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Decreased NR1 Phosphorylation and Decreased NMDAR Function in Hibernating Arctic Ground Squirrels

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Abstract

Heterothermic mammals such as ground squirrels tolerate ischemia and N-methyl-D-aspartate (NMDA) better than homeothermic mammals such as rats both in vivo and in vitro, and this tolerance is enhanced in the hibernating state. However, the cellular mechanisms underlying this tolerance remain unclear. NMDA receptors (NMDAR) play a key role in excitotoxicity. The purpose of the current study was therefore to test the hypothesis that NMDAR are down-regulated in hibernating Arctic ground squirrels (hAGS; *Spermophilus parryii*). To address this hypothesis, we used Western blot analysis to investigate NMDAR phosphorylation, an activator of NMDAR function, and internalization in naïve hippocampal tissue from hAGS, interbout euthermic AGS (ibeAGS), and rats. Furthermore, we used fura-2 calcium imaging to examine NMDAR function in cultured hippocampal slices from hAGS, ibeAGS, and rats. We report that phosphorylation of the NMDAR1 (NR1) subunit is decreased in hippocampal tissue from hAGS and that the NMDAR component of Glu-induced increase in $[Ca^{2+}]_i$ is decreased in hippocampal slices from hAGS. Moreover, the fraction of NR1 in the functional membrane pool in AGS is less than that in rats.

Keywords

hibernation; hippocampal slices; excitotoxicity; stroke; fura-2

Stroke is a primary cause of disability in the United States, and studies in traditional laboratory animals such as rats have produced a poor yield of pharmacotherapies (Sareen, 2002). Heterothermic mammals (e.g., ground squirrels) tolerate experimental hypoxia and ischemia significantly better than homeothermic mammals (e.g., rats) both in vivo and in vitro (Frerichs et al., 1998; Drew et al., 2004; Dave et al., 2006; Ross et al., 2006). Tolerance to central nervous system injury is enhanced in the hibernating state (Frerichs et al., 1998; Zhou et al., 2001; Ross et al., 2006). In addition, arousal from hibernation enhances learning in ground squirrels (Mihailovic et al., 1968; Weltzin et al., 2006). N-methyl-D-aspartate receptors (NMDAR) play a key role in excitotoxicity and synaptic plasticity (Ascher and Nowak, 1986; Choi, 1995). Down-regulation of NMDAR has been shown to contribute to hypoxia and ischemia tolerance in developing brains of rats and piglets (Mishra et al., 2001; Fritz et al., 2002) and to long-term anoxia tolerance in brains of

Western painted turtles (*Chrysemys picta*; Bickler, 1998). The objective of this study was, therefore, to test the hypothesis that NMDAR function is down-regulated in hibernating AGS.

Functional NMDAR are heteromeric and formed by NMDAR1 (NR1) subunits in various combinations with NMDAR2A–D (NR2A–D) subunits (Carroll and Zukin, 2002). NR1 is required to form functional NMDAR, whereas NR2 subunits play regulatory roles. Phosphorylation of NR1 and NR2 subunits enhances receptor function (Liu and Zhang, 2000). Internalization or altered insertion in the plasma membrane can also alter NMDAR function (Ehlers, 2000; Carroll and Zukin, 2002). To test the hypothesis that NMDAR function is down-regulated in hibernation, we first used Western blotting analysis to investigate NMDAR phosphorylation and internalization in naïve hippocampal tissue from hibernating Arctic ground squirrels (hAGS; *Spermophilus parryii*), interbout euthermic AGS (ibeAGS), and rats. Next, we used an in vitro hippocampal slice preparation and fura-2 Ca^{2+} imaging to assess NMDAR function. NMDAR function was assessed at a time point in culture when hippocampal slices from hAGS tolerate oxygen nutrient deprivation and NMDA-induced excitotoxicity better than slices from ibeAGS (Ross et al., 2006).

Intracellular calcium is a ubiquitous second messenger integrating many cellular processes, including neuronal development, gene expression, synaptic plasticity, and neuronal death (Siesjo, 1990). Intracellular calcium overload is essential for neuronal injury associated with ischemia and hypoxia (Budd, 1998). During ischemia and hypoxia, a decrease in ATP leads to loss of ion homeostasis, an increase in extracellular glutamate, and activation of an excitotoxic cascade that leads to neuronal damage (Choi, 1995). Intracellular calcium overload originates from increased conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA), NMDAR, and voltage-gated calcium channels (VGCC) as well as from release from intracellular stores, such as endoplasmic reticulum (ER; Tymianski et al., 1993). NMDAR have attracted significant attention because of their high calcium permeability and key role in excitotoxicity (Ascher and Nowak, 1986; Choi, 1995). Therefore, we used calcium imaging to assess NMDAR function in hibernation.

MATERIALS AND METHODS

Animals

The Institutional Animal Care and Use Committee of the University of Alaska Fairbanks approved all animal procedures. AGS were trapped on the northern slope of the Brooks Range, Alaska, approximately 20 miles south of the Toolik Field Station of UAF (68°38' N, 149°38' W; elevation 809 m) under permit from the Alaska Department of Fish and Game. Upon arrival at the university, AGS were quarantined for at least 14 days. All AGS were housed individually in cages at an ambient temperature (T_a) of approximately 18°C, fed approximately 40 g of Mazuri Rodent Chow per day, and kept on natural lighting for 64° latitude where the light:dark cycle changed from 20 hr:4 hr to 16 hr:8 hr before AGS were moved to “winter” conditions. In mid-August, AGS were fed 10–15 sunflower seeds each day for 2 weeks and were then moved to environmental chambers to facilitate hibernation. Chambers were set to a T_a of 2°C and 4 hr:20 hr light:dark cycle. While housed in environmental chambers, all AGS were fed rodent chow ad libitum, although animals do not eat when hibernating.

Groups of AGS (hAGS and ibeAGS) were matched for season, frequency, and duration of torpor bouts, as well as age, sex, and body weight. Most of the AGS were female juvenile (5–9 month of age) that were not sexually mature. Both hAGS and ibeAGS used in the current study had experienced at least three regular hibernation bouts lasting a minimum of 4 days each and three periods of interbout euthermia lasting approximately 24 hr each. hAGS

were chosen if they were at least 3 days into their current torpor bout, their respiratory rate was under five breaths per minute, and wood shavings placed on the animal's back upon entry into hibernation remained undisturbed. ibeAGS were chosen if their respiratory rate was more than 80 breaths per minute, body temperature was greater than 35.5°C, and if animals responded quickly to touch. All ibeAGS were hibernating 12–16 hr prior to slice preparation and tissue collection and had aroused naturally from hibernation in the past 12–16 hr.

Female, juvenile (28–34 day old) Wistar rats were used as positive controls. Rats were obtained from Simonsen Laboratories (Gilroy, CA), quarantined for at least 7 days, and kept at a T_a of approximately 21°C until euthanasia.

Tissue and Protein Lysate Preparation

Hippocampal tissue was collected from female 28–34-day-old rats, and hAGS and ibeAGS of both sexes. Animals were lightly anesthetized, and brains were removed quickly after decapitation. Hippocampi were dissected, quickly frozen in liquid N₂, and stored at –80°C.

For total protein lysate preparation, Approximately 20–30 mg of hippocampal tissue was homogenized in 200–300 µl (10× volumes) of ice-cold 1% NP-40 lysis buffer (50 mM Tris-HCl, pH 7.6, 0.02% sodium azide, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, 150 mM NaCl) with protease/phosphatase inhibitors, including 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, 1 µg/ml aprotinin, and 1 µg/ml antipain with a motor-driven polytron homogenizer for 30–40 seconds. The homogenate was left on ice for 40 min and then centrifuged at maximal speed for 10 min in a microcentrifuge. The supernatant was collected and termed *total protein lysate*.

Crude Membrane/Cytosolic Preparation

According to the methods of Ehlers (2000), approximately 50–60 mg of hippocampal tissue was homogenized in 500–600 µl (103 volume) of cold homogenization buffer (0.32 M sucrose, 10 mM HEPES, pH 7.4, 2 mM EDTA) with protease/phosphatase inhibitors, including 0.1 mM phenylmethylsulfonyl fluoride, 1.5 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM sodium orthovanadate, and 10 µg/ml antipain in a glass-Teflon homogenizer for 10–15 strokes. The homogenate was centrifuged at 1,000g for 15 min to remove the pelleted nuclear fraction (P1). Supernatant (S1) was then centrifuged at 200,000g for 15 min in a TLA100.2 rotor in a Beckman TL-100 ultracentrifuge (Beckman Coulter, Fullerton, CA) to yield a crude cytosolic fraction (S2) and a crude membrane pellet (P2). The crude membrane pellet (P2) was resuspended with homogenization buffer and centrifuged again at 200,000g for 15 min to yield the washed crude membrane pellet (P2). The membrane fraction was prepared by resuspending P2 in 200–250 µl (approximately 5× volume) of HEPES-lysis buffer (50 mM HEPES, pH 7.4, 2 mM EDTA) and protease/phosphatase inhibitors.

Western Blotting

Protein concentration was determined by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Twenty micrograms of protein was separated on 8% SDS-PAGE gels and then transferred to nitrocellulose membranes. Rat brain microsomal protein preparation (catalog No. 12-144; Upstate, Lake Placid, NY) was used as a positive control for pNR1 and NR1 detection in all experiments. After blocking with 5% milk in TBS (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl) for 1 hr, the membranes were incubated with the primary antibody (mouse anti-NR1 monoclonal antibody; 1:1,000; catalog No. MAb 363; Chemicon, Temecula, CA; or rabbit anti-phospho-NR1 polyclonal antibody pNR1, against Ser897 in NR1 subunit; 1:1,000, catalog No. 06-641; Upstate) in TBST (TBS and 0.1% Tween 20) with 1% bovine serum albumin overnight at 4°C with gentle agitation. The membranes were

washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG, 1:2,000; and anti-rabbit IgG, 1:2,000; Bio-Rad) for 1 hr. Immunoreactive bands were visualized by using enhanced chemiluminescence (ECL; Perkin-Elmer, Boston, MA). Membranes were stripped by incubation with 10 mM Tris-HCl (pH 2) and 150 mM NaCl for 30 min. Equal loading was then confirmed by reprobing with mouse antitubulin 3 monoclonal antibody (1:1,000; catalog No. MAb1637; Chemicon); rabbit anti-Na⁺,K⁺/ATPase -1 polyclonal antibody (1:5,000; catalog No. 06-170; Upstate); or mouse antiactin monoclonal antibody (1:5,000; catalog No. 5316; Sigma-Aldrich, St. Louis, MO) diluted in TBST with 1% bovine serum albumin. Each membrane was reprobed a maximum of twice. Scans of ECL exposures were analyzed in ImageQuant 5.2 software (Amersham Biosciences, Piscataway, NJ). Because hibernation is known to affect membrane properties (Azzam et al., 2000), relative efficiency of protein extraction from membrane and cytosolic fractions in hAGS, ibeAGS, and rats was assessed by comparing the ratio of band intensity of membrane and cytosolic markers.

Hippocampal Slice Preparation

We used fura-2 calcium imaging in a semichronic organotypic hippocampal slice preparation to determine whether NMDAR function is altered in slices from hAGS. NMDAR function was assessed at 24 hr in culture, a point when slices from hAGS tolerate NMDA better than slices from ibeAGS (Ross et al., 2006). Hippocampal slices were prepared from female hAGS, ibeAGS, and 28–34-day-old female Wistar rats. Prior to slice preparation, 12-mm-diameter Millicell-CM inserts (Millipore, Bedford, MA) were placed in a 24-well plate and equilibrated with 0.5 ml of Neurobasal Adult media supplemented with antioxidant-free B-27 serum substitute (Gibco, Grand Island, NY), 0.025 mM glutamate, 0.5 mM glutamine, and 1% streptomycin-penicillin (Sigma) for 1 hr at 37°C. Animals were anesthetized with 5% halothane and maintained at 3% halothane mixed with 100% oxygen, delivered at a flow rate of 1.5 liters/min while body weight and rectal temperatures were measured. After decapitation, brains were rapidly removed into ice-cold Hibernate Adult medium (Brain Bits, Springfield, IL). Hippocampi were dissected, embedded in 3% agar, and cut at a thickness of 300 µm with a vibraslicer (World Precision Instruments, Inc., Sarasota, FL). One slice was placed on each insert, and slices were cultured at 37°C in a 5% CO₂ US Autoflow CO₂ water-jacketed humidified incubator (NuAire, Plymouth, MN).

Ca²⁺ Imaging Recording

After 24 hr in culture, slices were loaded by replacing Neurobasal with 0.5 ml perfusion buffer (mM) NaCl 101, KCl 4.6, CaCl₂ 1.8, MgCl₂ 0.81, HEPES 10, and dextrose 21), with 10 µM fura-2 acetoxymethyl (fura-2AM; Molecular Probes, Eugene, OR), 0.12% dimethyl sulfoxide, 0.02% pluronic acid (Molecular Probes), and 0.5% bovine serum albumin added. Slices were loaded at 37°C for 1 hr in the dark. Equal loading and esterase activity were evaluated by comparing 510 nm emission fluorescence intensity when fura-2 was excited at 380 nm (F380) in slices during stable, resting conditions. After loading, individual slices were transferred to a heated chamber mounted on the stage of a Zeiss Axiovert S-100 inverted microscope, in which the temperature of the chamber was maintained at 37°C by a Delta T4 Culture Dish Controller (Biopetechs, Butler, PA). Individual slices were perfused for another 30 min at 1.2 ml/min with a peristaltic pump before imaging to allow for esterase activity (Biopetach). The CA1 area of the hippocampal slices was excited alternately at wavelengths of 340 and 380 nm with a high-speed wavelength-switching device (Lambda 10-C; Sutter, Novato, CA). Fluorescence intensity at 510 nm was recorded through a ×40, 0.60 LD Achromplan objective with a photometric KAF 1400-G2 low-light-level digital camera. Data were recorded in Metafluor imaging analysis software 6.1 (Meridian Instrument Co., Kent, WA).

$[Ca^{2+}]_i$ was calculated by using the following equation: $[Ca^{2+}]_i = K_d \times S_f \times (R - R_{min}) / (R_{max} - R)$. Calibration of K_d (259 nM) has been described in detail by Vanterpool et al. (2005). In brief, K_d was determined with a standard calibration buffer kit (Molecular Probes). R_{max} was measured by application of 10 μ M ionomycin (Molecular Probes) in perfusion buffer, and R_{min} was measured by application of calcium-free buffer. For calcium-free buffer, 1.8 mM $CaCl_2$ in perfusion buffer was replaced with 10 mM EGTA. S_f was defined as the ratio of fluorescence intensity at 380 nm in free calcium vs. saturated calcium. Ionomycin (10 μ M) and glutamate (1 mM) were dissolved in perfusion buffer and were pulse delivered to slices. To investigate the contribution of NMDAR to glutamate-induced increase in $[Ca^{2+}]_i$, slices were perfused with D, L-2-amino-5-phosphonovalerate (AP5) in a final concentration of 200 μ M (Sigma-Aldrich). AP5, dissolved in perfusion buffer, was applied for 15–20 min prior to and during application of a second pulse of glutamate. In some experiments, a second pulse of glutamate was applied in the absence of AP5.

Statistical Analysis

Data were expressed as group means $\pm$ SEM. Results were analyzed via one-way or two-way analysis of variance (ANOVA) and the Student-Newman-Keuls method for post hoc comparisons (Sigmastat version 3.0; Sysstat Software Inc., Chicago, IL) or paired *t*-test where indicated; $P < 0.05$ was considered significant.

RESULTS

Phosphorylation of NR1 Was Decreased in Naïve Hippocampal Tissue From hAGS Compared With ibeAGS

Phosphorylation of NR1 (pNR1) is known to enhance NMDAR activity (Liu and Zhang, 2000). Therefore, we used Western blots to examine the phosphorylation state of NR1 to determine whether NMDAR function may be altered in hibernation. Because pNR1 was not detectable in total protein lysate, we compared the ratio of pNR1 over NR1 in membrane fractions prepared from hAGS, ibeAGS, and rats. pNR1/NR1 was significantly lower in hAGS compared with ibeAGS and rats ($P < 0.05$, effect of group; Fig. 1), suggesting that NMDAR function is down-regulated in hAGS. Purity of fractions was assessed by using Na^+, K^+ -ATPase and tubulin as membrane and cytosolic markers, respectively. We found that the membrane fraction is free of cytosolic components and that the cytosolic fraction contains low levels of membrane components (Fig. 2, top panel). Furthermore, the ratio of Na^+, K^+ -ATPase vs. tubulin in membrane and cytosolic fractions among groups was not significantly different (10.9 ± 2.1 , 11.6 ± 3.1 , and 9.8 ± 1.2 for hAGS, ibeAGS, and rats, respectively; $n = 3$ animals per group; $P > 0.05$), suggesting that the efficiency of protein extraction was similar among groups. Moreover, ibeAGS and hAGS did not differ in NR1 abundance in the membrane fraction as shown by similar ratios of NR1 vs. Na^+, K^+ -ATPase ($P > 0.05$; Fig. 2, bottom panel).

Notably, the ratio of NR1/ Na^+, K^+ -ATPase in the membrane fraction was significantly lower in AGS than in rats ($P < 0.05$, effect of group; Fig. 2), and the ratio of NR1/actin in total protein lysate was significantly higher in AGS than in rats ($P < 0.05$, effect of group; Fig. 3). The higher NR1/actin ratio in AGS suggests that greater NR1 abundance in the cytosolic fraction could not be due to lower cytosolic actin levels. Indeed, densitometric analysis showed that the intensity of the actin bands was similar among groups ($450,356 \pm 60,603$, $443,344 \pm 8,218$, and $450,570 \pm 22,124$ for hAGS, ibeAGS, and rats, respectively, $n = 4$ animals per group, $P > 0.05$). Moreover, NR1 abundance in the membrane fraction relative to NR1 abundance in total protein lysate was significantly lower in AGS than in rats but was similar in hAGS and ibeAGS ($P < 0.001$, effect of group; Fig. 4).

Glutamate-Induced $[Ca^{2+}]_i$ Increase is Similar in hAGS and ibeAGS

Emission fluorescence intensity when excited at 380 nm was similar among groups (664 ± 30 , 815 ± 70 , and 768 ± 51 for rats, hAGS, and ibeAGS slices, respectively; $n = 12-17$ slices per group; $P > 0.05$). Similar fluorescence intensity confirms equal loading and esterase activity.

The resting $[Ca^{2+}]_i$ in hippocampal slices prepared from hAGS, ibeAGS, and rats was 95.2 ± 7.6 nM, 89.7 ± 3.4 nM, and 93.3 ± 6.5 nM, respectively, and there was no significant difference among groups. Glutamate induced a significant increase in $[Ca^{2+}]_i$ in all groups compared with resting levels of Ca^{2+} ($P < 0.001$, main effect of treatment). Glutamate increased $[Ca^{2+}]_i$ to 385.6 ± 29 nM, 378.9 ± 37.2 nM, and 510.8 ± 37.9 nM in hAGS, ibeAGS, and rats, respectively. No difference was found in glutamate-induced $[Ca^{2+}]_i$ increase between hAGS and ibeAGS, however, the glutamate-induced $[Ca^{2+}]_i$ in slices from rats was greater than in slices from hAGS or ibeAGS ($P < 0.05$, group $\times$ treatment; Fig. 5).

NMDAR Function Is Suppressed in Hippocampal Slices From hAGS

To address the role of NMDAR in the glutamate-induced $[Ca^{2+}]_i$ increase, we treated slices with AP5, a competitive NMDAR antagonist. In the presence of AP5, glutamate-induced $[Ca^{2+}]_i$ increase was significantly attenuated in hippocampal slices prepared from rats and ibeAGS. In contrast, AP5 did not attenuate the glutamate-induced rise in $[Ca^{2+}]_i$ in hippocampal slices prepared from hAGS ($P < 0.05$, group $\times$ treatment; Fig. 6), suggesting that NMDAR did not contribute significantly to glutamate-induced increase in $[Ca^{2+}]_i$ in slices from hAGS.

In the absence of AP5, a second pulse of glutamate, which was applied 5–10 min after the first pulse, induced a similar $[Ca^{2+}]_i$ increase as the first pulse of glutamate ($116\% \pm 16\%$, 100% , $81\% \pm 19\%$ for hAGS, ibeAGS, and rats, respectively; $n = 1-2$ per group).

DISCUSSION

Here we report decreased phosphorylation of the NR1 subunit in hippocampal tissue from hAGS, which is consistent with the observed down-regulation of NMDAR function found in hAGS hippocampus. Decreased NMDAR function may contribute to tolerance to NMDA excitotoxicity and oxygen nutrient deprivation observed in hippocampal slices from hAGS (Ross et al., 2006) as well as tolerance to traumatic brain injury in vivo (Zhou et al., 2001).

NMDAR function is activated by phosphorylation and inactivated by dephosphorylation. Protein kinase C, cAMP-dependent protein kinases (PKA), and protein tyrosine kinases increase NMDAR channel activity, and protein tyrosine phosphatases and serine/threonine phosphatases decrease NMDAR channel activity (Liu and Zhang, 2000). Our data show that phosphorylation of NR1 (Ser897) is decreased in hAGS compared with ibeAGS and rats. Ser897 is phosphorylated mainly by PKA, suggesting that activity of PKA may be lower or serine phosphatase activity may be higher in hAGS compared with ibeAGS and rat. Prolonged exposure to freezing or anoxia is known to suppress PKA activity in wood frog (*Rana sylvatica*) livers or in turtle (*Trachemys scripta elegans*) livers (Holden and Storey, 1996; Mehrani and Storey, 1995). Nonetheless, although cold, hAGS do not freeze like wood frogs, nor are they anoxic while hibernating (Ma et al., 2005).

Our results show that NR1 phosphorylation in hAGS was significantly decreased compared with ibeAGS and rats in naïve hippocampal tissue. Decreased phosphorylation is consistent with decreased NMDAR function. Decreased phosphorylation of NMDAR in turtle brain, newborn piglet brain, and 1- or 3-week-old rats is thought to contribute to anoxia and hypoxia/ischemia tolerance via maintenance of critical $[Ca^{2+}]_i$ (Bickler, 1998; Fritz et al.,

2002; Vij et al., 2005). Therefore, down-regulation of NMDAR via decreased phosphorylation could be a common mechanism adopted by hypoxia/ischemia-tolerant animals.

Although NMDAR are considered relatively stable compared with AMPAR, NMDAR can move in and out of the plasma membrane at a slow rate (Nong et al., 2004), and NMDAR trafficking affects synaptic NMDAR function (Hardingham and Bading, 2002). NR1 abundance in both membrane fractions and total protein lysates is similar in hAGS and ibeAGS; in addition, the efficiency of protein extraction is similar among groups, suggesting that internalization of NR1 does not contribute to down-regulation of NMDAR in hAGS. Although some membrane components are found in cytosolic fractions, NR1 was not detected in cytosolic fraction from either hAGS or ibeAGS. These results further support the interpretation that internalization of NR1 does not play a role in decreased NMDAR function in hAGS. Notably, the proportion of NR1 expressed in the functional membrane pool in AGS is less than in rats. These data suggest that increased internalization or decreased insertion of NR1 may occur in AGS compared with rats.

Intracellular calcium is a crucial ion involved in neuronal development, gene expression, synaptic plasticity, and neuronal death. The resting level of $[Ca^{2+}]_i$ reported here for rats is 94.6 nM, which is similar to that in other studies (Harris-White et al., 1998; Pottorf et al., 2000; Raley-Susman et al., 2001; Colwell, 2001; Michel et al., 2002), and the resting level of $[Ca^{2+}]_i$ in AGS is similar to that in rats. Thus, a difference in resting $[Ca^{2+}]_i$ cannot explain enhanced neuroprotection in the hibernating state (Zhou et al., 2001; Ross et al., 2006).

Effects of AP5 indicate that NMDAR do not contribute significantly to the glutamate-induced $[Ca^{2+}]_i$ increase in slices from hAGS, suggesting that NMDAR function is down-regulated in slices from hAGS. These results are consistent with decreased NR1 phosphorylation in hibernation. However, because phosphorylation state may change in vitro, further work is necessary to determine whether decreased phosphorylation in hippocampal slices suppresses NMDAR function in vitro. Nonetheless, NMDAR function assessed using fura-2 calcium imaging is decreased in hippocampal slices cultured for 24 hr. At 24 hr in culture, hippocampal slices from hAGS fail to show significant increase in cell death after oxygen nutrient deprivation or application of 500 μ M NMDA plus 20 mM KCl. This is in contrast to slices from ibeAGS and rats that show typical excitotoxic cell death at this time (Ross et al., 2006). Thus, decreased NMDAR function at 24 hr in culture likely contributes to tolerance to oxygen nutrient deprivation and NMDA excitotoxicity in slices from hAGS and is consistent with decreased NMDAR function in hibernation.

Although decreased NMDAR function results in a lower Ca^{2+} influx through NMDAR in hAGS, glutamate-induced $[Ca^{2+}]_i$ increase is similar in hAGS and ibeAGS, suggesting that the rise of $[Ca^{2+}]_i$ in hAGS may come from other sources, such as through AMPAR, VGCC, or Ca^{2+} release from intracellular stores. Igelmund (1996) suggested that AMPAR are up-regulated in hippocampal slices from hibernating golden hamsters (*Mesocricetus auratus*) compared with slices from warm-acclimated hamsters. Therefore, the similarity of the glutamate-induced $[Ca^{2+}]_i$ increase between hAGS and ibeAGS may be due to the down-regulation of NMDAR and up-regulation of other sources of Ca^{2+} . VGCC could be up-regulated in hibernation, although this is unlikely because of reported down-regulation of Q-type calcium channels in synaptosomes prepared from hibernating 13-lined ground squirrel (Gentile et al., 1996).

Excessive Ca^{2+} accumulation has been implicated as a major trigger for neuronal injury. However, excitotoxic injury depends on the Ca^{2+} source (Tymianski et al., 1993) as well as

Ca^{2+} concentration (Hartley et al., 1993; Eimerl and Schramm, 1994; Lu et al., 1996). Ca^{2+} influx through NMDAR is more toxic than that through other sources (Tymianski et al., 1993; Hedrick et al., 2005). Thus, although Glu-induced $[\text{Ca}^{2+}]_i$ was similar in slices from hAGS and ibeAGS, the contribution of NMDAR to Glu-induced increase in $[\text{Ca}^{2+}]_i$ was less in slices from hAGS, which is consistent with enhanced protection from oxygen nutrient deprivation and NMDA (Ross et al., 2006).

We used rats as a positive control when there was no significant difference found between hAGS and ibeAGS. Differences between rats and AGS in NR1 abundance in the membrane fraction and Glu-induced increases in $[\text{Ca}^{2+}]_i$ may or may not be related to the ability of AGS to hibernate. Nonetheless, these differences are consistent with enhanced tolerance to OND in AGS compared with rats (Ross et al., 2006) and deserve further study. Liu et al. (1997) found that the rate of Ca^{2+} uptake by cardiac sarcoplasmic reticulum in ground squirrels was significantly higher than that in rats. Therefore, a higher rate of Ca^{2+} uptake via sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA) is another potential explanation for lower Glu-induced $[\text{Ca}^{2+}]_i$ increase found in hippocampal slices from AGS.

In summary, we report that NR1 phosphorylation is decreased in hippocampal tissue from hAGS, which is consistent with the decreased NMDAR function found in the hippocampal slices from hAGS. Furthermore, the fraction of NR1 in the functional membrane pool in AGS is smaller than that in rats. Modulation of NMDAR may play an important role in the neuroprotection observed in hAGS (Zhou et al., 2001; Ross et al., 2006).

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Fig. 1. pNR1/NR1 is decreased in hAGS compared with rat and ibeAGS, suggesting that suppressed NMDAR function is due to decreased phosphorylation of NR1 in hAGS. **Top:** Representative scan of pNR1 and NR1 in membrane fraction; h, hAGS; ibe, ibeAGS; R, rat. **Bottom:** Results of densitometric analysis of Western blots. Horizontal bars indicate significant difference between groups: $P < 0.05$, $n = 3-4$ animals per group.

Fig. 2.

NR1 is most abundant in the membrane fraction. Purity of fractions was confirmed by using Na⁺,K⁺-ATPase, and tubulin as membrane and cytosolic markers. **Top:** Representative scan of NR1, Na⁺,K⁺-ATPase, and tubulin in membrane and cytosolic fractions. Membrane fraction is free of cytosolic components; cytosolic fraction contains low levels of membrane components. NR1 cannot be detected in cytosolic fraction. h, hAGS; ibe, ibeAGS; R, rat; Pos, positive control. **Bottom:** NR1 in the membrane fraction is similar in hAGS and ibeAGS. NR1 abundance in the membrane fraction of both hAGS and ibeAGS is lower than in rats. Bottom panel shows results of densitometric analysis of Western blots. Horizontal bars indicate significant difference between groups: $P < 0.05$, $n = 3$ animals per group.

Fig. 3.

NR1 abundance in total protein lysate is higher in AGS than in rat; hAGS and ibeAGS are not different (**bottom**). **Top:** Representative scan of NR1 and actin in the total protein lysate. h, hAGS; ibe, ibeAGS; R, rat; Pos, positive control. Bottom panel shows results of densitometric analysis of Western blots. Horizontal bars indicate significant difference between groups: $P < 0.05$; $n = 4$ animals per group.

Fig. 4.

Ratio of NR1 band densities in membrane fraction and in total protein lysate shows that the fraction of NR1 abundance in the membrane fraction (NR1_{mem}) relative to total protein lysate (NR1_{total}) is lower in AGS than in rats but is similar in hAGS and ibeAGS. Horizontal bars indicate significant difference between groups: $P < 0.001$; $n = 3-4$ animals per group.

Fig. 5.

hAGS, ibeAGS, and rat have similar resting $[Ca^{2+}]_i$, and glutamate induces a significant increase in $[Ca^{2+}]_i$ in all groups. Both ibeAGS and hAGS show less glutamate-induced $[Ca^{2+}]_i$ increase compared with rat. No difference is found between hAGS and ibeAGS; $n = 12-17$ slices per group. Horizontal bars indicate significant difference between groups: $P < 0.05$.

Fig. 6.

NMDAR function is suppressed in hAGS. **Top:** AP5 attenuated the second pulse of glutamate-induced $[Ca^{2+}]_i$ response to 41% and 42% of the initial glutamate-induced response, respectively, in slices from rats and ibeAGS ($P < 0.05$) and to 78% of the initial response in slices from hAGS ($P > 0.05$), suggesting that NMDAR are suppressed in hibernation ($n = 8-17$ slices per group). Horizontal bars indicate significant difference between groups: $P < 0.05$. **Bottom:** Representative traces of a Ca^{2+} imaging experiment using fluorescence dye fura-2 in a hippocampal slice prepared from ibeAGS. White horizontal bar indicates perfusion with normal perfusion buffer (PB); black horizontal bar indicates perfusion with AP5+PB. Arrowheads indicate the time points at which a pulse of glutamate was given. Different traces indicate different regions of interest within the CA1 region.