NMDA and kainate receptor expression, long-term potentiation, and neurogenesis in the hippocampus of long-lived Ames dwarf mice

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Abstract In the current study, we investigated changes in *N*-methyl D-aspartate (NMDA) and kainate receptor expression, long-term potentiation (LTP), and neurogenesis in response to neurotoxic stress in long-living Ames dwarf mice. We hypothesized that Ames dwarf mice have enhanced neurogenesis that enables retention of spatial learning and memory with age and promotes neurogenesis in response to injury. Levels of the NMDA receptors (NR)1, NR2A, NR2B, and the kainate receptor (KAR)2 were increased in Ames dwarf mice, relative to wild-type littermates. Quantitative assessment of the excitatory postsynaptic potential in Schaffer collaterals in hippocampal slices from Ames dwarf mice showed an increased response in high-frequency induced LTP over time compared with wild type. Kainic acid (KA) injection was used to promote neurotoxic stress-induced neurogenesis. KA mildly increased the number of doublecortin-positive neurons in wild-type mice, but the response was significantly enhanced in the Ames dwarf mice. Collectively, these data support our hypothesis that

the enhanced learning and memory associated with the Ames dwarf mouse may be due to elevated levels of NMDA and KA receptors in hippocampus and their ability to continue producing new neurons in response to neuronal damage.

Keywords Ames dwarf · Hippocampus · NMDA receptors · LTP · Neurogenesis

Introduction

Spatial memory is an important type of memory that helps individuals to cope with the environment. The hippocampus is the major brain region implicated in spatial memory performance (Rolls 2000; O'Keefe and Nadel 1978). Studies of human subjects with hippocampal damage provide evidence that this brain region plays a critical role in spatial memory (Abrahams et al. 1997; Astur et al. 2002; Feigenbaum et al. 1996; Goldstein et al. 1989; Maguire et al. 1996; Rosenbaum et al. 2000).

N-methyl D-aspartate (NMDA) receptor activation plays a central role in the acquisition of spatial memory (Morris et al. 1986; Tsien et al. 1996). Pharmacological blockade of NMDA receptors or deletion of NMDA receptor subunit 1 (NR1) leads to a substantial impairment of spatial memory (Danysz et al. 1995; Morris et al. 1990; Tsien et al. 1996). In addition, it has been found that like NMDA receptors, kainic acid receptors are also involved in long-term

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changes in synaptic transmission (Bortolotto et al. 1999; Contractor et al. 2001).

Long-term potentiation (LTP) is generally considered as a cellular and molecular mechanism for learning and memory (deToledo-Morrell et al. 1988; Gallagher and Nicolle 1993; Landfield and Lynch 1977; Moore et al. 1993; Shankar et al. 1998; Ward et al. 1999a, b). Similar to impairment of spatial memory, NR1 knockout mice exhibit impairment of LTP (Nakazawa et al. 2003). Several lines of evidence also suggest the involvement of NMDA receptors in the formation of spatial memory and in the regulation of neurogenesis in adults (Arvidsson et al. 2001; Luk et al. 2003; Deisseroth et al. 2004; Joo et al. 2007). Multiple studies have demonstrated that kainic acid (KA)-induced hippocampal injury enhances neurogenesis in adult rodent brain (Choi et al. 2007; Dong et al. 2003; Kwon et al. 2008; Yang et al. 2008), and newly formed neurons in the dentate gyrus are involved in learning and memory circuits (Zhao et al. 2006; Toni et al. 2007; Ge et al. 2007). Thus, the role of increased neurogenesis in reducing cognitive dysfunction after injury cannot be ruled out. Moreover, increased dentate neurogenesis has been positively correlated with spatial learning and memory performance (Drapeau et al. 2003; Kempermann et al. 2004; Shors et al. 2001).

Ames dwarf mice are long-lived and express a mutated Prop-1 gene resulting in impairment of anterior pituitary development that leads to a deficiency of circulating growth hormone, thyroid stimulating hormone, and prolactin (Brown-Borg et al. 1996). The lack of plasma GH results in severely reduced plasma levels of insulin-like growth factor 1. Despite decreased circulating insulin-like growth factor (IGF)-1, these animals have been shown to exhibit normal and, in some instances, enhanced cognitive function (Kinney et al. 2001a, b). A study reported that the local, hippocampal-synthesized IGF-I might be able to maintain central nervous system function independently of regulation by the hypothalamic–pituitary axis (Sun et al. 2005a). Recently, it has also been shown that old aged Ames dwarf mice have elevated levels of IGF-1 protein in the hippocampus, whereas the levels of the corresponding mRNAs are as high as in normal mice (Sun et al. 2005a). Ames dwarf mice also have higher levels of hippocampal neurogenesis that might contribute to the delay of cognitive loss during aging in long-lived dwarf mice (Sun and Bartke 2007; Sun et al. 2005b).

Recently, we have found that Ames dwarf mice have better hippocampal-based spatial memory compared with age-matched wild-type mice at 3 months of age, and the Ames mice retain this memory capability following an oxidative insult with KA (Sharma et al. 2010). The enhanced learning and memory observed in the Ames mice in our study is likely due to enhanced antioxidant status, enhanced neurogenesis, and/or to the differential expression and sensitivity of kainate (kainate receptor (KAR)1, KAR2, GluR5, GluR6/7) and NMDA (NR1, NR2A, NR2B) receptors in Ames dwarf as compared with wild-type mice. However, the data reported are only correlative and definite mechanisms underlying differences between spatial memory in Ames dwarf and wild-type mice are not clear.

In an effort to identify possible mechanisms for the enhanced spatial memory of the Ames mice, the present study was designed to determine the differences in NMDA and KA receptor expression and LTP between dwarf and wild-type mice. An additional aim was to determine the extent of neurogenesis in the dentate gyrus of Ames dwarf mice following neuronal damage with KA.

Material and methods

Experimental animals

Ames dwarf mice (*df/df*) were bred and maintained at the animal facilities of the University of North Dakota under controlled conditions of 12:12-h light/dark cycle and temperature ($22\pm 1^\circ\text{C}$) with ad libitum access to food (8640 Teklad 22/5 rodent diet with 22.6% crude protein, 5.2% fat, Harlan Laboratories) and water (standard laboratory conditions). The Ames dwarf mice used in this study were derived from a closed colony with a heterogeneous background (over 25 years). Homozygous (*df/df*) or heterozygous (*df/+*) dwarf males were mated with carrier females (*df/+*) to generate dwarf mice. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at the University of North Dakota. Three-month-old animals were utilized in this study. Wild-type female mice were not included to avoid potential hormonal effects. Both sexes of Ames dwarf mice were used since female

dwarfs do not exhibit estrous cycles and are therefore not influenced by sex-steroid hormones

RNA extraction and RT-PCR

Gene expression of the NMDA receptor subunits NR1, NR2A, and NR2B and the kainic acid receptor subunits KAR1, KAR2, GluR5, GluR6, and GluR7 were evaluated in one half of the hippocampus isolated from 3-month-old Ames dwarf ($n=6$) and age-matched wild-type mice ($n=6$) using quantitative real-time RT-PCR techniques. The brain was removed and the hippocampus was flash frozen in liquid nitrogen and stored at -80°C until further processing for Western blots and RT-PCR. Total RNA was extracted from the hippocampal tissue using Ultraspec RNA (Biotech), based on previously described methods (Chomczynski and Sacchi 1987). Equal amounts of RNA for the gene of interest and the reference gene, $\beta 2$ -microglobulin (Lupberger et al. 2002), were utilized to perform one-step real-time quantitative PCR using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and were assayed using a SmartCycler instrument (Cepheid, Sunnyvale, CA, USA).

Gene expression was quantified using the comparative C_T (threshold cycle) method (Heid et al. 1996). ΔC_T was obtained for each sample/gene by the following calculation: $\Delta C_T = C_{T,X} - C_{T,R}$, where $C_{T,X}$ is the threshold number for target gene amplification and $C_{T,R}$ is the threshold number for reference gene amplification. The amount of target (in all the treatment groups) was normalized to an endogenous reference gene (beta 2 microglobulin ($\beta 2M$); light chain of class I major histocompatibility complex) and relative to the control group (wild-type mice). The RT-PCR primers used were as shown in Table 1.

Western blot

Proteins extracted from the remaining one half of the frozen hippocampal tissue by homogenization on ice in CPE buffer (Brown-Borg and Rakoczy 2000) were used for Western blot analysis. Protein quantification of the samples was performed using the Bradford assay (Bradford 1976). Extracted protein samples (50 μg) were separated on 7.5% Criterion™ Precast Gels (Bio-Rad). Following electrophoresis, proteins

Table 1 Primer pairs utilized for real-time RT-PCR

Gene	Primer 5'–3'
$\beta 2M$	For—AAG TAT ACT CAC GCC ACC CA Rev—AAG ACC AGT CCT TG
NR1	For—CAG GAG CGG GTA AAC AAC AGC AAC Rev—GAC AGC CCC ACC AGC AGC CAC AGT
NR2A	For—AGC CCC CTT CGT CAT CGT AGA Rev—CAG AAG GGG AAA CAG TGC CAT TA
NR2B	For—TCC GCC GTG AGT CTT CTG TCT ATG Rev—CTG GGT GGT AAA GGG TGG GTT GTC
KAR1	For—CAG CCC AGT GTG TTT GTG A Rev—AAC ACC CTG GCA ATT CCC TC
KAR2	For—TCT TGG GCT TTT CCA TGT TCA Rev—CAA ACT CCG GGT AGA AGG GAT
GluR5	For—TCA AAA TCC GCC AGC TTC C Rev—TGA GCA GAG GTT TGG CGT CT
GluR6	For—TTC CTG AAT CCT CTC TCC CCT Rev—CAC CAA ATG CCT CCC ACT ATC
GluR7	For—CCG CAA GTC TGA TAG GAC CC Rev—CAG TAG CCC TCG AAC CGG T

were transferred to polyvinylidene difluoride membrane and were then blocked in 5% nonfat powdered milk for 1 h at room temperature. Membranes were incubated overnight at 4°C with antibodies to NR1, NR2A, or NR2B (1:200, Santa Cruz) and KAR2 (1:200, Millipore), GluR5 (1:500, Alomone), or GluR 6/7 (1:200, Millipore). Three 15-min washes in $1\times$ Tris buffered saline with 0.05% Tween 20 (TTBS) were applied before incubation with appropriate secondary antibody. Another series of three washes for 15 min each with $1\times$ TTBS was followed by a 5-min incubation in a chemiluminescent substrate (Bio-Rad). The membrane was then exposed to radiographic film (Fisher Scientific) and relative density quantified using a UVP Bioimaging system (Upland). Ponceau S staining for total protein was used as a loading control.

Long-term potentiation

A separate group of 3-month-old wild-type ($n=4$) and Ames dwarf mice ($n=4$) was anesthetized with isoflurane and decapitated. The brains were removed and placed in ice-cold buffered saline solution that contained 130 mM NaCl, 24 mM NaHCO_3 , 3.5 mM KCl, 1.25 mM NaH_2PO_4 , 0.5 mM CaCl_2 , 5 mM MgCl_2 , and 10 mM glucose saturated

with 95% O₂ and 5% CO₂ (pH 7.4). Horizontal slices (400 µm) containing the hippocampus were cut using a vibrating blade microtome (VT 1000S; Leica, Wetzlar, Germany). Slices were initially incubated in buffered saline solution at 35°C for 40 min for recovery and then kept at room temperature (~23°C) until use.

Slices were perfused in the above solution except the concentration of CaCl₂ was raised to 2.5 mM and the concentration of the MgCl₂ was reduced to 1.5 mM. Field excitatory postsynaptic potentials (fEPSPs) were recorded by placing a glass pipette containing the above extracellular solution in the stratum radiatum of the CA1 region and an insulated bipolar tungsten electrode ~200 µm from the recording electrode in the radiatum to stimulate the Schaffer collateral fibers. The stimulation intensity was adjusted to generate approximately 30–40% of a maximal response. The evoked fEPSPs were recorded with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA, USA) at 0.1 Hz in Clampex 9.2. After stabilization, 10 min of basal fEPSPs were recorded. LTP was induced by application of high-frequency stimulation of the Schaffer collateral fibers (1 s of 100 Hz, repeated three times at an interval of 10 s). The initial slopes of the fEPSPs were measured in Clampfit 9.2, and LTP was expressed as the percentage of the basal level (the average of initial slopes from the basal fEPSPs before the induction protocol was treated as 100%). All of the LTP was expressed as the mean ± standard error of the mean (SEM) unless indicated otherwise. Statistical analyses were made by two-tailed *t* tests and one-way analysis of variance (ANOVA).

Kainic acid injections and immunohistochemistry

To evaluate potential mechanisms leading to the resistance or enhanced recovery from oxidative insult in the CNS, we determined the regenerative capacity following an excitotoxic insult that specifically targets the hippocampus. Wild-type mice were randomly divided into three groups (*n*=4/group) to receive one of the following treatments: normal saline (WT-SAL), KA 15 mg/kg (WT-KA 15), or KA 30 mg/kg (WT-KA 30). Ames dwarf mice (*n*=3/group) were randomly divided to receive either normal saline (DF-SAL) or KA 15 mg/kg (DF-KA 15). These doses were based on

our previous study that showed that 30 mg/kg KA in wild-type mice and 15 mg/kg in Ames dwarf mice produces an equal amount of neuronal loss as well as an equal intensity of seizures due to differences in their body size (Sharma et al. 2010). Animals were perfused with phosphate-buffered saline (PBS; 0.1 M sodium phosphate buffer, pH 7.4, containing 0.9% saline) containing 4% paraformaldehyde, pH 7.4, 16 days after saline or KA injection. The 16-day time point was chosen because it was reported that the absolute number of newly born neurons in the KA-lesioned hippocampus was significantly higher at this time point relative to controls (Hattiangady et al. 2004, 2008). Brains were removed and placed in 30% sucrose for cryoprotection. Coronal sections (30 µm thick) were obtained using a freezing sliding microtome (Leica 3000R) and collected into PBS. Sections were then preserved in a cryoprotectant solution consisting of 0.1 M PBS, pH 7.2 (50%, *v/v*), sucrose (30%, *w/v*), polyvinylpyrrolidone (1%, *w/v*), and ethylene glycol (30%, *v/v*) at –20°C until staining (Watson et al. 1986).

Sections (every tenth free-floating section) from different mice were processed in parallel. Sections were rinsed in 0.1 M PBS for 2 h (to remove the cryoprotectant solution). After extensive rinsing, sections were incubated in 0.1 M PBS containing 3% normal goat serum, 1% BSA, and 0.1% Triton-X 100 for 2 h at room temperature. Sections were then incubated overnight at 4°C with the rabbit anti-doublecortin (anti-DCX; 1:1600, Abcam). Thereafter, sections were incubated with secondary biotinylated antibody (goat anti-rabbit, 1:500) for 1 h. Endogenous peroxidases were blocked by incubation in 3% H₂O₂ followed by incubation in streptavidin–horseradish peroxidase complex (Vector Labs), according to the manufacturer's instructions (Vector Labs). Sections were developed with diaminobenzidine as the substrate, mounted onto slides, dried overnight, dehydrated, and coverslipped with Permount (Fisher Scientific).

Quantification of DCX-positive cells using design-based stereology

Non-biased quantification of DCX-positive nuclei was performed using the optical fractionator method as originally described by West and Gundersen (1990) and adapted from previous studies (Hattiangady et al.

2008; Rao et al. 2006). DCX-positive cells in the dentate granular cell layer were counted in every 10th section through the entire hippocampus using the StereoInvestigator software (Microbrightfield Inc., Wiliston, VT, USA) on an Olympus BX51WI with a motorized *x*, *y*, and *z* stage. In each animal, DCX-positive cells were counted from 50–500 randomly and systematically selected frames (each measuring 30×30 μm , 0.0009 mm^2 area) in every serial section using the ×40 objective. For cell counting, the contour of the granular cell layer was delineated and the grid size set following standard manufacturer's instructions. In brief, the number and location of counting frames and the counting depth for that section were determined by entering parameters for the grid size (120×35 μm), the thickness of top guard zone (5%), and the optical dissector height (14 μm). The guard zones were set at 4 μm above and below the counting depth for each section with regional thickness and variation in section integrity taken into consideration. The cell bodies of DCX-positive cells were counted if they were entirely within the 14- μm depth counting frame. The StereoInvestigator software calculated the total number of DCX-positive cells per GCL by utilizing the optical fractionator formula: $N=1/\text{ssf}.1/\text{asf}.1/\text{hsf}.\Sigma Q^-$. For the calculations, ssf = section sampling fraction, which was 10 in our study as every 10th section was sampled; area sampling fraction (asf), which is calculated by dividing the area sampled with total area of the dentate hilus (i.e., the sum of DH areas sampled in every 10th section); hsf=height sampling fraction, which was calculated by dividing the height of the counting frame (14 μm) with the section thickness at the time of analysis (30 μm); and ΣQ^- denotes the total count of particles sampled for the entire DG. The sampling was optimized for maximal efficiency, with a final mean coefficient of error of less than 10%.

Statistical analysis

The RT-PCR and Western blotting data were analyzed using unpaired Students *t* tests. One-way ANOVA was used to analyze LTP data while the neurogenesis data were analyzed by two-way ANOVA (factors: genotype and treatment; GraphPad Prism). All data are presented as mean±SEM, and the *n* values are listed in the legends.

Results

NMDA receptor subunit gene expression

The mRNA levels of the NMDA subunits NR1, NR2A, and NR2B were assessed in the hippocampi of wild-type and Ames dwarf mice. The levels of NR1 and NR2A mRNA were 66% and 83% higher, respectively, in Ames dwarf as compared with wild-type mice ($p<0.05$; Fig. 1). There was no significant difference in the expression of NR2B mRNA between Ames dwarf and wild-type mice.

NMDA receptor subunit protein levels

The protein levels of NR1, NR2A, and NR2B were determined in hippocampal tissues from untreated wild-type and Ames dwarf mice. Both NR2A and NR2B protein levels were higher (49 and 68%, respectively) in Ames dwarf mice as compared with wild-type mice ($p<0.05$; Fig. 2). The protein levels of the NR1 subunit were not significantly different between Ames dwarf and wild-type mice.

KA receptor gene expression

The mRNA levels of KAR1, KAR2, GluR5, GluR6, and GluR7 were assessed in the hippocampi of both genotypes. The expression levels of KAR2 were higher in the Ames dwarf as compared with wild-type mice ($p=0.0556$; Fig. 3). Following KA injection, KAR2, GluR5, GluR6, and GluR7 decreased significantly.

KA receptor subunit protein levels

The protein levels of KAR2, GluR5, and GluR6/7 were assessed in the hippocampi of untreated animals of both genotypes (Fig. 4). The levels of KAR2 were 75% higher in Ames dwarf as compared with wild-type mice. The protein levels of GluR5 and GluR6/7 were not different between genotypes. Antibodies to subunits GluR6 and GluR7 were not available separately nor was an antibody for KAR1 found.

Long-term potentiation

The fEPSP slope was measured and presented as a percentage of baseline 10 min after the induction

Fig. 1 NMDA receptor mRNA expression in the hippocampus of wild-type and Ames dwarf mice. The mRNA levels of NR1, NR2A, and NR2B were assessed in the hippocampi of both genotypes. The levels of NR1 and NR2A were higher in the

Ames dwarf as compared with wild-type mice. Data are presented as mean ± SEM. *Indicates significance ($p < 0.05$) between the wild-type and Ames dwarf mice. $n = 5$ –6/genotype

protocol. The baseline slope was the average of slopes from the last 10 min before HFS. Ames dwarf mice showed higher LTP as compared with their wild-type counterparts, but differences did not reach statistical significance (EPSP slope $p = 0.2921$; Fig. 5). The number of slices exhibiting LTP was greater in Ames mice ($n = 14/19$) compared with wild-type mice ($n = 8/14$).

Neurogenesis following excitotoxic challenge

In both genotypes, DCX immunolabeling revealed newly formed neurons in the GCL throughout the dentate gyrus. Basal numbers of new neurons in the GCL were similar between dwarf and wild-type mice (Fig. 6). The number of DCX-positive neurons

increased following KA treatment in both genotypes. Comparison of the absolute number of DCX-positive neurons between saline and KA-treated groups demonstrated that there was a 147% increase ($p < 0.01$) in newly born cells in Ames dwarf mice following KA, but no significant increase in wild-type mice following this challenge (Fig. 6). In addition, the increase in neurogenesis in KA-treated dwarf mice was 60% and 113% greater when compared with both the WT-KA 15 ($p < 0.05$) and WT-KA 30 ($p < 0.05$), respectively.

Discussion

Ames dwarf mice live 50–70% longer than their wild-type siblings (Brown-Borg et al. 1996). The mechanisms

Fig. 2 NMDA receptor protein levels in hippocampal tissues of untreated wild-type and Ames dwarf mice. The protein levels of NR1, NR2A, and NR2B were assessed in the hippocampi of both genotypes. The levels of NR2A and NR2B were higher in

the Ames dwarf as compared with wild-type mice. Data are presented as mean ± SEM. *Indicates significance ($p < 0.05$) between the wild-type and Ames dwarf mice. $n = 5$ –6/genotype

Fig. 3 Kainate receptor mRNA expression levels in hippocampus of wild-type and Ames dwarf mice. The mRNA levels of KAR1, KAR2, GluR5, GluR6, and GluR7 were assessed in the hippocampi of both genotypes. The expression levels of

KAR2 were higher in the Ames dwarf as compared with wild-type mice. Following KA injection, KAR2, GluR5, GluR6, and GluR7 mRNA decreased. Data are presented as mean $\pm$ SEM. In both genotypes, $n=5-6$ for each treatment group

underlying this significant extension in life span are unclear but likely related to the deficiency of circulating

GH. Earlier studies have reported that Ames dwarf mice exhibit better cognition despite the lack of

Fig. 4 Kainate receptor protein levels in hippocampus of untreated wild-type and Ames dwarf mice. The protein levels of KAR2, GluR5, and GluR6/7 were assessed in hippocampi of both genotypes. The levels of KAR2 were higher in Ames

dwarf as compared with wild-type mice. Data are presented as mean $\pm$ SEM. *Indicates significance ($p < 0.05$) between the wild-type and Ames dwarf mice. $n=5-6$ /genotype

Fig. 5 Long-term potentiation: The excitatory post synaptic potential (EPSP) slope, measured as a percentage of baseline, is shown for the last 10 min before HFS and 20 min after HFS. The Ames dwarf (closed circles; $n=4$ mice, slices—14) tended to show higher LTP as compared with wild-type mice (open squares; $n=4$ mice, slices—8). Data are presented as mean $\pm$ SEM

peripheral GH and IGF-1 (Kinney et al. 2001a; Kinney et al. 2001b). Some recent studies have suggested that the absence of an age-related decline in cognition in dwarfs when compared with wild-type mice may be due to locally produced IGF-1 in the hippocampus and increased neurogenesis found in older dwarf mice (Sun et al. 2005a, b; Sun and Bartke 2007). In a previous report from our lab, no differences in baseline gene expression of hippocampal IGF-1 or the IGF-1 receptor were observed between 3-month-old Ames dwarf and wild-type mice (Sharma et al. 2010). Hippocampal IGF-1 protein levels were also not different in the three age groups of wild-type and dwarf mice evaluated. Behaviorally, we observed significantly better hippocampal-based spatial learning and memory in Ames mice compared with wild-type mice at 3 months of age, as assessed by both the Barnes maze and the T-maze (Sharma et al. 2010). Regarding IGF-1, however, the tissues were not perfused prior to removal in this previous study; thus, the potential contribution of peripheral IGF-1 to local CNS levels of this protein in the wild-type mice cannot be ruled out and may have prevented the detection of a difference between the two genotypes (Sharma et al. 2010; Adams et al. 2009).

There is still a possibility that locally produced IGF-1 is responsible for the enhanced memory in young Ames dwarf mice. However, our previous work led us to believe that there may be additional mechanisms involved in the enhanced hippocampal-

based spatial memory observed in dwarf mice as compared with wild-type mice (Sharma et al. 2010). There are various reports that have linked different subunits of NMDA receptors with spatial memory, LTP, and neurogenesis (Cao et al. 2007; Clayton et al. 2002; Arvidsson et al. 2001; Nacher and McEwen 2006). Therefore, in the present study, we examined the level of NMDA receptor subunits, glutamate and kainate receptor subunits, LTP, and the KA-induced neurogenesis in Ames dwarf mice as compared with wild-type mice.

We found that gene expression levels of NR1 and NR2A and protein levels of NR2A and NR2B were higher in Ames dwarf mice as compared with their wild-type counterparts. This evidence suggests that the higher expression of NMDA receptors may contribute to the enhanced learning and memory observed in dwarf mice. In addition, the kainate receptor subunit protein, KAR2, was significantly higher in the dwarf hippocampus. This is in agreement with a recently published study showing that hypothyroidism increases the expression of kainate receptors in the hippocampus. The increased expression of these receptors in hypothyroid animals was thought to be responsible for the heightened sensitivity of these animals to the effects of kainic acid. Indeed, Ames dwarf mice are hypothyroid (due to a deficiency in thyrotropin) and are very sensitive to kainic acid, as a lower concentration in dwarf mice

Fig. 6 Quantification of neurogenesis using double-cortin (DCX) as a marker of committed, proliferating cells at the end of neurogenesis. The *top panel* (4A–J) shows the dentate gyrus immunolabeling with DCX. Mice treated with KA showed increased neurogenesis in both genotypes. Stereological quantification of DCX-positive neurons is graphed for wild-type and Ames dwarf mice. The number of DCX-positive neurons was significantly higher in Ames dwarf mice with KA 15 mg/kg as compared with age-matched saline injected animals (** $p<0.01$) and when compared with wild-type mice with KA doses of 15 mg/kg ($\#p<0.05$) and 30 mg/kg ($\#p<0.05$). Data are presented as mean $\pm$ SEM ($n=4$ for wild-type mice in each treatment group, $n=3$ for Ames dwarf mice in each treatment group). *Scale bars* represent 200 μ m from **a–e** and 50 μ m from **f–j**. The experimenter was masked to treatment groups while conducting cell counts

(15 mg/kg) produced effects similar to those observed in wild-type mice receiving a dose of 30 mg/kg. In addition, the mRNA levels of glutamate and kainate receptors decreased following kainic acid treatment in Ames and wild-type mice, perhaps reflecting the lower numbers of neurons expressing these receptors (Gine et al. 2010).

As the NMDA receptors are the major receptors involved in LTP and LTP is thought to represent the molecular basis of memory, we evaluated LTP in hippocampal slices from both Ames and wild-type mice. We found that, similar to the increased NMDA receptor expression, there appeared to be a trend for increased LTP in Ames dwarf mice, but the differ-

ences were not statistically significant. The number of slices exhibiting LTP was adequate for statistical comparison (14 in dwarf and eight in wild type); however, an increase in sample size may have shown the difference between the genotypes. In addition, our experiment showed that more slices exhibited LTP in Ames dwarf (74%) compared with wild-type (57%) mice suggesting better molecular memory in Ames dwarf mice. Further studies are needed to determine if hippocampal slices treated with KA show preservation of LTP in Ames dwarf as compared with wild-type mice.

One study showed that in liver-specific IGF1-deficient mice (exhibit 75% reduction in circulating IGF-1 levels), LTP was absent but was restored following treatment with IGF-1 (Trejo et al. 2007). This further suggests that IGF-1-deficient, Ames dwarf mice maintain brain IGF-1 levels and therefore long-term potentiation.

Our previous study examining spatial memory demonstrated that dwarf mice, when subjected to a KA-induced oxidative insult leading to subsequent neuronal damage, retained their spatial memory at pre-KA levels. In contrast, wild-type mice exposed to a KA challenge that caused a similar level of neuronal loss appeared to suffer from memory loss as assessed by the Barnes maze. We therefore considered the possibility that increased neurogenesis and neuronal replacement in Ames dwarf mice following KA led to the preservation of memory. Multiple studies have demonstrated a critical role for NMDA receptors in hippocampal-dependent spatial memory (Adams et al. 2001) and in the regulation of developmental processes such as proliferation, migration, or neurite outgrowth (especially NR1 and NR2A; Le Greves et al. 2002, 2006). We examined hippocampal tissues of 3-month-old Ames dwarf and wild-type mice with DCX immunostaining 16 days following KA injections to determine the levels of neurogenesis in the dentate gyrus. Doublecortin is a specific marker of newly formed neurons in the adult dentate gyrus; hence, quantification of the absolute number of DCX-positive neurons reveals the status of active neurogenesis. Importantly, we found that numbers of DCX-positive neurons increased significantly in Ames mice at 3 months of age as compared with wild-type mice following KA, a treatment that causes selective neuronal degeneration (Coyle 1987). The rationale for using the 3-month-old mice was based on previous reports showing that the

plasticity and neurogenic response of hippocampal stem cells in response to kainate-induced injury is lost by middle age (Hattiangady et al. 2008). In addition, the DCX immunolabeling in our study revealed that more cells in the Ames dwarf mice were vertically oriented with elongated dendrites, a characteristic associated with more mature neurons in the dentate gyrus. Since DCX expression is generally associated with the final stages of neurogenesis (Gleeson et al. 1999; von Bohlen Und Halbach 2007; Rao and Shetty 2004), the Ames dwarf mice may have a larger pool of newly formed neurons to integrate synaptically, providing a cellular resource for improved cognitive function. If this hypothesis is correct, future studies are required to demonstrate a causative link associated with the number of dendritic spines and synapses formed and the improved cognition associated with newly born neurons.

In conclusion, we found that long-living Ames mice exhibit elevated levels of NMDA and kainate receptors in the hippocampus, a finding that may contribute to the enhanced learning and memory observed in these mice based on our current understanding in this area. In addition, Ames dwarf mice are capable of enhanced neurogenesis following KA, a hippocampal-targeted neurotoxic challenge, and thus potentially forming new synaptic connections to preserve memory, a response that was not apparent in wild-type mice. Further studies are needed to confirm the integration of newly formed neurons in the hippocampal synapses of these mice as well as identification of regulatory factors involved in synaptic integration. Results from these investigations will assist in developing novel therapeutic strategies to enhance functional neurogenesis in response to pathologic stress or injury.

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