparations (n=9–22). Statistical significance was determined by two-way ANOVA with Bonferroni's post test for multiple comparisons: *$p < 0.05$, ***$p < 0.001$.

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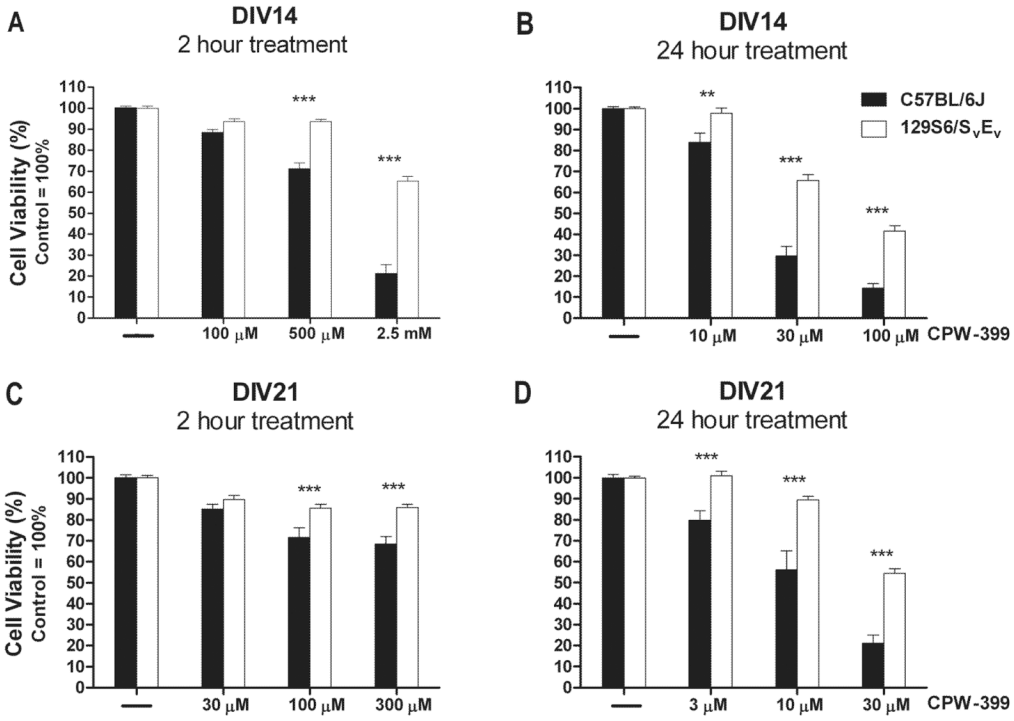

Figure 3. Cerebellar granule cells from C57BL/6J mice are more sensitive to cell death mediated by the non-desensitizing AMPA receptor agonist CPW-399

CPW-399-mediated cell death was investigated in primary cultures of cerebellar granule cells from wild type C57BL/6J (filled bars) and 129S6/S_vE_v (open bars) mice. Cultures were prepared from seven-day-old pups and treated with CPW-399 at the indicated concentrations after (A, B) two or (C, D) three weeks of *in vitro* development. The NMDA receptor antagonist MK-801 was added to all treatments at a 50 μM concentration to prevent transactivation of NMDA receptors. Twenty-four hours after the cessation of the two-hour treatment or the commencement of the 24-hour treatment, cell viability was assessed via MTT assay. Columns and bars represent mean ± S.E.M. of four separate experiments with four separate culture preparations (n=8–22). Statistical significance was determined by two-way ANOVA with Bonferroni's post test for multiple comparisons: **p < 0.01, ***p < 0.001.

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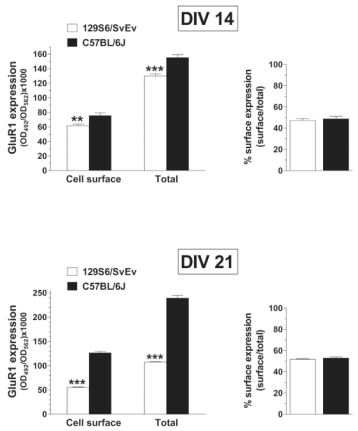

Figure 4. Cerebellar granule cells from C57BL/6J mice express more GluR1 on their cell surfaces

AMPA receptor subunit GluR1 expression was investigated via *in situ* ELISA on primary cultures of cerebellar granule cells from wild type C57BL/6J (filled bars) and 129S6/S_vE_v (open bars) mice. Cultures were prepared from seven-day-old pups and assayed for total and surface expression of GluR1 after 14 (A) or 21 (B) days of *in vitro* development. Relative surface expression was determined by absorbance ratio (n=8). Percentage of GluR1 expressed on the cell surface was determined by dividing surface expression absorbance values by the corresponding total expression absorbance values. Statistical significance was determined by unpaired *t* test.

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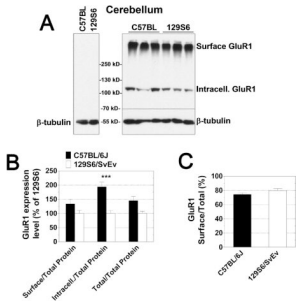

Figure 5. Significantly higher intracellular expression level of the GluR1 AMPA receptor subunit in the cerebellum of C57BL/6J mice

The surface and intracellular expression levels of the GluR1 AMPA receptor subunit in acutely isolated cerebellar slices from one-month-old C57BL/6J and 129S6/S_vE_v male mice were determined by surface crosslinking and Western blotting. **(A)** Representative Western blots of cerebellar samples. (*left*) The membrane-impermeable cross-linking agent, BS^3, does not cross-link the intracellular protein, β-tubulin. (*right*) Besides surface (cross-linked, high M.W.) GluR1 and intracellular GluR1, β-tubulin was also detected in each lane. **(B)** The expression levels of GluR1 in each lane were normalized to the total protein levels (total protein level in each lane was determined by Ponceau S staining). Total GluR1: surface + intracellular. GluR1 levels in C57BL/6J samples were expressed as percentage of levels in 129S6/S_vE_v samples (GluR1 levels in 129S6/S_vE_v samples were set as 100%). There was a significantly larger intracellular pool of GluR1 in the C57BL/6J strain as determined by 2-way ANOVA with Bonferroni's test for pairwise multiple comparison. Surface cross-linked samples from eight mice were analyzed for each genotype. Columns and bars represent mean ± S.E.M. **(C)** The cerebellar ratios of Surface/Total GluR1 were similar, not statistically different (by unpaired t-test) in the two mouse strains.

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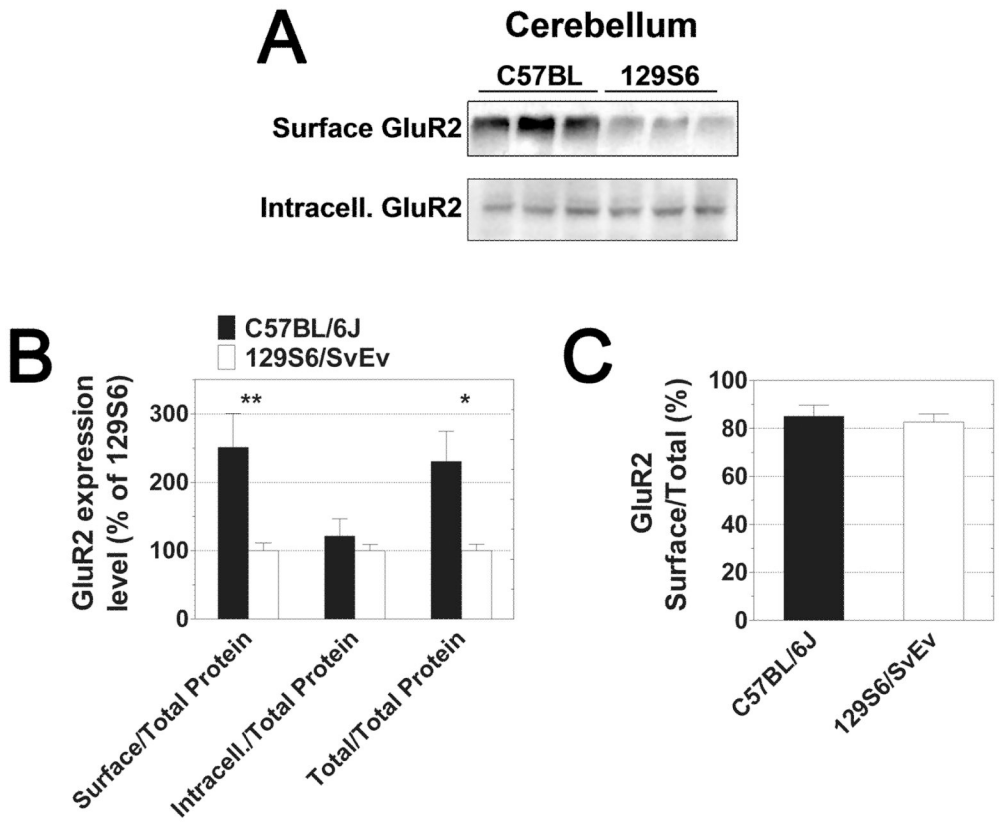

Figure 6. Significantly higher surface and total expression levels of the GluR2 AMPA receptor subunit in the cerebellum of C57BL/6J mice

The surface and intracellular expression levels of the GluR2 AMPA receptor subunit in acutely isolated cerebellar slices from one-month-old C57BL/6J and 129S6/S_vE_v male mice were determined by surface crosslinking and Western blotting. **(A)** A representative Western blot of cerebellar samples shows surface (cross-linked) and intracellular GluR2. **(B)** The expression levels of GluR2 in each lane were normalized to the total protein level (determined by Ponceau S staining); Total GluR2: surface + intracellular. GluR2 levels in C57BL/6J samples were expressed as percentage of levels in 129S6/S_vE_v samples (GluR2 levels in 129S6/S_vE_v samples were set as 100%). There were significant differences in surface and total GluR2 cerebellar expression between the two mouse strains as determined by 2-way ANOVA with Bonferroni's test for pairwise multiple comparison. Surface cross-linked samples from eight mice were analyzed for each genotype. Columns and bars represent mean ± S.E.M.

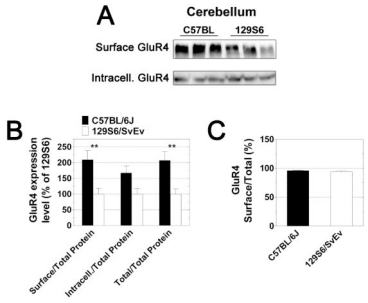

Figure 7. Significantly higher surface and total expression levels of the GluR4 AMPA receptor subunit in the cerebellum of C57BL/6J mice

The surface and intracellular expression levels of the GluR4 AMPA receptor subunit in acutely isolated cerebellar slices from one-month-old C57BL/6J and 129S6/S_vE_v male mice were determined by surface crosslinking and Western blotting. **(A)** A representative Western blot of cerebellar samples shows surface (cross-linked) and intracellular GluR4. **(B)** The expression levels of GluR4 in each lane were normalized to the total protein level (determined by Ponceau S staining); Total GluR4: surface + intracellular. GluR4 levels in C57BL/6J samples were expressed as percentage of levels in 129S6/S_vE_v samples (GluR4 levels in 129S6/S_vE_v samples were set as 100%). There were significant differences in surface and total GluR4 cerebellar expression between the two mouse strains as determined by 2-way ANOVA with Bonferroni's test for pairwise multiple comparison. Surface cross-linked samples from five mice were analyzed for each genotype. Columns and bars represent mean ± S.E.M.

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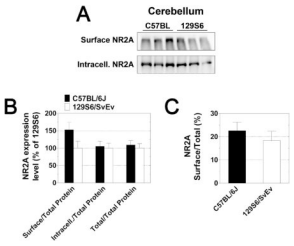

Figure 8. Similar expression level of the NR2A NMDA receptor subunit in the cerebellum of C57BL/6J and 129S6/S_vE_v mice

The surface and intracellular expression levels of the NR2A NMDA receptor subunit in acutely isolated cerebellar slices from one-month-old C57BL/6J and 129S6/S_vE_v male mice were determined by surface crosslinking and Western blotting. **(A)** A representative Western blot of cerebellar samples shows surface (cross-linked) and intracellular NR2A. **(B)** The expression levels of NR2A in each lane were normalized to the total protein level (determined by Ponceau S staining); Total NR2A: surface + intracellular. NR2A levels in C57BL/6J samples were expressed as percentage of levels in 129S6/S_vE_v samples (NR2A levels in 129S6/S_vE_v samples were set as 100%).There was no difference in NR2A cerebellar expression between the two mouse strains as determined by 2-way ANOVA with Bonferroni's test for pairwise multiple comparison. Surface cross-linked samples from eight mice were analyzed for each genotype. Columns and bars represent mean ± S.E.M.

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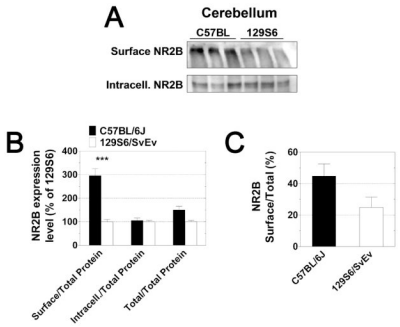

Figure 9. Significantly higher surface expression of the NR2B NMDA receptor subunit in the cerebellum of C57BL/6J mice

The surface and intracellular expression levels of the NR2B NMDA receptor subunit in acutely isolated cerebellar slices from one-month-old C57BL/6J and 129S6/S_vE_v male mice were determined by surface crosslinking and Western blotting. **(A)** A representative Western blot of cerebellar samples shows surface (cross-linked) and intracellular NR2B. **(B)** The expression levels of NR2B in each lane were normalized to the total protein level (determined by Ponceau S staining); Total NR2B: surface + intracellular. NR2B levels in C57BL/6J samples were expressed as percentage of levels in 129S6/S_vE_v samples (NR2B levels in 129S6/S_vE_v samples were set as 100%). Statistical significance was determined by 2-way ANOVA with Bonferroni's test for pairwise multiple comparison. Surface cross-linked samples from eight mice were analyzed for each genotype. Columns and bars represent mean ± S.E.M.; ***$p<0.001$, **$p<0.01$.