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Prenatal NAP+SAL Prevents Developmental Delay in Down Syndrome through affects on NMDA and GABA receptors

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Abstract

OBJECTIVE—Down Syndrome affects 1/800 infants. Prenatal NAP+SAL prevents developmental delay in Ts65Dn mice. We investigated if this finding involves NMDA and GABA receptor subunits.

STUDY DESIGN—Pregnant Ts65Dn mice were treated with placebo or NAP+SAL on gestational days 8–12. After developmental delay prevention was shown, 4 trisomic (Ts), 4 control and 3 Ts+NAP+SAL adult brains (from 3 litters) were collected. Calibrator-normalized RT-PCR was performed using NR2A, NR2B, GABA_Aα5 and GABA_Aβ3 primers with GAPDH standardization. Statistics included ANOVA and Fisher PLSD with P<0.05 as significant.

RESULTS—NR2A, NR2B and GABA_Aβ3 levels were decreased in Ts vs control (all P<0.05). Prenatal NAP+SAL increased NR2A, NR2B and GABA_Aβ3 to levels similar to control (all P<0.05). A significant difference in GABA_Aα5 levels was not found.

CONCLUSION—Prenatal NAP+SAL increases NR2A, NR2B and GABA_Aβ3 expression in adult Down Syndrome mice to levels similar to controls. This may explain how NAP+SAL improve developmental milestone achievement.

Keywords

Down Syndrome; Ts65Dn; NMDA; GABA; learning; NAP and SAL

INTRODUCTION

Down Syndrome (DS), due to a triplication of critical regions of chromosome 21, affects 1/800 newborn infants and is the most common genetic etiology of mental retardation.¹ DS is associated with characteristic physical features and abnormal central nervous system

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function, particularly developmental delay and hypotonia in the newborn period, mental retardation evident in childhood, and early onset of Alzheimer's disease.

Previously, we looked at the role of two neuroprotective peptides, NAPVSIPQ (NAP) and SALLRSIPA (SAL), in preventing developmental delay in the Ts65Dn mouse model of DS. 2 NAP and SAL are 8 and 9 amino acid peptide fragments, respectively, which mimic the activity of their parent proteins, activity dependent neuroprotective protein (ADNP) and activity dependent neurotrophic factor (ADNF). ADNP and ADNF are released by glial cells in the brain³ and are regulated by vasoactive intestinal peptide (VIP).^{4,5} These peptides are neuroprotective *in vivo* against diverse neuronal insults, including excitotoxicity, closed head injury, ischemic brain injury, apolipoprotein E deficiency and alcohol-induced microcephaly, fetal death and growth restriction.^{6,7} NAP and SAL have also been shown to prevent learning deficits in a mouse model of Fetal Alcohol Syndrome (FAS)⁸ as well as enhance learning in control mice.⁹

In Down Syndrome, there is a known glial deficit resulting in decreased ADNP levels² and an up-regulatory increase of VIP.¹⁰ Recently, we demonstrated that trisomic (Ts) mice that were prenatally treated with NAP+SAL achieved developmental milestones at the same time as the controls in 3 of 4 motor (righting, vibrissa placing and forelimb grasp) and 1 of 4 sensory (weak tactile stimulation) milestones ($p < 0.01$) and there was a trend towards better performance in the Ts+peptides compared to the Ts group in forelimb placing and weight. Prenatal treatment with NAP+SAL also reversed the deregulation of ADNP and VIP in whole brain samples of these offspring.²

Our objective was to delineate if the prevention of developmental milestone delay by prenatal administration of NAP+SAL was mediated through alterations in the NMDA and GABA receptor subunits, NR2A, NR2B, GABA_Aα5 and GABA_Aβ3, which are fundamental to long term potentiation, the electrophysiological model of learning.

MATERIALS AND METHODS

We used the Ts65Dn mouse model which closely mimics the genetic abnormality in DS. The Ts65Dn mice contain a trisomic segment of mouse chromosome 16 which is orthologous to the "critical region" of human chromosome 21 and the mice exhibit a number of features characteristic of DS, including impairment of hippocampal function as evidenced by impaired spatial memory¹¹⁻¹⁶ and context discrimination.¹⁷ In the newborn period, Ts65Dn mice mimic the human condition in developmental delay in motor and sensory milestones.¹⁴ In adulthood, Ts65Dn mice have a deficit in short and long term memory and learning, as well as early onset of the neuropathology of Alzheimer disease.¹⁴ Microscopically, Ts65Dn neonates have fewer granule cells in the hippocampus, reduced long term potentiation (LTP) and abnormal synaptic plasticity.¹⁸⁻²⁰

Ts65Dn female mice (The Jackson Laboratory, Bar Harbor, ME) were kept in a 12-hour light/12-hour dark regimen, with food and water available at all times with 6% fat diet. The mice received humane animal care in compliance with the National Institutes of Health (NIH) guidelines for care and use of experimental animals. The protocol was approved by

National Institute of Child Health and Human Development (NICHD) Animal Care and Use Committee. Females were mated with B6EiC3SnFi male mice; the presence of vaginal plug was checked twice daily and the day of its appearance was considered day 0 (E0) of pregnancy.

Ts65Dn pregnant females were treated by intraperitoneal injection on days E8, 9, 10, 11 and 12 (a typical mouse gestation is 18-21 days long) with saline (placebo; n=6) or NAP (20 µg) and SAL (20 µg) (SynPep, Dublin, CA) (n=4). This time period was chosen based on previous studies that showed that this is a critical time for VIP action during *in utero* development.²¹

After delivery, the litters were coded and each animal was labeled with standard markings. As previously reported, offspring were weighed and tested from postnatal day 5 to 21 for motor and sensory milestones with standardized tests by operators blinded to the pup's treatment and genotype.² After completion of the developmental tests, tail tips were collected and sent to Jackson laboratory for genotyping.

In order to evaluate NAP+SAL's mechanism of action we explored whether NAP+SAL altered expression of the NR2A, NR2B, GABA_Aα5 and GABA_Aβ3 subunits. Whole brains from 4 trisomic (Ts), 4 control and 3 Ts+peptides offspring from separate litters were microdissected separately and immediately frozen in dry ice. Each group represented at least 3 litters and the mice were 40 weeks old at time of dissection.

RNA extraction was performed by homogenizing each brain sample using a sonicator (Janke & Kunkel, Wilmington, NC) and the samples were processed with SV Total RNA Isolation System (Promega, Madison, WI). A 5-µL aliquot was obtained for spectrophotometric determination of RNA content. The remaining sample portion was stored at - 80°C. Using 5ug of total RNA, the reverse transcriptase (RT) reaction was performed to synthesize cDNA (Applied Biosystems, Foster City, CA) with a final volume of 150 uL. Each sample was run in duplicate. Negative controls included RT reactions omitting RNA or reverse transcriptase.

The primers used for RT-PCR included the primer pairs for NR2A, NR2B and GABA_Aα5, GABA_Aβ3 and GAPDH. The NR2B primer pair (synthesized by Integrated DNA Technologies, Coralville, IA; GenBank accession number NM_008171) was designed using Primer 3 software and the sequence was 5'-CCG CAG CAC TAT TAGA GAA CA-3' (sense) and 5'-ATC CAT GTG TAG CCG TAG CC-5' (antisense). NR2A primer pair sequence (synthesized by Integrated DNA Technologies, Coralville, IA; GenBank accession number NM_008170) was 5'-GGA AGT TGG ACG CTT TCA TC-3' (sense) and 5'-TCT TCC ATC TCA CCG TCA CC-3' (antisense) and was designed by TIB Molbiol (Adelphia, NJ). The GABA_Aα5 primer pair was designed and synthesized by TIB Molbiol (Adelphia, NJ) and its sequence was 5'-GGC AGA CAG TAG GCA CTG AG-3' (sense) and 5'-TCT TCC ATC TCA CCG TCA CC-3' (antisense) (GenBank accession number BC_062112). The GABA_Aβ3 primer sequence was 5'-CGA GTT GCC CTT GGG ATT AC-3' (sense) and 5'-GAT ATT CCC GTG AGC ATC CAC -3' (antisense) (GenBank accession number 14402; designed and synthesized by TIB Molbiol, Adelphia, NJ). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene located on mouse chromosome 6,

was used as an internal standard due to its stability in cells (Integrated DNA Technologies, Coralville, IA). GAPDH primer sequence was 5'-TGC ACC ACC AAC TGC TTA (sense) and 5'-GGA TGC AGG GAT GAT GTT C (antisense).

Using Fast Start DNA Master SYBR Green 1 dye-base detection by Roche (Indianapolis, IN) NR2A, NR2B, GABA_Aα5, GABA_Aβ3 and GAPDH expression were measured by real-time polymerase chain reaction (PCR) using Roche's LightCycler with relative quantification software and melting point analysis to assess specificity of amplified genes. To eliminate the risk of cross-contamination, LightCycler Uracil-DNA Glycosylase (Roche) was added to the master mix. Each sample was run in duplicate and relative quantification was performed by using calibrator-normalized data with efficiency correction. Results are presented as a normalized ratio of NR2A, NR2B, GABA_Aβ3 or GABA_Aα5-to-GAPDH. Statistical analysis included ANOVA and Fisher PLSD with P<0.05 considered significant.

RESULTS

Using real-time PCR, we found that gene expression of NR2A, NR2B and GABA_Aβ3 levels were decreased in whole brain samples from the adult trisomic mice (Ts65Dn) compared to the control (wild type) animals (all P<0.05, Figure 1). Prenatal treatment with NAP+SAL prevented the Down Syndrome-associated decrease in NR2A, NR2B and GABA_Aβ3 with levels similar to control and significantly higher than the Ts animals (all P<0.05) (Figure 1). There was no difference in GABA_Aα5 levels between the three groups.

COMMENT

We found that prenatal administration of NAP+SAL at a critical point in gestation has long lasting effects as it increases NR2A, NR2B and GABA_Aβ3 expression in adult trisomic (Ts) mice to levels similar to wild type controls. This may in part explain the mechanism behind the previously reported prevention of developmental milestone delay in the mouse model of DS.

NMDA receptors (NMDAR) are tetrameric, assembled from the NR1 subunit, which is an essential component of all NMDAR complexes, along with various combinations of NR2 or NR3 subunits. The NR2B subunit promotes long term potentiation (LTP) and is abundant in states of high firing, such as early development. The NR2A subunit, however, is known to be less plastic and is abundant in adulthood.²² The functional properties of the receptor depend on the specific subunit composition and stoichiometry in which subunits combine to form a channel.²² NMDARs are regulated by the inhibitory tone of the neurotransmitter, GABA, and the GABA_A receptor, whose subunits include GABA_Aα5 and GABA_Aβ3.²³ Both of these subunits have been shown to be key components in LTP and learning.^{24,25} Our findings of Down Syndrome-induced alterations in the expression of NR2A, NR2B and GABA_Aβ3 levels in adult mice and reversal of these alterations with prenatal NAP+SAL to levels similar to controls may provide insight into NAP and SAL's mechanism of action.

NAP and SAL are known to have long-lasting effects after a short exposure. Previously, we demonstrated that prenatal exposure with these peptides prevented alcohol-induced learning deficits and deregulation of NMDA and GABA receptor subunits in adult offspring in a

mouse model of FAS.8,26 This may occur by the peptides altering a variety transcription regulation pathways that ultimately may influence LTP.

Although there are various antenatal screening and diagnostic tests for DS in humans, there is no known prenatal intervention to alleviate the learning deficits, developmental delay or early Alzheimer's disease associated with this condition. Once a child is born with DS, treatment focuses mainly on early intervention programs, such as physical therapy, occupational therapy, and speech therapy. Since DS can be diagnosed prenatally, an intervention during pregnancy which may improve cognitive function is an attractive option.

References

1. Cunningham, FG, Leveno, KJ, Bloom, SL, Hauth, JC, Gilstrap, LC, Wenstrom, KD, editors. Williams Obstetrics. 22. New York: McGraw-Hill; 2005. Genetics; 290
2. Toso L, Cameroni I, Schmidt C, Abebe D, Bissell S, Spong CY. Prevention of developmental delays in a Down Syndrome model. *Obstetrics & Gynecology*.
3. 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 Mar; 72(3):1283–93. [PubMed: 10037502]
4. Giladi E, Hill JM, Dresner E, Stack CM, Gozes I. Vasoactive intestinal peptide (VIP) regulates activity-dependent neuroprotective protein (ADNP) expression in vivo. *J Mol Neurosci*. 2007; 33(3):278–83. [PubMed: 17952637]
5. Gozes I, Zamostiano R, Pinhasov A, Bassan M, Giladi E, Steingart RA, et al. A novel VIP responsive gene. Activity dependent neuroprotective protein. *Ann N Y Acad Sci*. 2000; 921:115–8. [PubMed: 11193814]
6. Brenneman DE, Spong CY, Hauser JM, Abebe D, Pinhasov A, Golian T, et al. Protective peptides that are orally active and mechanistically nonchiral. *J Pharmacol Exp Ther*. 2004; 309:1190–1197. [PubMed: 15007105]
7. Spong CY, Abebe D, Gozes I, Brenneman DE. Prevention of fetal demise and growth restriction in a mouse model of fetal alcohol syndrome. *J Pharmacol Exp Ther*. 2001; 297(2):774–9. [PubMed: 11303069]
8. Vink J, Auth J, Abebe D, Brenneman DE, Spong CY. Novel peptides prevent alcohol-induced spatial learning deficits and pro-inflammatory cytokine release in a mouse model of fetal alcohol syndrome. *Am J Obstet Gynecol*. 2005; 193(3 Pt 1):825–9. [PubMed: 16150281]
9. Toso L, Endres M, Vink J, Abebe DT, Brenneman DE, Spong CY. Learning enhancement with neuropeptides. *Am J Obstet Gynecol*. 2006 Apr; 194(4):1153–8. [PubMed: 16580319]
10. Hill JM, Ades AM, McCune SK, Sahir N, Moody EM, Abebe DT, et al. Vasoactive intestinal peptide in the brain of a mouse model for Down syndrome. *Exp Neurol*. 2003; 183(1):56–65. [PubMed: 12957488]
11. Reeves RH, Irving NG, Moran TH, Wohn A, Kitt C, Sisodia SS, et al. A mouse model for Down Syndrome exhibits learning and behavior deficits. *Nat Genet*. 1995; 11:177–184. [PubMed: 7550346]
12. Demas GE, Nelson RJ, Krueger BK, Yarowsky PG. Spatial memory deficits in segmental trisomic Ts65Dn mice. *Behav Brain Res*. 1996; 82:85–92. [PubMed: 9021073]
13. Demas GE, Nelson RJ, Krueger BK, Yarowsky PG. Impaired spatial working and reference memory in segmental trisomy (Ts65Dn) mice. *Behav Brain Res*. 1998; 90:100–201.
14. Holtzman DM, Santucci D, Kilbridge J, Chua-Couzens J, Fontana DJ, Daniels SE, et al. Developmental abnormalities and age-related neurodegeneration in a mouse model of Down syndrome. *Proc Natl Acad Sci*. 1996; 93:13333–13338. [PubMed: 8917591]
15. Escorihuela RM, Vallina IF, Martinez-Cue C, Baamonde C, Dierksen M, Tobena A, et al. Impaired short- and long-term memory in Ts65Dn mice, a model for Down syndrome. *Neurosci Lett*. 1998; 247:171–174. [PubMed: 9655620]

16. Sago H, Carlson EJ, Smith DJ, Rubin EM, Crnic LS, Huang TT, et al. Genetic dissection of region associated with behavioral abnormalities in mouse models for Down syndrome. *Pediatr Res*. 2000; 48:606–613. [PubMed: 11044479]
17. Hyde LA, Frisone DF, Crnic LS. Ts65Dn mice, a model for Down syndrome, have deficits in context discrimination learning suggesting impaired hippocampal function. *Behav Brain Res*. 2001; 118:53–60. [PubMed: 11163633]
18. Belichenko PV, Masliah E, Kleschevnikov AM, Villar AJ, Epstein CJ, Salehi A, et al. Synaptic structural abnormalities in the Ts65Dn mouse model of Down Syndrome. *J Comp Neurol*. 2004; 480:281–298. [PubMed: 15515178]
19. Contestabile A, Fila T, Ceccarelli C, Bonasoni P, Bonapace L, Santini D, et al. Cell cycle alteration and decreased cell proliferation in the hippocampal dentate gyrus and in the neocortical germinal matrix of fetuses with Down syndrome and in Ts65Dn mice. *Hippocampus*. 2007; 17(8):665–78. [PubMed: 17546680]
20. Best TK, Siarey RJ, Galdzicki Z. Ts65Dn, a mouse model of Down syndrome, exhibits increased GABAB-induced potassium current. *J Neurophysiol*. 2007; 97:892–900.
21. Spong CY, Lee SJ, McCune SK, Gibney G, Abebe DT, Alvero R, et al. Maternal regulation of embryonic growth: the role of vasoactive intestinal peptide. *Endocrinology*. 1999; 140(2):917–24. [PubMed: 9927324]
22. Perez-Otano I, Ehlers D. Learning from NMDA receptor trafficking: clues to the development and maturation of glutamatergic synapses. *Neurosignals*. 2004; 3:175–89.
23. Freund TF, Buzsaki G. Interneurons of the hippocampus. *Hippocampus*. 1996; 6:347–470. [PubMed: 8915675]
24. Barker JL, Behar T, Li YX, Liu QY, Ma D, Maric D, et al. GABAergic cells and signals in CNS development. *Perspect Dev Neurobiol*. 1998; 5:305–322. [PubMed: 9777645]
25. Mehta AK, Ticku M. An update on GABAA receptors. *Brain Res*. 1999; 29:196–217.
26. Toso L, Poggi SH, Roberson R, Woodard J, Park J, Abebe D, et al. Prevention of alcohol-induced learning deficits in fetal alcohol syndrome mediated through NMDA and GABA receptors. *Am J Obstet Gynecol*. 2006 Mar; 194(3):681–6. [PubMed: 16522397]

Figure 1.

NR2A, NR2B and GABA_Aβ3 expression are decreased in adult Ts mice. Prenatal NAP +SAL reversed these alterations to levels similar to control. On the X-axis are the three study groups: Ts in black diagonal striped bars, wild type control in black bars, and Ts+NAP+SAL in white bars. On the Y-axis is the primer/GAPDH expression (10²).