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Adult naked mole-rat brain retains the NMDA receptor subunit GluN2D associated with hypoxia tolerance in neonatal mammals

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ABSTRACT

Adult naked mole-rats show a number of systemic adaptations to a crowded underground habitat that is low in oxygen and high in carbon dioxide. Remarkably, brain slice tissue from adult naked mole-rats also is extremely tolerant to oxygen deprivation as indicated by maintenance of synaptic transmission under hypoxic conditions as well as by a delayed neuronal depolarization during anoxia. These characteristics resemble hypoxia tolerance in brain slices from neonates in a variety of mammal species. An important component of neonatal tolerance to hypoxia involves the subunit composition of NMDA receptors. Neonates have a high proportion of NMDA receptors with GluN2D subunits which are protective because they retard calcium entry into neurons during hypoxic episodes. Therefore, we hypothesized that adult naked mole-rats retain a protective, neonatal-like, NMDA receptor subunit profile. We used immunoblotting to assess age-related changes in NMDA receptor subunits in naked mole-rats and mice. The results show that adult naked mole-rat brain retains a much greater proportion of the hypoxia-protective GluN2D subunit compared to adult mice. However, age-related changes in other subunits (GluN2A and GluN2B) from the neonatal period to adulthood were comparable in mice and naked mole-rats. Hence, adult naked mole-rat brain only retains the neonatal NMDA receptor subunit that is associated with hypoxia tolerance.

1. Introduction

Brain tissue is especially vulnerable to oxygen deprivation in almost all species. However, brain slices from naked mole-rats (*Heterocephalus glaber*) show an exceptional resistance to the negative effects of hypoxia [11]. This is consistent with evolving in a chronically low oxygen environment. Naked mole-rats are indigenous to East Africa where they live in sealed subterranean burrows in colonies of up to around 300 individuals [6]. Since many animals breathe the same poorly ventilated air, especially when huddled together in their nests, oxygen becomes depleted while CO_2 becomes elevated [1].

Naked mole-rats have several peripheral adaptations to survive in their hypoxic environment. Their hemoglobin has a higher affinity for oxygen than most other mammals and their weight-specific metabolic rate is about one-third less than that of other rodents [7,10]. Also, their peripheral nerves are insensitive to acidosis, which is useful in their environment high in CO_2 [17].

In a previous study, we assessed central resistance to hypoxia in hippocampal slices from adult naked mole-rats and laboratory mice. We found that synaptic transmission in slices from naked mole-rats was maintained in low oxygen concentrations that caused transmission to decrease or cease in slices from mice [11]. In addition, neuronal depolarization after total oxygen deprivation was greatly delayed in slices from naked mole rats compared to slices from mice. Moreover, we recently found that hippocampal neurons in slices from naked mole-rat brain had an attenuated increase in internal calcium concentration during a 10 min hypoxia exposure when compared to neurons from mice [12]. This is important because too much internal calcium accumulation leads to irreversible damage or cell death. The hypoxia tolerance of adult naked mole-rat brain tissue resembles the tolerance observed in neonatal brain slices from a variety of common mammalian species [2]. This led us to hypothesize that adult naked-mole rat brain may retain features that confer hypoxia tolerance in neonates [11].

Neonatal mammalian brains display a number of hypoxia tolerant mechanisms [2]. An important one involves NMDA receptors and intracellular calcium [5]. Neurons experience a cascade of cellular processes during oxygen deprivation: failure to regenerate ATP leads to loss of ion concentration gradients and cellular depolarization, followed by massive glutamate release due to reversal

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of sodium-dependent glutamate transporter activity [3,9,19]. This rise in glutamate over-activates glutamate receptors, importantly NMDA receptors, allowing toxic amounts of cations, especially calcium, to enter the cells. Intracellular calcium activates calpains, calcium-sensitive proteases that are major participants in neuronal degeneration [15].

Therefore, NMDA receptors play a large role in hypoxic injury. These receptors are tetrameric assemblies including two GluN1 subunits and two subunits selected from GluN2A, GluN2B, GluN2C, and GluN2D [8]. The GluN2 subunits determine the biophysical and pharmacological activity of the receptor [16,20,22,23]. Importantly, receptors that include GluN2D subunits are particularly resistant to hypoxia [5]. Under hypoxia these receptors have a greatly reduced channel open time compared to receptors without GluN2D subunits. Consequently, less calcium enters the cells through NMDA receptors with GluN2D subunits. The resulting lower internal calcium concentration could prevent calcium-mediated cell injury, and is thought to be an important mechanism of hypoxia resistance in neonatal mammals [5].

The subunit profile of NMDA receptors in hippocampus and cortex changes as an animal develops and ages. GluN2B and GluN2D are highly expressed in neonatal brains while GluN2A and GluN2C are relatively scarce. As the animal matures, the expression pattern changes: GluN2B and GluN2D decrease while GluN2A and GluN2C increase [14]. During the developmental change in the GluN2 subunit profile, there is a corresponding decrease in tolerance to hypoxia [21]. In mice, these changes occur during the first postnatal month. Thus neonatal mice (e.g. P6) show a relatively high proportion of GluN2D, and during hypoxia, a relatively attenuated accumulation of intracellular calcium *in vitro*, and a robust tolerance to hypoxia *in vivo*. In contrast, mice 3–4 weeks old and older show a relatively low proportion of GluN2D, a much greater accumulation of intracellular calcium during hypoxia, and a much lower tolerance to hypoxia *in vivo*.

Since adult naked mole-rats show both a neonatal-like attenuated calcium response and tolerance to hypoxia, we hypothesized that they retain a protective, neonatal-like NMDA receptor subunit profile. If true, we should observe less prominent age-related changes in subunits compared to the changes observed in mice. We used subunit-specific antibodies to assess age-related changes in NMDA receptors in mice and naked mole-rats. The results show that adult naked mole-rat brain retains much more of the hypoxia-protective GluN2D subunit compared to adult mice. However, age-related changes in other subunits (GluN2A and GluN2B) were comparable to those in mice. Hence, adult naked mole-rat brain only retains the NMDA receptor subunit that is associated with hypoxia tolerance in neonates.

2. Methods and materials

2.1. Animals

Experiments were performed on male and female C57Bl/6 mice (bred from stock obtained from Charles River Laboratories, Wilmington, MA) and naked mole-rats of both sexes (born in colonies maintained in our laboratories), housed under normoxic laboratory conditions. Experiments were conducted on mice and naked mole-rats at postnatal day 6 and adulthood (2–3 months for mice; 1–3 years for naked mole-rats). Animal protocols were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

2.2. Western immunoblotting

Whole brains (without cerebellum) were freshly excised from both mouse and naked mole-rat at postnatal day 6 (P6) and at P > 60 (adults) and homogenized in ice-cold buffer containing 50 mM Hepes, 125 mM NaCl, 100 mM sucrose, 2 mM KAcetate, 10 mM EDTA, 10 mM N-ethylmaleimide, 10 μg/mL leupeptin, 10 μmg/mL pepstatin A, 2 μg/mL aprotinin, and 2 00 mM PMSF. The homogenate was centrifuged at a low speed (1000 × *g*, 10 min), then the supernatant was re-centrifuged at a high speed (20000 × *g*, 10 min), and the pellet was re-suspended in homogenizing buffer. Proteins were precipitated in methanol at 4 °C, re-suspended in SDS–PAGE buffer with 1% DTT for a final concentration of 2 μg protein/μL, and boiled for 5 min. Proteins were resolved on 7.5% Tris HCl gels and transferred (40 V, 20 min, 4 °C then 15 V, overnight, 4 °C) to PVDF membranes in transfer buffer (12 mM Tris HCl, 6 mM NaAcetate, 0.3 mM EDTA disodium, pH 7.5). Blots were blocked for 1 h at room temperature in 1% skim milk blotting buffer, then incubated overnight at 4 °C with primary antibody directed against GluN1 (1:500, Antibodies Incorporated), GluN2A (1:5000; Chemicon International), GluN2B (1:500, Antibodies Incorporated), or GluN2D (1:1000, Chemicon International). After incubation, blots were washed with PBS (two cycles of a quick wash followed by a 5-min wash repeated twice), and then incubated with the appropriate secondary antibody in 1% skim milk blotting buffer. After the blot was again washed with PBS, it was incubated for 5 min in a chemiluminescence reagent mixture (ECL plus, GE Healthcare) and exposed to film for 5–10 min depending on signal strength. Four different blots were run for each antibody; each blot contained a sample from a neonate and an adult mouse and a neonate and an adult naked mole-rat. All blots were re-blotted with β-actin as a loading control.

Western blots were scanned and analyzed quantitatively by densitometry with ImageJ software. Data were statistically analyzed using a two-tailed unpaired *t*-test with significance level set to 5%. For GluN2A, which increased in an age-dependent manner, we designated the density of the band from adult as 100%. We then determined relative density as a percentage of the band from neonate relative to the band from adult. For GluN2B, GluN2D and GluN1, which decreased in an age-dependent manner, we designated the density of the band from neonate as 100% and determined relative density as a percentage of the band from adult to the band from neonate.

3. Results

We used immunoblotting with subunit-specific antibodies to measure age-related changes in the NMDA receptor subunits, GluN2A, GluN2B, GluN2D, and GluN1, in mice and naked mole-rats. Previous studies have shown that in mice GluN2B and GluN2D decrease with age whereas GluN2A increases while GluN1 remains relatively stable [14]. Our results for mice were consistent with this. For naked mole-rats, we also found a variety of age-related changes. The most robust species difference we found was for GluN2D, with mice showing a decrease in expression of ∼90% (from P6 to *P* > 60) but naked mole-rat showing a decrease of only ∼33%. There were no statistical differences in age-related changes for GluN2A or GluN2B. Quantitative statistics are presented in the paragraphs below.

Fig. 1 shows examples of Western blots for each receptor subunit that we examined for both species and for both age groups. The important comparisons were within subunit and species. The absolute density of bands labeled with the same antibody cannot be compared across species because of possible differences in antibody affinity. Similarly, the density of bands labeled with different antibodies (i.e., different receptor subunits) cannot be compared within or between species. As the examples in the top row of Fig. 1 show, both mice and naked mole-rats showed a higher density for GluN2A in adults compared to their neonate counterparts. In contrast, the other three receptor subunits showed a decrease

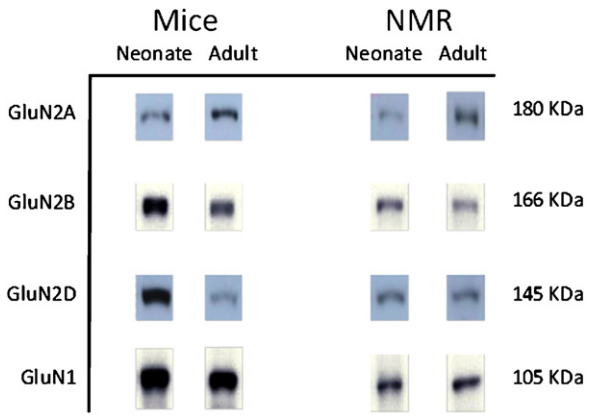

Fig. 1. Example western blots from the four NMDA receptor subunits examined. For each subunit, examples from a neonate and an adult are shown for both mice and naked mole-rats (NMR). A change in the density of the blots between neonate and adult corresponds to an age-related change in expression of that subunit.

in density from the neonatal period to adulthood in both species (Fig. 1).

To make quantitative comparisons of these age-related changes, we calculated the average percentage difference in the optical density of immunoreactive bands in samples from neonates and adults within subunit type and species. For GluN2A in mice, we calculated that the density of bands from neonates was 35.2 + −13.5% that of adults (Fig. 2A). Similarly, the density of bands from neonatal naked mole-rats was 56.0 + −15.55% that of adults (Fig. 2A). These quantitative results correspond to the age-related increases shown for the examples in Fig. 1. We used a *t*-test to compare the age-related change between species (35.2% mice and 56.0% naked mole-rats) and found no significant difference ($t = 1.02$, df = 6, $p = 0.35$).

The remaining subunits that we tested showed age-related changes in the opposite direction from GluN2A. For GluN2B (Fig. 2B), the density of the bands decreased from neonates to adults for both species, and there was not a significant species difference ($t = 0.67$, df = 6, $p = 0.53$). The labeling of GluN2B in adult mice was 64.6 + −3.6% that of neonates whereas GluN2B labeling in adult naked mole-rats was 71.4 + −9.0% that of neonates.

The density of bands for GluN2D also decreased from neonates to adults in both species. However, as mentioned above, GluN2D showed the most robust species difference (Fig. 2C). For adult mice, GluN2D labeling was only 12.7 + −0.6% that of neonates whereas in adult naked mole-rats, the GluN2D density was 66.1 + −5.5% that of neonates; this species difference was highly significant ($t = 9.74$, df = 6, $p = 0.0001$).

We also measured age-related changes in the NMDA receptor subunit GluN1. This subunit is found in all NMDA receptors and thus gives a measure of overall changes in the quantity of NMDA receptors. GluN1 showed the smallest age-related change among subunits for both mice and naked mole-rats, as expected (Fig. 2D). However, the species difference was significant. Adult mice showed a decrease to 75.6 + −4.7% that of neonates and naked mole-rats showed a decrease to 93.1 + −2.1% ($t = 2.60$, df = 5, $p = 0.048$).

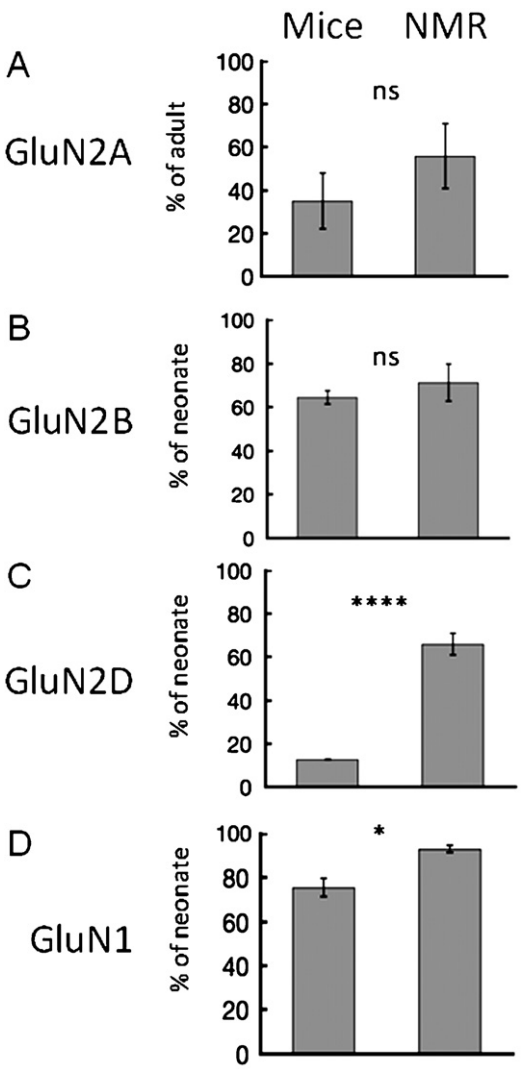

Fig. 2. Analysis of age-related changes in NMDA receptor subunit expression. A. GluN2A increased from neonate to adult in both species. Hence, we designated the adult band as having a density of 100%. The bars indicate the average percentage of adult density shown by neonates. B., C., and D. GluN2B, GluN2D, and GluN1 decreased from neonate to adult in both species (opposite from GluN2A). Therefore, we designated the neonatal band as having a density of 100%. The bars indicate the average percentage of neonatal density shown by adults. Each bar in Fig. 2 was derived from an $N = 4$, except for the bar corresponding to GluN1 for naked mole-rat, which was $N = 3$. Error bars are standard error. $^{*}p < 0.05$, $^{****}p < 0.0001$.

4. Discussion

The present study shows that adult naked mole-rats retain relatively high brain levels of GluN2D, an NMDA receptor subunit associated with hypoxia tolerance in neonatal rats and mice. To the best of our knowledge this is the first demonstration of a constitutive neurotransmitter receptor adaptation in the brain of a hypoxia-tolerant mammalian species. Because the animals we tested were maintained under normoxia, this characteristic is not induced by hypoxia but is inherent to this species (much like their high affinity hemoglobin and low metabolic rate).

Other hypoxia-tolerant animals are known to alter their neuronal NMDA receptors in a variety of ways that are induced by hypoxic challenge (acute hypoxia). In turtle neurons, it has been shown that NMDA receptors are internalized after they are exposed to hypoxia for several days [4]. NMDA receptors in the hibernating Arctic Ground squirrel had significantly less phosphorylation of the GluN1 subunit of the NMDA receptor during hibernation. Phosphorylation of GluN1 is known to enhance NMDA receptor activity [18,24,25]. As already mentioned, neonatal rats and mice have more GluN2D; receptors containing this subunit have lower channel activity (higher percentage of time in the closed state) than others during hypoxia exposure. The NMDA receptor modifications observed in all of these model systems would work to prevent intracellular calcium accumulation during exposure to hypoxic conditions. Therefore, it is not surprising that naked mole-rats would also show alterations in their NMDA receptors. It is

possible that naked mole-rats living under chronic hypoxia, their normal environment in the wild [1], would show additional modulation of NMDA receptors, resulting in an even greater attenuation of the calcium response.

There are two additional aspects of the present study that are interesting and worthy of speculation. The first aspect concerns the age-related reduction in GluN1 which reflects a reduction in NMDA receptors. Naked mole-rats show significantly reduced reduction of GluN1 into adulthood compared to mice. The other aspect concerns what consequences, other than hypoxia tolerance, might be associated with retaining GluN2D into adulthood in naked mole-rats. NMDA receptors with GluN2D have a longer open time when stimulated (under normoxic conditions) compared to NMDA receptors with other GluN2 subunits [5]. But how this might affect behavior is unknown and we have not observed any obvious deficits in the day-to-day behaviors of the mole-rats in our laboratories. Because NMDA activity is associated with learning and memory, it is noteworthy that naked mole-rats can learn an operant conditioning task for discriminating odorants as quickly and as well as rats [13].

5. Conclusions

Compared to mice, naked mole-rats retain much more of the important neonatal subunit, GluN2D, that is protective during hypoxia. Naked mole-rats do not retain the entire neonatal NMDA profile into adulthood. GluN2A increased and GluN2B decreased with age as in mice. The retention of GluN2D in adult naked mole-rats is consistent with other brain features that suggest a slowed or arrested maturation in this species [11].

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