Journal of Neuroscience Research

Journal of Neuroscience Research 91:934–942 (2013)

Do Reductions in Brain *N*-Acetylaspartate Levels Contribute to the Etiology of Some Neuropsychiatric Disorders?

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N-acetylaspartate (NAA) is recognized as a noninvasive diagnostic neuronal marker for a host of neuropsychiatric disorders using magnetic resonance spectroscopy (MRS). Numerous correlative clinical studies have found significant decreases in NAA levels in specific neuronal systems in an array of neuropsychiatric and substance-abuse disorders. We have recently identified the methamphetamine-induced neuronal protein known as "shati" as the NAA biosynthetic enzyme (aspartate *N*-acetyltransferase [Asp-NAT]; gene *Nat8l*). We have generated an *Nat8l* transgenic knockout mouse line to study the functions of NAA in the nervous system. We were unable to breed homozygous *Nat8l* knockout mice successfully for study and so used the heterozygous mice ($Nat8l^{+/-}$) for initial characterization. MRS analysis of the $Nat8l^{+/-}$ mice indicated significant reductions in NAA in cortex (−38%) and hypothalamus (−29%) compared with wild-type controls, which was confirmed using HPLC (−29% in forebrain). The level of the neuromodulator *N*-acetylaspartylglutamate (NAAG), which is synthesized from NAA, was decreased by 12% in forebrain as shown by HPLC. Behavioral analyses of the heterozygous animals indicated normal behavior in most respects but reduced vertical activity in open-field tests compared with age- and sex-matched wild-type mice of the same strain. $Nat8l^{+/-}$ mice also showed atypical locomotor responses to methamphetamine administration, suggesting that NAA is involved in modulating the hyperactivity effect of methamphetamine. These observations add to accumulating evidence suggesting that NAA has specific regulatory functional roles in mesolimbic and prefrontal neuronal pathways either directly or indirectly through impact on NAAG synthesis. © 2013 **Wiley Periodicals, Inc.**

Key words: magnetic resonance spectroscopy; dopamine; methamphetamine; NAA; *N*-acetylaspartylglutamate; NAAG; locomotor activity; HPLC

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N-acetylaspartate (NAA) is one of the most concentrated metabolites in the human brain, and yet more than 50 years after its discovery the physiological functions served by NAA remain controversial, and no definitive function has been identified (Moffett et al., 2007; Benarroch, 2008). Some investigators have proposed that NAA is involved in housekeeping functions, including neuronal osmoregulation (Baslow, 2002, 2003), whereas other investigators have focused on the role of NAA in providing some of the acetyl coenzyme A (acetyl CoA) needed for oligodendrocyte maturation and myelin lipid synthesis during brain development (Chakraborty et al., 2001; Madhavarao et al., 2005; Wang et al., 2009; Arun et al., 2010). Additional lines of research have tied NAA to neuronal energy metabolism (Patel and Clark, 1979; Clark, 1998; Clark et al., 2006; Arun et al., 2009) and possibly other functions related to acetyl CoA synthesis and utilization (Ariyannur et al., 2010a,b; Arun et al., 2010). However, none of these proposed cellular functions implicates NAA as having an important role in higher brain functions, including cognition and control of behaviors and emotions.

Because of its exceptionally high concentration in the brain, the regional levels of NAA can be evaluated noninvasively by proton magnetic resonance spectroscopy (MRS). Numerous MRS studies over the last 2 decades have shown that NAA is decreased in specific neuronal

Contract grant sponsor: Uniformed Services University of the Health Sciences, contract grant number: R0703W.

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Received 25 January 2013; Revised 4 March 2013; Accepted 13 March 2013

Published online 30 April 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.23234

systems in a number of neurological and psychiatric disorders and substance-abuse conditions (Tsai and Coyle, 1995; Moffett et al., 2007; Brugger et al., 2011; Maddock and Buonocore, 2012). NAA appears as the most prominent peak in magnetic resonance spectrograms of the healthy human brain and is found almost exclusively in the nervous system. It is synthesized in neurons by the enzyme aspartate *N*-acetyltransferase (Asp-NAT). However, because most researchers have associated NAA with basic biological functions such as osmoregulation, myelin lipid synthesis, and acetyl CoA metabolism, it has been assumed that NAA does not play any important role in neural activity involved in psychological processes. However, increasing numbers of findings suggest that NAA may also play some role in behavior.

In ongoing efforts to investigate the role of NAA in brain functions, we have characterized the biosynthetic enzyme of NAA and have shown that the amphetamine-induced brain protein called "shati" (corresponding gene *Nat8l*) is the NAA biosynthetic enzyme Asp-NAT (Ariyannur et al., 2010b). This molecular identification has also been reported by another research group, who found a deletion and reading frame shift in the *Nat8l* gene in the only known patient with severe NAA deficiency (Wiame et al., 2010). We generated an *Nat8l* gene knockout mouse line for study, but attempts at breeding between heterozygote pairs were unsuccessful at three separate animal facilities. The reasons for the impaired fecundity in these mice are at present unknown. In this preliminary study, we compared several neurochemical and behavioral parameters between $Nat8l^{+/-}$ mice and age- and sex-matched wild-type mice of the same background strain to begin accessing what phenotypic characteristics are associated with reduced NAA synthesis in the brain.

MATERIALS AND METHODS

Generation of Heterozygous Asp-NAT Gene (*Nat8l*) Knockout Mice

Targeted gene deletion of *Nat8l* (NCBI gene ID 269642, MGI 2447776) was performed by making a parental allele $Nat8l^{tm1(KOMP)Vlcg}$ at chromosome 5 using the specified BACvec obtained from the mouse bMQ BAC library with an additional inserted neomycin selection cassette driven by the human ubiquitin C gene promoter. The cloned parental embryonic stem cell (VGB6) of the C57BL/6NT mouse strain was then injected into C57BL/6NTac pseudopregnant mice (Taconic), and subsequent generations were screened for germline transmission using the VelociGene genotyping strategy, which looks for PCR products of the deleted segment of the gene as well as reporter/selection markers of the insert. In the final clone, 4,377 bp were removed from 5′ end of the gene locus. The project was carried out on contract at the Mouse Biology Program at the University of California, Davis, which is an NIH-funded Knockout Mouse Repository.

Analysis of NAA by MRS and by HPLC

In vivo MRS was performed on a Bruker BioSpec system (Bruker NMR, Billerica, MA). The system consists of a 7-Tesla, 20-cm horizontal bore, superconducting magnet (Magnex Scientific, Abingdon, United Kingdom) with a Biospec 70/20 console and computer workstation with Paravision software (version 5.1) for imaging and spectroscopy. An Autopac mouse and rat positioning and physiological monitoring system, an 86-mm quadrature transmit coil, and a phase array mouse head coil (four channels) were used for MRS acquisition. The mice were anesthetized with isoflurane (1–2%) during positioning and scanning. High-resolution structural MRI and MRS were acquired, which provide both anatomical and metabolic information at selected brain regions of interest. T2-weighted high-resolution images (RARE, 0.30 × 0.30 × 0.25 mm, TE = 15 and 45 msec, matrix = 128 × 128 × 100) were acquired in the coronal, sagittal, and horizontal orientations with whole-brain coverage. Localized single-voxel PRESS MRS (Bottomley, 1987) was applied with TR = 2,500 msec, TE = 20 msec, number of averages = 256, and voxel size of 24–30 mm^3. The scan time for each voxel was approximately 11 min. All MRS spectra were corrected for eddy currents and water-scaled for absolute quantitation (institutional units) of NAA, choline (Cho), and creatine (Cr) using LCModel (6.2, S.W. Provencher). Image processing and quality control aspects of MRS spectra were performed using custom functions created in Matlab (R2011b; Mathworks, Nattick, MA) and LCModel.

For high-performance liquid chromatography (HPLC) analysis, animals were anesthetized and euthanized by decapitation, and brains were removed immediately, cooled on ice, and divided into forebrain (cortex, diencephalon, and midbrain) and hindbrain (cerebellum and brainstem). NAA and NAAG concentrations were determined by HPLC using a method described previously (Arun et al., 2008).

Behavioral Studies

Open-field activity. Open-field activity was measured to provide information about gross motor movement, general health, depressive-like behavior, and anxiety-like behavior. The six parameters used in the present experiment were horizontal activity, vertical activity, total distance, movement time, center time, and center time as a proportion of movement time. Horizontal activity, total distance, and movement time provide information about gross motor performance and general health. Vertical activity provides an index of depression wherein rodents that exhibit more vertical movement escape activity are interpreted as displaying less depression-like behavior (Grippo et al., 2003; Prut and Belzung, 2003; Sarkisova et al., 2008). Center time provides an index of anxiety (Jones et al., 2008). There is an inverse relationship between center time and anxiety. Center time as a proportion of movement time is a way to assess the animal's anxiety with respect to its movement patterns.

Mice were housed with continuous access to food and water and were maintained under a normal day–night cycle. Open-field activity was measured using an Omnitech Electronics Digiscan infrared photocell system (Test box model RXYZCM, 16 TAO; Accuscan Electronics), located in a room constructed to minimize sound. Mice were placed singly in a 20 × 20 × 30 cm clear Plexiglas arena with a Plexiglas lid with ventilation. Sixteen paired photocell arrays measured horizontal and vertical locomotor activity. Data were automatically gathered and transmitted to computer via an Omnitech model

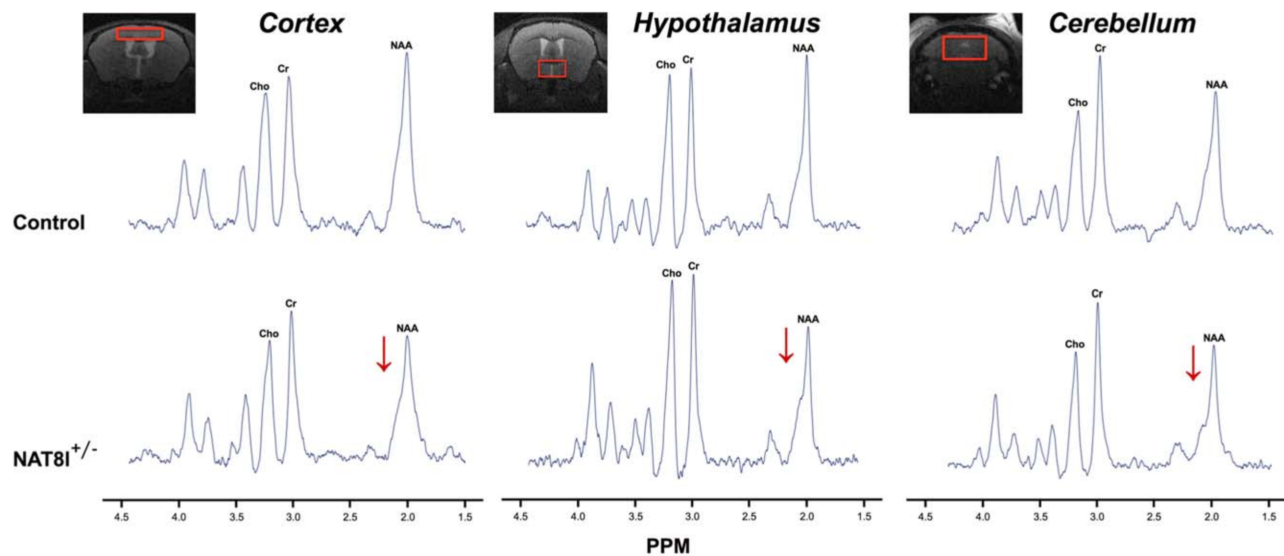

Fig. 1. MRS spectra in several brain regions of wild-type and $Nat8l^{+/-}$ mice. Representative MRS spectra taken from a wild-type mouse and an $Nat8l^{+/-}$ mouse. MRS spectra were processed using baseline correction, peak alignment, and curve fitting to obtain peak areas of NAA (2.0 ppm), creatine (Cr; 3.0 ppm), and choline (Cho; 3.2 ppm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DCM-I-BBU analyzer. Activity was monitored for 1 hr during the light cycle, and each mouse was tested three separate times. Once subjects had been placed in the test areas, the experimenter turned off the lights and left the room. In total 14 mice were studied during this experiment, four heterozygous males, four wild-type males, two heterozygous females, and four wild-type females.

Effect of methamphetamine administration on locomotion. We followed a procedure similar to that used in a previous study in which antisense oligonucleotides were used to suppress expression of *Nat8l*, the gene for Asp-NAT (Niwa et al., 2007). Four heterozygous males and four age-matched wild-type males were habituated for 1 hr in the open-field activity arena for 2 days, and then methamphetamine was administered once per day for 5 days (1 mg/kg, S.C.). Methamphetamine was withdrawn for the next 4 days, and then mice were challenged with a lower dose of methamphetamine (0.3 mg/kg, S.C.) on day 10. Open-field activity was measured for 1 hr immediately after the methamphetamine administration on days 1, 3, 5, and 10. Different activity-related parameters were compared between wild-type and heterozygous mice.

Statistical Analysis

Data are presented as mean $\pm$ SEM. Parametric data were analyzed by ANOVA with repeated measures, multivariate analysis of variance (MANOVA), or analysis of covariance (ANOCOVA) followed by Dunnett's multiple-comparisons posttest. Nonparametric data were analyzed using a Wilcoxon signed rank test for paired data or a Kruskal-Wallis test (nonparametric ANOVA). Metabolite concentrations were analyzed with PSPP (0.7.5; GNU) using unpaired *t*-tests. An associated probability value of $P < 0.05$ was considered significant.

RESULTS

Attempted Breeding of Heterozygous ($Nat8l^{+/-}$) Mice

Breeding was attempted at several animal facilities (USUHS, MD, UC Davis, and Taconic Farms), without success. Initially, four female heterozygote mice were paired with wild-type males to obtain additional breeding pairs. No pups were forthcoming in 6 months, so rederivation (in vitro fertilization) was performed using sperm from a single male heterozygote. Four heterozygotic breeding pairs were obtained from this effort, and they were used for breeding, again without success. In view of these difficulties, the efforts to generate homozygous pups by this approach were discontinued with this line. The remaining heterozygotes (four males and two females) along with wild-type counterparts (C57BL/6NT strain) were brought to our animal facility and used for behavioral and biochemical studies toward initial characterization of their phenotypes.

The reasons for the reproductive difficulties in this knockout mouse line remain unclear. There was no evidence of nonspecific chromosomal abnormalities during the knockout procedures, because both the ova and the sperm were viable based on the pregnancies obtained and success in the rederivation using sperm from one male. Several findings indicate that the loss of functional Asp-NAT is not embryonic lethal. There has been a documented case of a child with a mutation in the *NAT8L* gene wherein no functional protein is expressed (Wiame et al., 2010). The child exhibits delayed myelination, ataxia, lack of expressive speech, behavioral abnormalities, cognitive impairment, epilepsy, and secondary microcephaly (Burlina et al., 2006).

TABLE I. Changes in the Concentrations of NAA, Creatine (Cr), and Choline (Cho) and the NAA/Cr Ratio as Detected by MRS[a]

	[NAA]			NAA/Cr		
	% Avg. diff	% ± SEM	*P* value	% Avg. diff	% ± SEM	*P* value
Cortex	−38	9/10	0.0142	−47	8/11	0.0036
Hypothalamus	−29	3/4	0.0004	−38	4/2	0.0001
Cerebellum[b]	−11	3/6	0.1309	−29	6/6	0.0188

	[Cr]			[Cho]		
	% Avg. diff	% ± SEM	*P* value	% Avg. diff	% ± SEM	*P* value
Cortex	17	9/10	0.0142	10	12/10	0.5552
Hypothalamus	11	2/4	0.0747	2	6/3	0.8017
Cerebellum[b]	25	8/2	0.0572	3	9/3	0.8056

[a]% Avg. diff, average percentage difference [control − *Nat8l* KO$^{+/-}$/control]; SEM, standard error of mean [control value/*Nat8l*$^{+/-}$ value]; *P* value, unpaired *t*-test *P* value between control and *Nat8l*$^{+/-}$ KO mice (n = 4, all males).
[b]Cerebellum *Nat8l*$^{+/-}$ KO mouse (n = 3, all males).

Very recently, another research group has successfully bred homozygous *Nat8l* knockout mice and shown that they have specific behavioral abnormalities, demonstrating that the loss of Asp-NAT function is not lethal but does have phenotypic consequences that include alterations in behavior (Furukawa-Hibi et al., 2012).

NAA and NAAG Levels Are Decreased in the Brain of Heterozygous Asp-NAT (*Nat8l*) Gene Knockout Mice

NAA levels were analyzed by MRS in *Nat8l*$^{+/-}$ and wild-type mice (Fig. 1). Scans of cortex and hypothalamus showed significant decreases of 29% and 38%, respectively, in absolute NAA concentration and 38% and 47% in NAA/Cr relative to age-matched C57BL/6NTac control mice (Table I). In the cerebellum, there was a significant decrease in the NAA/Cr ratio relative to controls (29%), but the decrease in absolute NAA concentration there (~11%) did not reach statistical significance. Among the other metabolites examined, Cr was significantly increased by 17% in cortex. Decreased NAA levels were confirmed in whole forebrain (prosencephalon through mesencephalon) and whole hindbrain (metencephalon and myelencephalon) by HPLC analysis with reductions of approximately 29% in forebrain and 33% in hindbrain (Fig. 2). Because the neuronal dipeptide NAAG is synthesized from NAA and glutamate, we used HPLC to measure NAAG levels in the *Nat8l*$^{+/-}$ mice and found the levels to be significantly reduced by approximately 12% in forebrain and 9% in hindbrain (Fig. 2).

Open-Field Activity Studies

Horizontal activity was similar among all mice (Fig. 3A), showing that all were in good health and able to move normally. In contrast, there were significant differences in vertical activity (Fig. 3B). Wild-type mice (1,715.21 ± 177.91) showed significantly more vertical activity than did the *Nat8l*$^{+/-}$ mice (905.17 ± 217.97; $F[1,10] = 8.29$, $P = 0.016$, $\eta^2 = 0.453$). These findings suggest that the *Nat8l*$^{+/-}$ mice showed more depression-related behaviors than did the wild-type mice. Alternatively, these findings may indicate that the mice exhibiting less vertical activity were less motivated to escape the enclosure or had more difficulty balancing on their hind legs. Among males, the heterozygous mice (1,129.67 ± 303.98) exhibited only about half the level of vertical activity compared with wild-type mice (2,192.67 ± 303.98; $F[1,6] = 6.11$, $P = 0.048$, $\eta^2 = 0.505$). Despite the small sample (n = 4 males per group), the effect sizes and statistical power of the vertical activity findings were robust.

Methamphetamine Effect on Behavior

It has previously been shown that treatment of mice with antisense oligonucleotides of the *Nat8l* gene enhanced methamphetamine-induced acute responses and behavioral sensitization (Niwa et al., 2007, 2008a). It was therefore of interest to test the effect of methamphetamine on the *Nat8l*$^{+/-}$ mice. Heterozygous and wild-type mice increased horizontal activity with repeated administration of methamphetamine over time. This behavioral response was greater among the *Nat8l*$^{+/-}$ mice compared with wild-type mice ($F[2,10] = 6.47$, $P = 0.016$, $\eta^2 = 0.564$; Fig. 4).

With methamphetamine administration, vertical activity changed over time in both groups ($F[2,10] = 12.50$ [covarying for baselines], $P = 0.002$, $\eta^2 = 0.714$; Fig. 5). *Nat8l*$^{+/-}$ mice showed less vertical activity (307.75 ± 80.41) than did wild-type mice prior to drug administration (622.94 ± 80.41; $F[1,6] = 7.68$, $P = 0.032$, $\eta^2 = 0.561$). On the first day of drug administration, the *Nat8l*$^{+/-}$ mice reduced vertical activity (−22.39 ± 209.72) compared with their own baseline values, whereas wild-type mice increased vertical activity (1,090.89 ± 209.72) compared with their baseline values ($F[1,5] = 12.85$ [covarying for baselines], $P = 0.016$, $\eta^2 = 0.720$). In addition, there was a significant time-by-group interaction, with wild-type mice showing a large decrease in activity over time ($F[2,10] = 12.15$, $P = 0.002$, $\eta^2 = 0.708$ [covarying for baseline]).

All mice decreased the ratio of center time to overall movement time over the course of the study (Fig. 6). At

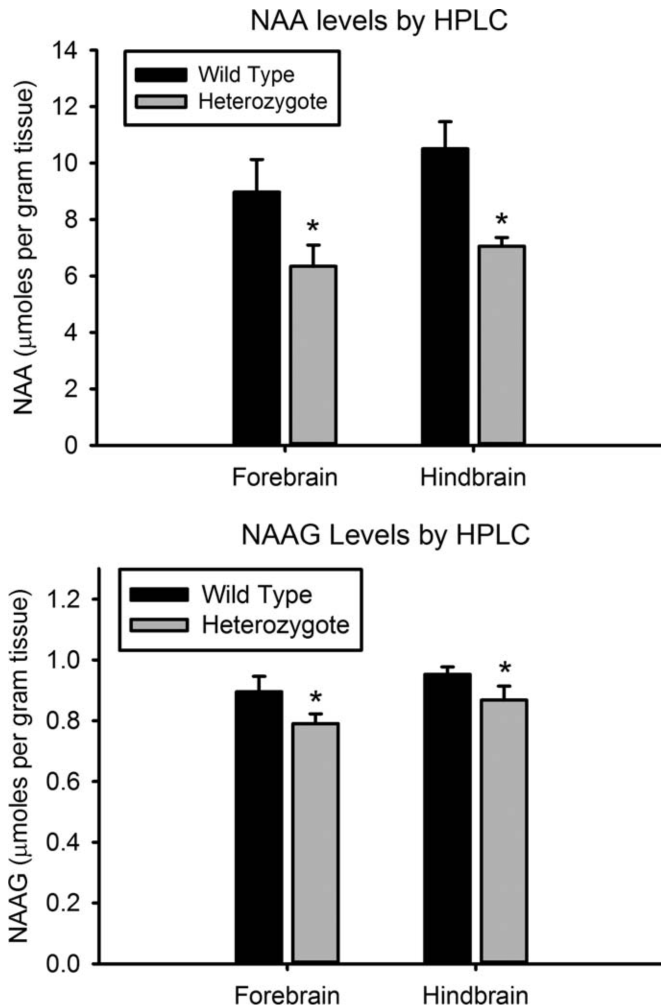

Fig. 2. Analysis of NAA and NAAG by HPLC. NAA levels in the forebrain and hindbrain were analyzed by HPLC as described in Materials and Methods. Values (±SD) of wild-type animals were compared with those of *Nat8l*$^{+/-}$ mice. NAA levels in the heterozygous mice were decreased by ~29% in the forebrain and ~33% in the hindbrain, including cerebellum and brainstem. NAAG levels were decreased by approximately 12% in forebrain and 9% in hindbrain. Statistical analysis was carried out using Student *t*-test. $^{\star}P < 0.01$ (n = 2 male mice per group; three samples per mouse).

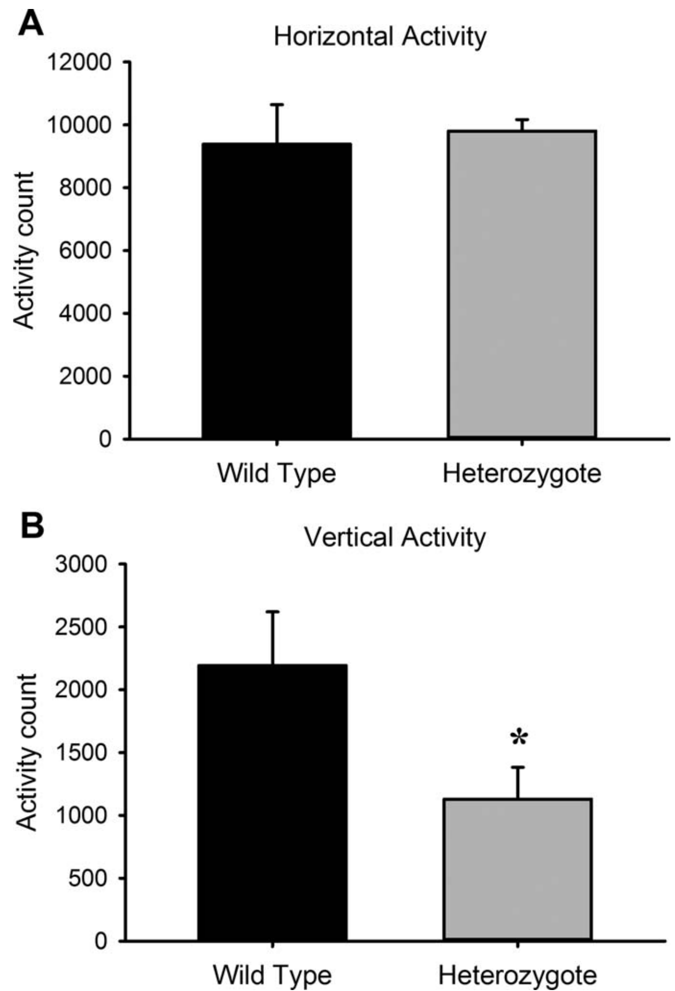

Fig. 3. Horizontal activity and vertical activity measurements. Open-field activity was studied as described in Materials and Methods using four heterozygous males and four age-matched wild-type control males of the same mouse strain. **A** depicts the number of beam breaks the animals made in the horizontal field, representing general health and movement. There were no significant findings between groups. **B** depicts the number of beam breaks animals made in the vertical field, representing an inverse relationship with depression-related or similar behavioral deficits. The more beam breaks observed in the vertical dimension, the more the mice are trying to escape the enclosure, so less depressive-like or exploratory-like behavior is evident. The *Nat8l*$^{+/-}$ mice exhibited significantly less vertical activity than did the wild-type mice (F[1,10] = 8.29, $^{\star}P = 0.016$, $\eta^2 = 0.453$).

baseline, before methamphetamine treatment, the *Nat8l*$^{+/-}$ mice spent proportionally more time (26.67 ± 1.87) in the center of the arena than did the wild-type mice (10.70 ± 1.87; F[1,6] = 36.57, $P = 0.001$, $\eta^2 = 0.859$), suggesting that the heterozygous mice were less anxious or more exploratory. Throughout the study with methamphetamine administration, the *Nat8l*$^{+/-}$ mice spent a greater proportion of time in the center of the arena (15.29 ± 2.10) than did the wild-type mice (7.28 ± 2.10; F[1,6] = 7.22, $P = 0.036$, $\eta^2 = 0.546$).

DISCUSSION

Recent findings have begun to open up new directions in NAA research that may overturn the long-held belief that NAA is not involved in neuronal activities associated with behavior. In 2007, a cDNA microarray study showed that chronic methamphetamine administration led to substantially increased mRNA expression for an unknown protein in neurons of the nucleus accumbens in mice (Niwa et al., 2007). The authors identified the gene as *Nat8l*, and the corresponding protein was predicted to have a structural domain corresponding to the active site of an *N*-acetyltransferase enzyme. The authors found that *Nat8l* antisense oligonucleotide treatment enhanced acute methamphetamine responses, methamphetamine-induced

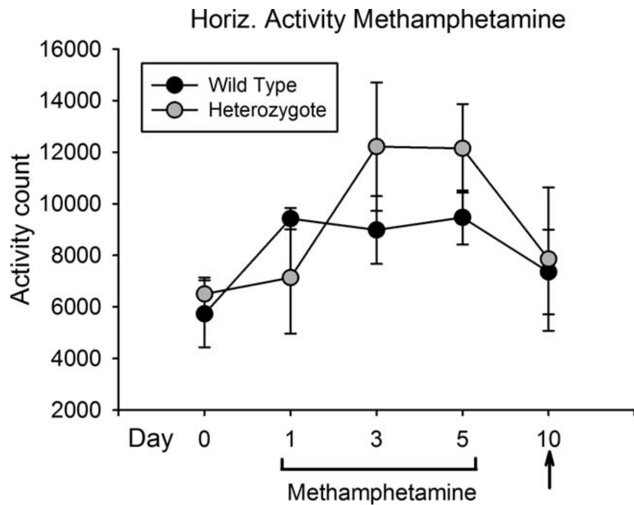

Fig. 4. Differential effect of methamphetamine on horizontal activity. The figure depicts the number of beam breaks in the horizontal field throughout the study. Day 0 shows horizontal activity without drug treatment. Methamphetamine was administered at 1 mg/kg on days 1–5, and then withdrawn for 4 days before challenging with 0.3 mg/kg methamphetamine on day 10 (arrow). On initial treatment with methamphetamine, the wild-type mice increased horizontal activity more than the $Nat8l^{+/-}$ mice, but, with subsequent administrations, the increase in horizontal activity was enhanced in the heterozygous animals. Responses to the lower dose challenge administration on day 10 were similar in the two groups ($F[2,10] = 6.47$, $P = 0.016$, $\eta^2 = 0.564$; n = 4 males per group).

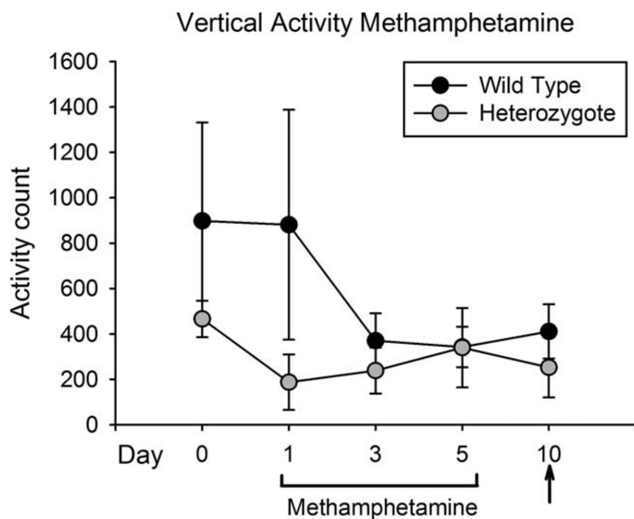

Fig. 5. Differential effect of methamphetamine on vertical activity. The numbers of beam breaks the animals made in the vertical field in response to methamphetamine over time are shown. The $Nat8l^{+/-}$ mice displayed less vertical activity than did wild-type mice, as expected from our previous open-field activity studies ($F[1,6] = 7.68$, $P = 0.032$, $\eta^2 = 0.561$). There was also a significant time-by-group interaction, with wild-type mice showing a large decrease in activity over time, covarying for baseline ($F[2,10] = 12.15$, $P = 0.002$, $\eta^2 = 0.708$; n = 4 males per group).

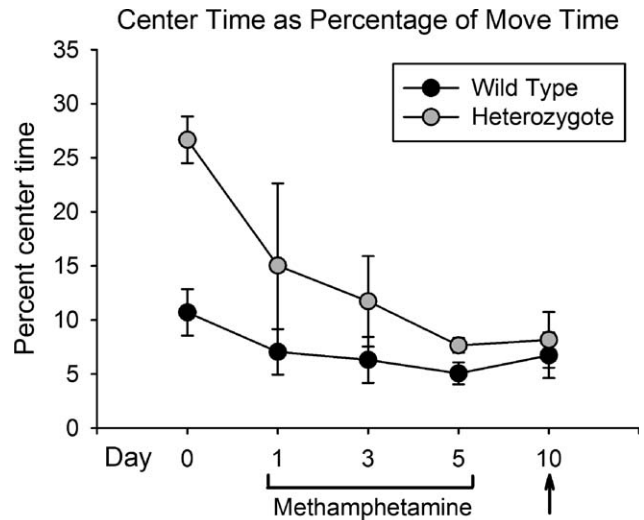

Fig. 6. Differential effect of methamphetamine on center time/movement time. Amount of time the animals spent in the center of the arena as a proportion of their total movement time throughout the study with methamphetamine treatment. All mice decreased the ratio of center time/movement time over time. At baseline, the $Nat8l^{+/-}$ mice spent proportionally more time in the center of the arena than did the wild-type mice ($F[1,6] = 36.57$, $P = 0.001$, $\eta^2 = 0.859$). Over the course of the experiment, the $Nat8l^{+/-}$ mice spent a greater proportion of time in the center of the arena than did the wild-type mice ($F[1,6] = 7.22$, $P = 0.036$, $\eta^2 = 0.546$; n = 4 males per group).

behavioral sensitization, and conditioned place preference. Microdialysis studies showed that blockade of *Nat8l* expression with antisense oligonucleotides potentiated methamphetamine-induced dopamine overflow in the nucleus accumbens and reduced dopamine uptake in the midbrain. These findings suggest that this unidentified protein inhibits the actions of methamphetamine by modulating dopaminergic neurotransmission. In 2010, two independent groups identified *Nat8l* as the gene encoding aspartate *N*-acetyltransferase (Asp-NAT), the enzyme that biosynthesizes NAA (Ariyannur et al., 2010b; Wiame et al., 2010). This molecular identification implicates the *Nat8l* gene product, Asp-NAT, as being responsible for the effects on dopamine release and uptake demonstrated previously (Niwa et al., 2007, 2008a,b). Currently, the only known function of Asp-NAT is the synthesis of NAA from acetyl coenzyme A and aspartate in neurons.

Very recently, Furukawa-Hibi and colleagues (2012) have generated an *Nat8l* knockout mouse line and successfully bred homozygous Asp-NAT-deficient mice. They found subtle behavioral differences, including increased vertical activity, increased grooming behavior, and decreased social interaction in unfamiliar surroundings. They also found increased brain-derived neurotrophic factor mRNA in the prefrontal cortex and hippocampus, decreased glial cell line-derived neurotrophic factor mRNA in the striatum and hippocampus, and decreased lipopolysaccharide-induced TNF-α factor mRNA in the striatum. The authors did not associate any of these changes with the loss of NAA synthesis in the

brain. However, based on the role of Asp-NAT in synthesizing NAA, it seems likely that the phenotypic differences in these mice were related to the lack of NAA and/or the loss of the related neuropeptide, NAAG, in the brain.

In our preliminary examination we found that heterozygous *Nat8l*$^{+/-}$ mice have significant forebrain decreases in NAA as detected by MRS and confirmed by HPLC analysis. Smaller but significant reductions were noted for NAAG, which is synthesized from NAA. Preliminary behavioral studies showed significant differences in behavior and responsiveness to methamphetamine between *Nat8l*$^{+/-}$ mice and age- and sex-matched controls of the same strain of mice. We tested a number of locomotion parameters and found no significant differences in most measures; however, vertical activity, an indication of exploratory and escape behaviors, was substantially lower in the *Nat8l*$^{+/-}$ mice. Interestingly, Furukawa-Hibi et al. (2012) found increased vertical activity in their homozygous (*Nat8l*$^{-/-}$) mice. The reasons for the opposite effects on vertical activity in the heterozygous and homozygous mice are unknown, but we observed behavior for a 1-hr period, whereas Furukawa-Hibi and colleagues observed behavior for only 5 min per session, which might have affected the outcomes. We also found that, in response to methamphetamine treatment, vertical activity, horizontal activity, and center time as a percentage of movement time were all significantly different in the *Nat8l*$^{+/-}$ mice compared with controls. The observations by Niwa and colleagues (2007) that decreased expression of *Nat8l* led to increased responsiveness to methamphetamine fits well with our findings in the current study, in which a 29% reduction in forebrain NAA levels was associated with significantly increased horizontal locomotor activity on days 3 and 5 of treatment with methamphetamine compared with wild-type mice of the same strain (Fig. 4).

Direct data on possible effects of NAA on neurochemical correlates of behavior such as neurotransmitter release or uptake are currently lacking, but a number of studies have hinted at this possibility. NAA levels in chronic schizophrenia patients have consistently been reported to be reduced in the frontal and temporal lobes and thalamus (Brugger et al., 2011). These small but significant reductions have been interpreted to indicate a loss of neuronal integrity or function in these brain regions. In an elegant series of studies in nonhuman primates with bilateral mesial temporal-limbic ablations and in schizophrenia patients, Bertolino and colleagues (1999, 2000) found that NAA levels in dorsolateral prefrontal cortex were inversely correlated with dopamine release after amphetamine administration. Lower levels of NAA correlated with higher levels of dopamine release. Because NAA has been considered primarily a surrogate marker for neuronal health and integrity, these findings were interpreted to mean that reduced NAA in prefrontal areas reflected underlying pathologies, which in turn resulted in abnormal dopamine release. However, if viewed in light of the fact that NAA may be directly or indirectly involved in regulating dopamine neurotransmission, the results imply more than correlation; they suggest some degree of causality. We have found that the antipsychotic medications clozapine and haloperidol, which work in part by blocking dopamine D2 receptors, act to increase significantly both NAA and NAAG synthesis in a dose-dependent manner in a model system for studying dopaminergic neuronal responses (Arun et al., 2008). The *Nat8l* knockdown studies by Niwa et al. showed that reduced NAA synthesis resulted in increased extracellular dopamine, whereas our results with clozapine and haloperidol showed that blocking dopamine receptors increases NAA synthesis. All of these findings highlight the functional linkages between extracellular dopamine levels and NAA synthesis.

In several studies, NAA has been associated with higher cognitive functions, including positive correlations with intelligence measures (Jung et al., 2009; Aydin et al., 2011), negative correlations with cognitive interference (Grachev et al., 2001), and concentration variations in specific brain regions associated with different personality traits (Ryman et al., 2011). These alterations in regional brain NAA levels have been interpreted to indicate differences in neuronal viability or function but have generally not led to the conclusion that NAA itself plays any role in cognition or behavior. However, if NAA is directly or indirectly involved in regulating dopaminergic transmission, then the reductions observed in specific brain regions in some disorders may contribute to the pathologies. This raises the possibility that *Nat8l* polymorphisms and single-allele defects may be potential etiological or exacerbating factors in some specific neurological disorders. The NCBI Short Genetic Variations database lists 142 verified human polymorphisms in the *Nat8l* gene. Among these, four nonsynonymous coding SNPs that result in residue changes in the protein sequence are listed in the database (rs147251131; G [Gly] → D [Asp], rs143685504; V [Val] → A [Ala], rs143186812; Q [Gln] → X [AMB], rs460589; A [Ala] → G [Gly]). These polymorphisms could potentially result in altered protein functionality with effects on higher brain functions.

The current findings add further support to the growing body of research indicating that reduced NAA levels in forebrain have functional effects on emotion and behavior. Therefore, the reduced NAA levels observed in specific brain areas in a number of clinical conditions may need to be reexamined as a potentially contributing factor in the etiology of some of these disorders. This is a substantially different view from the general consensus that NAA is simply a surrogate marker for neuronal function and integrity (Tsai and Coyle, 1995).

In addition to any direct actions that NAA may have in modulating neurotransmission, the modest reductions in NAAG that result from decreased brain NAA may also play a role in the behavioral and emotional abnormalities observed when brain NAA levels are reduced. In the current study with *Nat8l*$^{+/-}$ mice, NAA levels in forebrain were reduced by approximately 29%, and as a result forebrain NAAG levels were reduced by

approximately 12%. It is possible that some of the behavioral effects of reduced NAA are the result of an impact on NAAG synthesis. In the studies by Niwa and colleagues (2007), the treatment with *Nat8l* antisense nucleotides was performed continuously via cannula for 3 days before methamphetamine treatment and behavioral testing. We have found that NAAG synthesis from NAA is delayed; radiolabel incorporation into NAA can be observed within 1 hr, whereas incorporation into NAAG could not be observed until between 6 and 24 hr of radiolabel application (Arun et al., 2006). Because Niwa et al. treated animals for 3 days with *Nat8l* antisense nucleotides prior to behavioral testing, there was sufficient time for the drop in NAA to reduce NAAG levels substantially by way of reduced substrate availability for the NAAG biosynthetic enzyme. Recent investigations on the newly discovered NAAG synthase enzymes (RIMKLA and RIMKLB) showed that they have exceptionally high Km values for NAA. These findings indicate that the two NAAG synthase enzymes require very high concentrations of NAA to support NAAG synthesis; 1.48 mM for RIMKLA and 4.59 mM for RIMKLB (Collard et al., 2010). Therefore, modest reductions in NAA levels would be expected to have an impact on NAAG synthesis.

NAAG has been described as the most abundant neuroactive peptide in the CNS (Neale et al., 2000). NAAG levels in specific brains regions are increased in some neuropsychiatric disorders such as schizophrenia but are reduced in others such as major depressive disorder (Reynolds and Reynolds, 2011). Evidence indicates that NAAG is involved in neurotransmitter release modulation, so modest reductions in NAAG levels could have behavioral consequences. NAAG is synthesized from NAA and glutamate by the enzyme NAAG synthase (Becker et al., 2010), and increasing NAA levels leads to increased NAAG synthesis (Gehl et al., 2004; Arun et al., 2006; Becker et al., 2010; Collard et al., 2010). Our findings suggest that reduced NAA levels result in reduced NAAG synthesis. Because NAAG has been implicated in regulating glutamatergic activity (Zuo et al., 2012), the reduced NAAG levels observed in the current study could have led to dysregulated glutamate release, which might have been responsible for some of the observed behavioral effects.

Taken together, the data suggest that reduced NAA synthesis in neurons leads to altered neurotransmitter release regulation either directly or indirectly through a reduction in NAAG synthesis, or both. This emerging view of NAA as either directly or indirectly involved in regulation of neurotransmission, rather than acting only as a surrogate marker for neuronal health, opens up new avenues for research and potential opportunities for therapeutic interventions into a variety of neurological and psychiatric disorders involving reduced NAA levels and altered dopaminergic and glutamatergic transmission.

ACKNOWLEDGMENTS

All authors report no biomedical financial interests or potential conflicts of interest.

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