Activity-dependent neuroprotective protein (ADNP) exhibits striking sexual dichotomy impacting on autistic and Alzheimer’s pathologies

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INTRODUCTION

Autism spectrum disorder (ASD) affects ~1.5% children with a prevalence of three boys/one girl and with ~50% deficient or borderline intellectual function, in the US.¹ The high comorbidity of ASD with intellectual disability suggests common genes and pathways.²–⁴ Focusing on sexual dimorphism and cognitive functions and contrasting with ASD, there is a greater prevalence of Alzheimer’s disease (AD) in women compared with men, whereas men may have a higher risk of mild cognitive impairment, an intermediate stage between normal aging and dementia.⁵

Activity-dependent neuroprotective protein (ADNP), recently estimated to be de novo mutated in at least 0.17% of ASD cases,⁶–⁸ was discovered in our laboratory and identified as vital for brain formation.⁹–¹³ Partial deficiency in ADNP resulted in hyperphosphorylation of the MT-associated protein tau (leading to AD/ frontotemporal dementia—like tau pathology) paralleled by cognitive deficits. NAP (NAPVSIPQ), a snippet from ADNP,¹² enhancing tau-MT interaction¹⁴,¹⁵ and inhibiting, in part, tau aggregation,¹⁶–¹⁸ reversed ADNP deficiencies in vivo,¹² while increasing ADNP-MT end binding protein interaction through the shared SIP domain.¹⁹ Tau deposition was also associated with autism.²⁰ GSK3beta-deficient mice (the tau kinase overactivated in ADNP⁺/− mice¹²), showed improved social behavior vs control mice,²¹ suggesting that overactivation of GSK3beta and the resulting tau hyperphosphorylation impairs social behaviors, as seen in autism. In parallel, the MT-associated protein 2 (MAP2) expression was depleted in autistic patients.²² In this respect, ADNP silencing resulted in MAP2 depletion²³ and NAP treatment increased MAP2 expression.²⁴

We recently showed that ADNP⁺/− mice exhibited reduced hippocampal beclin1 (a key factor in the regulation of autophagy, which was also reduced in schizophrenia postmortem hippocampus compared with controls). At the protein level, ADNP co-immunoprecipitated with the MT-associated protein 1 light chain 3, another major regulator of the autophagy process, which was augmented by NAP;²⁶ protecting the autophagic flux.²⁶ Auto-phagy has been associated with autism,²⁷ for example, in mice haploinsufficient for ambra1 (a positive regulator of beclin1), showing autism-like behavior restricted to the female gender.²⁸ In addition, other converging mechanisms shared by schizophrenia and autism have been shown, including mutations affecting synaptic strength and cytoskeleton activity.²⁹

Importantly, ADNP expression, correlated with related proteins is deregulated in schizophrenia postmortem brains.³⁰ Complete gene expression microarray analysis identified altered transcript content in the ADNP⁺/− mice¹² compared with
ADNP+/−, including a soluble carrier transcript, involved in intracellular signaling cascade, transforming growth factor, involved in angiogenesis and Pax6, involved in neuronal migration and axogenesis, which are either mutated or altered in their expression in individuals with autism. In addition, ADNP haploinsufficiency was also associated with deficits in social memory.

From a diagnostic point of view, assessment of ADNP expression lymphocytes showed increased expression in patients suffering from schizophrenia compared with matched controls, which was reduced with disease progression only in female patients. This increased expression in schizophrenia is contrasted by decreased expression in peripheral blood mononuclear cells from multiple sclerosis patients, and, at the protein level, complete serum proteomics identified ADNP as the only protein decreasing in serum from AD patients compared with controls.

Given the male/female differences in ASD and AD, it is of interest to note that ADNP expression in the arcuate nucleus of the rodent hypothalamus, a brain area associated with appetite and sexual behavior, exhibited fluctuations during the estrous cycle, proestrus sections being the most ADNP-immunoreactive and estrous sections the least, whereas male arcuate nucleus ADNP-like immunoreactivity was significantly lower than that of the female estrous.

Here, we asked if ADNP is differentially expressed in the hippocampus of males and females and if changes in ADNP expression regulate key ASD and AD risk genes, leading to sex-specific cognitive and social differences. Better understanding of ADNP, which regulates >400 genes during the development, will pave the path to better targeted treatments.

MATERIALS AND METHODS

Animals

All procedures involving animals have been approved by the Animal Care and Use Committee of Tel Aviv University and the Israeli Ministry of Health. ADNP heterozygous mice on a mixed C57BL and 129/Sv background, a model for cognitive impairments, were housed in a 12-h light/12-h dark cycle facility with free access to rodent chow and water.

The procedure to generate ADNP+/− animals was described previously. From severe inbred mating problems, mating with ICR, an outbred mouse line, was implemented allowing for continuous breeding and excellent progeny. Genotyping was performed by Transnetyx (Memphis, TN, USA). ADNP+/− and littermates ADNP+/+ mice were compared. Six-month-old male or female mice were exposed daily (5 µl per nostril) to intranasal administration, of a vehicle solution, in the rodent hypothalamus, a brain area associated with appetite fluctuations during the estrous phase.

Hippocampal sexual dichotomy

ADNP+/− mice were 7–8 months of age at the time of testing. Mice used as novel (target mice) to be explored by the subject were mice from the 129/SvJ strain (in our colony), known for their docile nature (7–8 months old). The social approach task was previously reported. A plexiglas box was divided into three adjacent chambers, each 20 cm (length) × 40.5 cm (width) × 22 cm (height), separated by two removable doors. Steel wire pencil cups (10.16 cm diameter), 10.8 cm (height), www.kitchen-plus.com, were used as both containment for the target mice and as inanimate objects (weights prevented the mice from overturning the cups). Experiments were conducted in a dimly lit area during the light phase of the mouse. The brightness of the right and left chambers was measured with a light meter (MRC lab, Holon, Israel) and kept at ~6 ±0.5 lux before experiments were initiated, to avoid bias. Three sides of the box were covered to prevent the mice from using spatial cues. The long side facing the experimenter was left open for experimenter view.

Target mice (males for males and females for females) were placed inside the wire cup in one of the side chambers for three 10-min sessions on the day before the test for habituation. The next day, each subject mouse was tested in an experiment with three phases, each 10-min long (measured with a simple timer): I and II, the habituation phases (ensuring no bias), and III, the experimental phase, recorded with a video camera. No significant differences were noted between time periods spent in the different chambers in the habituation phase.

In phase III, an empty wire cup (novel object) was placed in the center of the right or left chamber and the cup containing the target mouse was placed in the center of the other chamber. Location of the empty wire cup (novel object) and the novel mice were counterbalanced to avoid confounding side preference. The doors were then removed and the timer started for 10-min started. During this phase, the experimental mouse and the novel mouse with the novel mouse with two silent stopwatches (first novel mouse exposure). The three-chamber apparatus was cleaned between mice.

The social approach task was used as habituation for the social memory task, 3 h after the first phase (3-min exposure), the mouse was placed back into the apparatus for another 3 min (second phase), during which one cup contained the familiar mouse and the other contained a novel mouse. The positions of the familiar and novel mouse during phases 1 and 2 were counterbalanced within and between groups to exclude the possibility of positional effects, but were kept the same for a given animal. The discrimination capacity (social memory) was analyzed using the formula: D2 = (b−a)/(a+b), as for the object recognition test.

Olor habituation–dishabituation

This test was performed as described.

Biochemical and immunochemical procedures

Male ADNP+/− and ADNP+/+ littermates (wild type) mice (5- to 6-months old) were decapitated, the hippocampus was dissected, frozen in liquid nitrogen and maintained frozen (~80 °C) until further processing. RNA and protein were extracted using Macherey-Nagel NucleoSpin RNA/Protein kit (Bethlehem, PA, USA). RNA purity and concentration were determined with a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The protein amount was estimated by the BCA-200 protein kit (Pierce, Rockford, IL, USA). An additional RNA quantification experiment on a limited amount of transcripts was carried out in older mice (9-month-old male mice). Before hippocampal RNA extraction, the 9-month-old mice (n = 5 per group) were treated with vehicle (defined above) and were subjected to behavioral tests as outlined in Figures 1 and 2.

Immunoprecipitation

Proteins (400–500 µg) were extracted from the hippocampus and diluted in lysis buffer for further immunoprecipitation using the CoIP kit (Pierce) protocol. Twenty microliters of A/G PLUS-Agarose beads were loaded to a column washed with coupling buffer at 90 g for 1 min. Ten micrograms of rabbit mouse ADNP antibody (Bethyl Laboratories, Montgomery, TX, USA) was kept in its home cage between phases 2 and 3. The time spent sniffing/touching each object was measured. Data were analyzed using the discrimination capacity formula: D2 = (b−a)/(a+b), where ‘a’ designated the time of exploration of the familiar object and ‘b’ designated the time of exploration of the novel object.
Figure 1. ADNP+/- differ from ADNP+/+ male mice: object recognition, social interactions and sexual dichotomy. Animal performance in the object recognition test is shown (n = 16–18 for each of the male groups; n = 11 for each of the female groups). (a, b) ADNP+/- male mice are deficient in object recognition. Two identical objects were first presented, and one of the identical objects was replaced by a novel object 3 h after sniffing the familiar object (short retention choice) (a) or on the following day (–24 h later, long retention choice) (b). Data are expressed as mean (±s.e.m.) by a relative discrimination index (D2 = (b–a)/(b+a); b = time (s) sniffing a novel object, a = time (s) sniffing a familiar object). Two-way analysis of variance (ANOVA) revealed no significant differences in the short retention choice (a). In the long retention choice (b), two-way ANOVA showed a significant effect of genotype only in the male group (F(1,49) = 5.022, P = 0.030). ADNP-deficient male mice spent significantly less time (<2-fold) in exploring the new object as compared with control mice (ADNP+/+). Fisher’s LSD post hoc test revealed a significant difference between ADNP+/- male mice compared with ADNP+/+ mice (**P < 0.01). (c) Sniffing time of empty cup and novel mouse—social recognition test. Data are expressed as mean (±s.e.m.) total time (s) spent exploring mice or objects. A three-chamber cage was used. Two-way repeated measure ANOVA with group as a fixed factor and sniffed item (that is, mouse vs cup) as repeated factor revealed no main effect for group (F(1,48) = 1.051, P = 0.378) on the sniffing time periods of a novel mouse and a cup. However, a main effect was found for the sniffed item (F(1,48) = 75.761, P < 0.001), indicating a strong preference for the novel mouse over the cup. In addition, an interaction effect between group × sniffed item was found (F(1,48) = 2.9296, P = 0.028). Fisher’s LSD post hoc test revealed significant differences between sniffing time period of the cup and mouse in all the groups (**P < 0.01 vs cup in the same group—male or female). Bonferroni post hoc test revealed significant differences between sniffing time period of the cup and mouse in all the groups (**P < 0.01) except for ADNP+/- females (P = 0.18). The sniffing time of mouse in the latter group was significantly lower than in the ADNP+/- males (**P < 0.05), with no change in the sniffing time of the cup (P > 0.99 after adjustment for multiple comparisons). (d) ADNP+/- mice displayed a significant decrease in social memory. Animal performance in the social memory test is shown (3 h after the original 3-min exposure). Data are expressed as mean (±s.e.m.) total time (s) spent exploring another mouse as designated by a relative discrimination index (b = time sniffing a novel mouse, a = time sniffing a familiar mouse). The total time allowed for sniffing in the second exposure was 3 min). In the social memory test, the ADNP-deficient male and female mice spent significantly less time exploring the novel mouse as compared with control mice (ADNP+/+). Two-way ANOVA showed a significant genotype effect (F(1,47) = 31.357, P < 0.001) in the social memory test (ADNP+/- vs ADNP+/+ mice), but no general sex effect (males vs females), (F(1,47) = 1.563, P = 0.217). Fisher’s LSD post hoc test revealed a significant genotype difference between the ADNP+/- and ADNP+/- in the male group (**P < 0.01) as well as in the female group (**P < 0.05); there was also a significant sex effect in the ADNP+/- group (**P < 0.01 male vs female). ADNP, activity-dependent neuroprotective protein; LSD, least significant difference.

Quantitative real-time RT-PCR

Equal amounts of total RNA (1 μg RNA/sample, obtained from 6-month-old mice) were subjected to reverse transcription (RT) using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA). Real-time PCR was performed using Powered SYBR Green PCR master mix (Kappa Technologies, Woburn, MA, USA) and ABI PRISM 7900 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA, USA). RNA expression levels were determined using specific mouse primers: elF4E sense 5′-TCTGGCTAGAGACCTGTGCA-3′, anti-sense 5′-AGTCCATATTCTATTTAACC-3′; ApoE primers sense 5′-ACGGTCTTGTGAGGATAC-3′, anti-sense 5′-ACAGTGGCGTGAGTCTT-3′; ADNP sense 5′-ACGAAAATCAAGCGATCCGG-3′, anti-sense 5′-GGACATCCCGAAGATGACTT-3′; ADNP2 sense 5′-GGAAAGAAGCGAGATAGCC-3′, anti-sense 5′-TCTGGTCCGAGCCCTCA-3′.
RESULTS

ADNP expression modulated behavior in a sexual-dependent manner: ADNP+/− male, but not female mice display deficits in object recognition.

ADNP haploinsufficient mice did not differ in their open field behavior38 (data not shown). In contrast, while there was no difference in the short-term retention choice (3 h, Figure 1a), in the long-term retention choice, ADNP+/− male mice spent greater than doubled time with the novel object, compared with ADNP++ male littersmates. Thus, a potential deficit in the memory or rather repetitive behavior and preference of the familiar condition was observed with ADNP deficiency (Figure 1b). In contrast to the males, there was no genotype difference in females representing a significant sex difference (Figure 1b).

ADNP+/− mice exhibit sex differences in social recognition.

When comparing the preference of an inanimate object to a mouse (male, in the case of males; and female, in the case of females), all males preferred animals over the empty cup (Figure 1c), exhibiting longer sniffing periods with the animal. A significant sex difference was discovered, with ADNP+/− females, showing only a trend of being more interested in the animal over the empty cup (contrasting ADNP+/− females) and being less socially interested (2-fold less interaction time with the other female mouse compared with males). Comparing males with females, it could be that the ADNP+/− males are deficient in learning to recognize the other animal and thus continue to persist increasing their interaction time, whereas the females may recognize quickly and thus exhibit less interested behavior.

ADNP+/− male mice display a significant preference to a familiar mouse rather than a novel one (decreased social memory).

In the social memory test, there was a significant sex difference in the control mice, with males preferring the novel mouse and females showing no preference (Figure 1d). This was not observed in the ADNP+/− mice, that is, ADNP+/− male mice behave like ADNP+/− females but unlike ADNP+/− males. Thus, as seen in the object recognition test, ADNP+/− male mice showed a highly significant preference to the familiar over the novel mouse, opposite to the ADNP+/− mice. Similarly, ADNP+/− female mice also preferred the familiar female. In previous experiments, inbred ADNP+/− male mice (not mated with the ICR mice12,33) also showed social memory deficits. However, these experiments could not be repeated in the same way (open arena), as the ICR background used here resulted in very aggressive mice, and the only possibility to perform mouse interaction tests was with mice enclosed in an isolated compartment.

Bioinformatics

ADNP Sequence analysis was used to identify the consensus KclYcnyLp and cekYkpgVLL—eIF4E binding sites as per published literature.44

Statistical analysis

Two-way repeated measures analysis of variance followed by Fisher’s least significant difference test, with group as a fixed factor (group) and sniffed item (that is, cup vs a novel mouse) as a repeated factor, was used to analyze the data in the social recognition task. In all other measurements, two-way analysis of variance followed by Fisher’s least significant difference was used. One-way analysis of variance or Student’s t-test were used when required. All the analyses were conducted with SigmaPlot software (Chicago, IL, USA) for Windows.

ADNP+/− male mice display a significant preference to a familiar mouse rather than a novel one (decreased social memory).
ADNP+/− mice show intact odor habituation–dishabituation. The odor habituation–dishabituation measured two parameters including (1) intact olfactory function and (2) olfactory memory. Here, sex comparisons reveal significant differences between males and females in the intact and deficient ADNP groups, with only males showing intact odor habituation–dishabituation and complete identity between the male ADNP+/− and ADNP−/− groups, that is, no genotype differences (Figure 2). Thus, the differences observed above could be attributed to emotional/cognitive disturbance dissociated from olfactory memory.

Mechanism associated with autism: eukaryotic translation initiation factor 4E (eIF4E)

A recent publication addressed central mechanisms of ASDs and eIF4E interaction.41 eIF4E binding sites44 (Figure 3a). These sequences were identified as putative eIF4E binding motif sequences on the ADNP protein sequence KclYcnyLpgVLL (Figure 3b). ADNP antibodies followed by western analysis with eIF4E antibody; lane 6: 500 μg of brain lysate of ADNP+/+ male mice and lane 7: 500 μg of brain lysate from ADNP+/* male mice show intact odor habituation–dishabituation and ADNP hippocampal expression exhibits a striking sexual dimorphism. Given the sex and genotype differences in ADNP+/− mice, we were interested to see if ADNP expression is sex-dependent in the hippocampus, a brain area directly associated with learning and memory. We revealed, for the first time, a significant 2-fold decreased ADNP expression in the female hippocampus, similar to the genotype-associated decrease in ADNP+/− males, with the genotype decrease (ADNP haplinsufficiency) in females being insignificant (Figure 4b). To reflect this sex difference to men, we have also reevaluated our previous data in postmortem human hippocampal tissue25,30 discovering the same sex difference, with males expressing ~25% more ADNP transcript than females (Figure 4c). In contrast to ADNP, no significant differences were found in the related ADNP2 (Figure 4d), agreeing with our previous results showing no effect of the ADNP-deficient genotype of ADNP2 expression.45 ApoE, the major risk gene for AD, which has been shown to be suppressed by ADNP during embryonic development,13 was twice decreased in males compared with females (Figure 4e), contrasting the increased hippocampal ADNP in males (Figure 4f). This sex difference observed in ADNP+/− females revealed increased ApoE transcript content which was further doubled in ADNP+/+ females (Figure 4e).

All the above-mentioned experiments were carried out in 5- to 6-month-old mice. A follow-up experiment utilized 9-month-old male mice showing no genotype effect for eIF4E in the older mice (Figure 4f). Given a significant decrease in eIF4E transcript levels between ADNP+/− ‘young’ male mice and ADNP+/+ ‘old’ male mice (P = 0.001, paired t-test, comparing Figure 4f to Figure 4a), a similar age-dependent decrease was also observed in ADNP+/− mice (P = 0.001, paired t-test).

Finally, abnormalities in neuroligins, (NLGNs), which are down-stream to eIF4E, have been suggested as causal for autism42 with
ADNP+/+ male and female mice, respectively, at 5 months of age (F(1,23) = 8.760, P = 0.010) and a significant effect of genotype (F(1,15) = 14.660, P = 0.002). Fisher’s LSD post hoc test revealed a significant 2-fold decrease in ADNP+/− male mice compared with control mice (***P < 0.001). In addition, Fisher’s LSD post hoc test revealed a significant 2-fold increase in ADNP+/− male mice compared with ADNP+/− female mice (P < 0.05). (c) Human ADNP: Hippocampal human ADNP used before was analyzed comparing males and females. As there was no difference at the ADNP transcript levels between normal and schizophrenia subjects, the cohorts were pooled to obtain 22 men and six women. Results showed a statistically significant increase in men (Student’s t-test, **P < 0.05). (d) ADNP2: two-way ANOVA showed no significant effect of sex (P = 0.977) and no significant effect of genotype (P = 0.498). (e) ApoE: two-way ANOVA showed a significant effect of sex (F(1,23) = 128.734, P < 0.001) and a significant effect of genotype (F(1,23) = 13.430, P = 0.001). Fisher’s LSD post hoc test revealed a significant 2-fold increase in ADNP+/− female mice compared with ADNP+/− female mice (**P < 0.001). In addition, Fisher’s LSD post hoc test revealed a highly significant decrease in male mice compared with female mice for both ADNP+/− and ADNP+/− (**P < 0.001). One-way ANOVA showed a significant decrease in ADNP+/− male mice compared with ADNP+/− mice (P < 0.05). (f) Nine-month-old male mice, eIF4E: no specific genotype effect, unlike (a) 5- to 6-month old. ADNP, activity-dependent neuroprotective protein; LSD, least significant difference.

**DISCUSSION**

A major surprising finding of the current work was the doubled hippocampal expression of ADNP in the male vs the female mouse which was found to mimic the postmortem human brain, followed by a two-fold decrease in the ADNP haploinsufficient male, and no significant decrease in the ADNP+/− female. The increase in ADNP expression paralleled enhanced behavioral performance in the ADNP intact males compared with females in the social memory task, presenting intact olfactory discrimination. In contrast, ADNP

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**Figure 4.** Autism-specific gene modulation in the hippocampus of ADNP+/− mice: sexual dichotomy. Hippocampal RNA from 5- to 6-month-old mice was analyzed by quantitative real-time PCR (n = 6–8 mice per group). (a) eIF4E: Two-way analysis of variance (ANOVA) showed no significant effect of sex (P = 0.692) and genotype (P = 0.137). However, as there was a marginal genotype trend, one-way ANOVA was also performed showing a significant increase in eIF4E transcripts in the ADNP+/− male mice compared with ADNP+/+ male mice (**P < 0.05). (b) ADNP: two-way ANOVA showed a significant effect of sex (F(1,15) = 8.760, P = 0.010) and a significant effect of genotype (F(1,15) = 14.660, P = 0.002). Fisher’s LSD post hoc test revealed a significant 2-fold decrease in ADNP+/− male mice compared with control mice (***P < 0.001). In addition, Fisher’s LSD post hoc test revealed a significant 2-fold increase in ADNP+/− male mice compared with ADNP+/− female mice (P < 0.05). (c) Human ADNP: Hippocampal human ADNP used before was analyzed comparing males and females. As there was no difference at the ADNP transcript levels between normal and schizophrenia subjects, the cohorts were pooled to obtain 22 men and six women. Results showed a statistically significant increase in men (Student’s t-test, **P < 0.05). (d) ADNP2: two-way ANOVA showed no significant effect of sex (P = 0.977) and no significant effect of genotype (P = 0.498). (e) ApoE: two-way ANOVA showed a significant effect of sex (F(1,23) = 128.734, P < 0.001) and a significant effect of genotype (F(1,23) = 13.430, P = 0.001). Fisher’s LSD post hoc test revealed a significant 2-fold increase in ADNP+/− female mice compared with ADNP+/− female mice (**P < 0.001). In addition, Fisher’s LSD post hoc test revealed a highly significant decrease in male mice compared with female mice for both ADNP+/− and ADNP+/− (**P < 0.001). One-way ANOVA showed a significant decrease in ADNP+/− male mice compared with ADNP+/− mice (P < 0.05). (f) Nine-month-old male mice, eIF4E: no specific genotype effect, unlike (a) 5- to 6-month old. ADNP, activity-dependent neuroprotective protein; LSD, least significant difference.
haploinsufficiency resulted in a severe cognitive impairment in the males with somewhat spared females in the object recognition test, mimicking the findings in children with ADNP mutations with the four female patients showing mild intellectual disability, while most of the males (five out of six) showing severe intellectual disability.6

It is also important to bear in mind that our colony, outbred with ICR mice, is still showing dramatic behavioral effects in the ADNP+/- males, which implicates a strong genotype effect, as predicted from human studies. These findings are also reproduced in the females, with ADNP+/- mice only insignificantly trending to prefer mice over objects in the social recognition test, unlike the ADNP++ females and males. The sex difference in hippocampal ADNP expression is even more striking when comparing it with previous findings in the arcuate nucleus of the hypothalamus showing an opposite finding, with ADNP decreased expression in males and regulation by the estrous cycle involving estrogen effects.36 In this respect, ADNP is part of the SWI/SNF chromatin remodeling complex47 including BAF57 that specifically regulates estrogen receptor α (ERα)-mediated transcription48 and ADNP was

Figure 5. Neuroligins show sex-dependent expression. Hippocampal RNA from 5- to 6-month-old mice was analyzed by quantitative real-time PCR (n = 6–8 mice per group). The 4 NGLN genes were compared. (a) NGLN1: two-way analysis of variance (ANOVA) showed a significant effect of sex (F(1,26) = 16.754, P < 0.001), but no significant effect of genotype (P = 0.561). Fisher’s LSD post hoc test revealed a significant difference between male mice compared with female mice for both ADNP+/+ and ADNP+/- (P < 0.05). (b) NGLN2: two-way ANOVA showed no significant effect of sex (P = 0.777) and no significant effect of genotype (P = 0.450). (c) NGLN3: two-way ANOVA showed a significant effect of sex (F(1,24) = 7.800, P = 0.010), but no significant effect of genotype (P = 0.771). Fisher’s LSD post hoc test revealed a significant difference between ADNP+/- male mice compared with ADNP+/- female mice (*P < 0.05). (d) NGLN4: two-way ANOVA showed a significant effect of sex (F(1,25) = 12.258, P = 0.002), but no significant effect of genotype (P = 0.482). Fisher’s LSD post hoc test revealed a significant difference between ADNP+/- male mice compared with ADNP+/- female mice (*P < 0.05). (e) Nine-month-old male mice, NGLN1: no specific genotype effect, like (a) 5- to 6-month old. (f) Nine-month-old male mice, NGLN2. (g) Nine-month-old male mice, NGLN3. (h) Nine-month-old male mice, NGLN4. ADNP, activity-dependent neuroprotective protein; LSD, least significant difference.
shown to regulate its own expression.\textsuperscript{13,45} Furthermore, the ADNP-interacting SWI/SNF member, BRG1, also interacts with endogenous androgen receptor-responsive promoters\textsuperscript{50} and both BRG1 and BAF57 are involved in the enhancement of androgen receptor activity.\textsuperscript{51} Our studies suggest direct interaction of ADNP in these processes, explaining in part the sexual divergence associated with ADNP-deficient genotype.

The human study on autistic children showing \textit{de novo} mutations in ADNP referred to ADNP as an SWI/SNF member\textsuperscript{6} with predominant nuclear localization, as indicated by our original studies.\textsuperscript{3} We are now showing an interaction of ADNP with the cytoplasmic elf4E and increased elf4E expression in the hippocampus of the 5- to 6-month-old ADNP\textsuperscript{57}−/− male mouse, which has been suggested as causal for ASD.\textsuperscript{52,53} Interestingly, this increased elf4E expression did not persist at 9 months of age, which was coupled to an overall decreased elf4E expression in the older mice. These findings may be related to the fact that overall protein synthesis decreases with brain aging\textsuperscript{2,53} coupled to the fact that autism is an early onset disorder associated with deregulation during development.

Besides elf4E, ADNP interacts with several other proteins, including PSF (a tau splicing factor)\textsuperscript{54} and both PSF and elf4E are phosphorylated by Mrk kinase.\textsuperscript{55} Thus, ADNP binding to either protein may affect the phosphorylation and activation state of elf4E, which is associated with the regulation of translation. Sexual dimorphism was also found at the level of neuroligin expression, shown to be regulated by elf4E.\textsuperscript{56} Further, dramatic sexual dimorphism was identified in the expression of ApoE suggesting a broader effect of ADNP on autism and AD-related genes. With ApoE being the major risk gene for AD, a connection is made for a higher AD risk in autistic individuals.

The highly significant increase of ApoE expression in the female hippocampus coupled to further increase in the ADNP+/−/− female may be correlated with increased prevalence of AD in women compared with men.\textsuperscript{5} Furthermore, neuroligin1 (doubled in the female mouse hippocampus compared with males) interacts with amyloid beta peptide to increase the formation of amyloid beta oligomers,\textsuperscript{50} a major pathology in AD. Interestingly, in older mice (9 vs 5–6 months of age), a significant reduction in the NLGN1 transcript was found suggesting that increases in neuroligin1 may be related to the initial stages of AD.

The second major pathology in AD, tau hyperphosphorylation and accumulation of tau neurofilibrillary tangles has been shown to be a part of the pathology of ADNP-deficient mice.\textsuperscript{3,5} In addition, an initial increase in ADNP expression, followed by an aging-associated dramatic decrease, predict tau pathology in mice with ADNP interacting with tau mRNA splicing.\textsuperscript{54,57} As indicated above, comprehensive proteomics identified ADNP as the only protein decreased in the serum of AD patients compared with controls.\textsuperscript{35}

The current study puts ADNP replacement therapy as a major drug target. The ADNP-derived, eight amino acid neuroprotective peptide NAP (davunetide), protected against ADNP-deficiency outcomes in the inbred ADNP\textsuperscript{57}+/− mouse, that is, tau pathology and cognitive impairments,\textsuperscript{12} and has shown positive indications of increasing cognitive scores in aging mild cognitive impairment patients.\textsuperscript{58–60} Although NAP (davunetide) showed activity in mild cognitive impairment, its clinical results in progressive supranuclear palsy patients were disappointing.\textsuperscript{61} In contrast, in schizophrenia patients, NAP (davunetide) treatment indicated protection of activities of daily living\textsuperscript{52} and brain cell function (magnetic resonance spectroscopy measurements).\textsuperscript{52} The current findings set the stage with a paradigm of ADNP deficiency that provides a new model for (1) better understanding of the pivotal role of ADNP in neurodevelopment and neuroprotection in males and females and paves the path to (2) optimizing and improving NAP (davunetide) and related compounds\textsuperscript{15,57} for potential future clinical development in ASD and prevention of later onset of AD, while paying attention to the dramatic sex differences toward precise medical intervention/rational translational psychiatry.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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**REFERENCES**

1. Maenner MJ, Rice CE, Arneson CL, Cunniff C, Schieve LA, Carpenter LA et al. Potential impact of DSM-5 criteria on autism spectrum disorder prevalence estimates. JAMA Psychiatry 2014; 71: 292–300.
2. Gillberg C, Billstedt E. Autism and Asperger syndrome: coexistence with other clinical disorders. Acta Psychiatr Scand 2000; 102: 321–330.
3. Pinto D, Pagnamenta AT, Klei L, Anney R, Moncho D, Regan R et al. Functional impact of global rare copy number variation in autism spectrum disorders. Nature 2010; 466: 368–372.
4. Talskowsi ME, Rosenfeld JA, Blumenthal I, Pillamamani V, Chiang C, Heibut A et al. Sequencing chromosomal abnormalities reveals neurodevelopmental loci that confer risk across diagnostic boundaries. Cell 2012; 149: 525–537.
5. Mielke MM, Vernuri P, Rocca WA. Clinical epidemiology of Alzheimer’s disease: assessing sex and gender differences. Clin Epidemiol 2014; 6: 37–48.
6. Helsmoortel C, Vulto-van Silfhout AT, Coe BP, Vandeweyer G, Rooms L, van den Ende J et al. A SWI/SNF-related autism syndrome caused by de novo mutations in ADNP. Nat Genet 2014; 46: 380–384.
7. O’Roak BJ, Vives L, Fu W, Egerton JD, Stanaway IB, Phelps IG et al. Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. Science 2012; 338: 1619–1622.
8. O’Roak BJ, Vives L, Giritjian S, Karakoc E, Krumm N, Coe BP et al. Sporadic autism exomes reveal a highly interconnected protein network of de novo mutations. Nature 2012; 485: 246–250.
9. Bassan M, Zamostiano R, Davidson A, Pinhasov A, Giladi E, Perl O et al. Complete sequence of a novel protein containing a femtomolar-activity-dependent neuroprotective peptide. J Neurochem 1999; 72: 1283–1293.
10. Zamostiano R, Pinhasov A, Gelber E, Steingart RA, Seroussi E, Giladi E et al. Cloning and characterization of the human activity-dependent neuroprotective protein. J Biol Chem 2001; 276: 708–714.
11. Pinhasov A, Mandel S, Torchinsky A, Giladi E, Pittel Z, Goldsweig AM et al. Activity-dependent neuroprotective protein: a novel gene essential for brain formation. Brain Res Dev Brain Res 2003; 144: 83–90.
12. Vuil-Shultzman I, Pinhasov A, Mandel S, Grigoridias N, Touloumi O, Pittel Z et al. Activity-dependent neuroprotective protein snippet NAP reduces tau hyperphosphorylation and enhances learning in a novel transgenic mouse model. J Pharmacol Exp Ther 2007; 323: 438–449.
13. Mandel S, Rechavi G, Gozes I. Activity-dependent neuroprotective protein (ADNP) differentially interacts with chromatin to regulate genes essential for embryogenesis. Dev Biol 2007; 303: 814–824.
14. Oz S, Iwashko-Pachima Y, Gozes I. The ADNP derived peptide, NAP modulates the tubulin pool: implication for neurotrophic and neuroprotective activities. PLoS One 2012; 7: e51458.
15. Quaisha S, Cowan CM, Mudher A. NAP (davunetide) rescues neuronal dysfunction in a Drosophila model of tauopathy. Mol Psychiatry 2013; 18: 834–842.
16. Shiryayev N, Jouroukhin Y, Giladi E, Polyzoidou E, Grigoriadis NC, Rosenmann H et al. NAPs protect memory, increases soluble tau and reduces tau hyperphosphorylation in a tauopathy model. Neurobiol Dis 2009; 34: 381–388.
17 Gozes I, Iram T, Maryanovsky E, Avir C, Rozenberg L, Schirer Y et al. Novel tubulin and tau neuroprotective fragments sharing structural similarities with the drug candidate NAP (Davuentide). J Alzheimers Dis 2014; 40: 523–536.
18 Gozes I, Schirer Y, Idan-Feldman A, David M, Fumman-Assaf S. NAP alpha-aminoisobutyric acid (IsoNAP). J Mol Neurosci 2014; 52: 1–9.
19 Oz S, Kapitansky O, Iwashko-Pachima T, Malishevich A, Giladi E, Skalka N et al. The NAP peptide of activity-dependent neuroprotective protein (ADNP) regulates dendritic spines through microtubule end binding proteins. Mol Psychiatry 2014; 19: 1115–1124.
20 Garbem JY, Neumann M, Trojanowski JQ, Lee VM, Feldman G, Norris JW et al. A mutation affecting the sodium/potassium exchanger, SLCA2, causes mental retardation with tau deposition. Brain 2010; 133: 1391–1402.
21 Latapy C, Rioux V, Guitton MJ, Beaulieu JM. Selective deletion of forebrain glycopeptide tau neuroprotective fragments sharing structural similarities with the drug candidate NAP (Davuentide). J Mol Neurosci 2014; 52: 1–9.
22 Esteves AR, Gozes I, Cardoso SM. The rescue of microtubule-dependent trafficking in adult autistic individuals. Neuropsychol Appl Neurobiol 2004; 30: 615–623.
23 Mandel S, Spivak-Pahis I, Gozes I. ADNP differential nucleus/cytoplasm localization in neurons suggests multiple roles in neuronal differentiation and maintenance. J Mol Neurosci 2008; 35: 127–141.
24 Smith-Swintosky VL, Gozes I, Brennan DE, D’Andrea MR, Plata-Salaman CR. Activity-dependent neurotrophic factor-9 and NAP promote neurite outgrowth in rat hippocampal and cortical cultures. J Mol Neurosci 2005; 25: 225–238.
25 Merenlender-Wagner A, Malishevich A, Shemer Z, Udalueva M, Gibbons A, Scarr E et al. Autophagy has a key role in the pathophysiology of schizophrenia. Mol Psychiatry 2013.
26 Esteves AR, Gozes I, Cardoso SM. The rescue of microtubule-dependent traffic recoveres mitochondrial function in Parkinson’s disease. Biochem Biophys Acta 2014; 1842: 7–21.
27 Di Nando A, Wertz MH, Kwiakowski E, Tsai PT, Leech JD, Greene-Colozzo E et al. Neuronal Tsc1/2 complex controls autophagy through AMPK dependent regulation ofULK1. Hum Mol Genet 2016; 23: 3865–3874.
28 Dere E, Dahm L, Lu D, Hammerschmidt K, Ju A, Tantra M et al. Heterozygous ambral deficiency in mice: a genetic trait with autism-like behavior restricted to the female gender. Front Behav Neurosci 2014; 8: 181.
29 Freyer M, Pocklington AJ, Kavanagh DH, Williams HJ, Dwyer S, Gromley P et al. More novo mutations in schizophrenia implicate synaptic networks. Nature 2014; 506: 179–184.
30 Dresner E, Agam G, Gozes I. Activity-dependent neuroprotective protein (ADNP) expression level is correlated with the expression of the sister protein ADNP2: a genetic model of schizophrenia. Eur Neuropsychopharmacol 2011; 21: 355–361.
31 Okada K, Hashimoto K, Iwata Y, Nakamura K, Tsuji M, Tsuichya KJ et al. Decreased serum levels of transforming growth factor-beta1 in patients with autism. Prog Neuropsychopharmacol Biol Psychiatry 2007; 31: 187–190.
32 Maekawa M, Iwayama Y, Nakamura K, Sato M, Toyota T, Ohnishi T et al. A novel missense mutation (Leu46Val) of PAX6 found in an autistic patient. Neurosci Lett 2009; 462: 267–271.
33 Shaya N, Pikman R, Giladi E, Gozes I. Protection against taupathy by the drug candidates NAP (davunetide) and D-SAL: biochemical, cellular and behavioral aspects. Curr Pharm Des 2011; 17: 2603–2612.
34 Bracht M, Kawabe K, Nyrendra M, Gilles LJ, Robins RA, Gran B et al. Expression of activity-dependent neuroprotective protein in the immune system: possible functions and relevance to multiple sclerosis. Neuroimmunomodulation 2009; 17: 120–125.
35 Yang MH, Yang YH, Lu CY, Jong SB, Chen LJ, Lin YF et al. Activity-dependent neuroprotector homeobox protein: a candidate protein identified in serum as diagnostic biomarker for Alzheimer’s disease. J Proteomics 2012; 75: 3617–3629.
36 Fumman S, Hill JM, Vulih J, Zaltzman R, Hauser JM, Brennan DE et al. Sexual dimorphism of activity-dependent neuroprotective protein in the mouse arcuate nucleus. Neurosci Lett 2005; 373: 73–78.
37 Alcalay RN, Giladi E, Pick CG, Gozes I. Intranasal administration of NAP, a neuroprotective peptide, decreases anxiety-like behavior in aging mice in the elevated plus maze. Neurosci Lett 2004; 361: 128–131.
38 Merenlender-Wagner A, Pikman R, Giladi E, Andrieux A, Gozes I. NAP (davunetide) enhances cognitive behavior in the STOP heterogeneous mouse—a microtubule-deficient model of schizophrenia. Peptides 2010; 31: 1368–1373.
39 McFarlane HG, Kusek GY, Yang M, Phoenix JL, Bolivar VJ, Crawley JN et al. Autism-like behavioral phenotypes in B6TBR-+/-f/f mice. Genes Brain Behav 2008; 7: 152–163.
40 Matsuoka Y, Jouroukhin Y, Gray AJ, Ma L, Hirata-Fukae C, Li HF et al. A neuronal microtubule-interacting agent, NAPVISIPQ, reduces tau pathology and enhances cognitive function in a mouse model of Alzheimer’s disease. J Pharmacol Exp Ther 2008; 325: 146–153.
41 Bernstein J, Shefer J, Eloy-Stein O. The translational repression mediated by the platelet-derived growth factor 2c-sis mRNA leader is relieved during megakaryocytic differentiation. J Biol Chem 1995; 270: 10559–10565.
42 Glogkas CG, Khourotsky A, Ran I, Rampakakis E, Nevarko T, Weatherill DB et al. Autism-related deficits via dysregulated elf4e-dependent translational control. Nature 2013; 493: 371–377.
43 Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C (T) method. Nat Protoc 2008; 3: 1101–1108.
44 Gosselin P, Martinez E, Morales J, Czjzek M, Giilla, Gaffeny I et al. Tracking a refined elf4e-binding motif reveals Angel1 as a new partner of elf4e. Nucleic Acids Res 2011; 41: 7783–7792.
45 Dresner E, Malishevich A, Avir C, Leibman BS, Alon S, Ofr I et al. Novel evolutionary-conserved role for the activity-dependent neuroprotective protein (ADNP) family that is important for erythropoiesis. J Biol Chem 2012; 287: 40173–40185.
46 Yu J, He X, Yao D, Li Z, Li H, Zhao Z et al. A sex-specific association of common variants of neuriligin genes (NLGN3 and NLGN4X) with autism spectrum disorders in a Chinese Han cohort. Behav Brain Funct 2011; 7: 13.
47 Mandel S, Gozes I. Activity-dependent neuroprotective protein constitutes a novel element in the SWI/SNF chromatin remodeling complex. J Biol Chem 2007; 282: 34448–34456.
48 Garcia-Pedropio JM, Kiskinis E, Parker MG, Belandia B. The SWI/SNF chromatin remodelling subunit BRG1 is a critical regulator of estrogen receptor function in breast cancer cells. J Biol Chem 2008; 283: 22656–22664.
49 Abromon K, Quian, JP, Buddel UI. Activity-dependent neuroprotective protein modulates its own gene expression. J Mol Neurosci 2012; 43: 39–49.
50 Dai Y, Ngo D, Jacob J, Forman LW, Fallaw DV. Prohibitin and the SWI/SNF ATPase subunit BRG1 are required for effective androgen antagonist-mediated transcriptional repression of androgen receptor-regulated genes. Carcinogenesis 2008; 29: 1725–1733.
51 Li X, Zhou C, Wu WH, Yang N, Qin H, Sun Z et al. ZM121 preferably enhances the transcriptional activity of androgen receptor with short polyglutamine tract. PLoS One 2011; 6: e25040.
52 Gozes I, Cronin BL, Moskwitz MA. Protein synthesis in rat brain microvessels regulated in the brains of mouse models of human tauopathies: toward davunetide. Autophagy 2013; 9.
53 Jarskog LF, Dong Z, Kangarlu A, Colibazzi T, Girgis RR, Kegeles LS et al. Effects of the neuroprotective peptide davunetide (AL-108) on cognition and functional outcomes in a double-blind, placebo-controlled phase 2/3 trial. Lancet Neurolog 2013; 12: 676–685.
54 Javitt DC, Buchanan RW, Keefe RS, Kern R, McMahon RP, Green MF et al. Effect of the neuroprotective peptide davunetide (AL-108) on cognition and functional capacity in schizophrenia. Schizophr Res 2012; 136: 25–31.
55 Jarskog LF, Dong Z, Kangarlu A, Colibazzi T, Girgis RR, Kegeles LS et al. Effects of davunetide on N-acetylaspartylase and choline in dosolateral prefrontal cortex in patients with schizophrenia. Neuropsychopharmacology 2013; 38: 1245–1252.

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