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Understanding the mechanism of learning enhancement:

NMDA and GABA receptor expression

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Abstract

OBJECTIVE—Administration of neurotrophic peptides NAP+SAL to aged mice resulted in significant learning enhancement. NMDA and GABA receptors are fundamental for learning as they are the major modulators of the Long Term Potentiation, the electrophysiologic mechanism for learning. Also, these receptors have been shown to be involved in NAP+SAL prevention of learning deficit in a mouse model for fetal alcohol syndrome when administered prenatally during development. Our objective was to test if NMDA and GABA receptors contribute to the learning enhancement induced by the peptides after adult administration.

STUDY DESIGN—Aged (14.5 months) males were treated for 10 consecutive days with placebo or D-NAP+D-SAL (20µg, via gavage). At the end of the treatment, brains were harvested. Calibrator-normalized relative real time PCR was performed using primers for GABA-Aβ3, GABA-Aα5, and the NMDA receptor subunits NR2A and NR2B with GAPDH standardization. Statistical analysis included ANOVA with p<0.05 significant.

RESULTS—Five control brains and 6 brains from animals treated with NAP+SAL were collected. There was no difference in GABA-Aβ3, GABA-Aα5, NR2A and NR2B subunits after adult administration of NAP+SAL as compared to the controls (p>0.05).

CONCLUSION—Postnatal treatment with NAP+SAL induced learning enhancement in aged mice with mechanism that does not involve alteration in NMDA and GABA receptor expression. Thus the mechanism of learning enhancement might be different for a developing fetus than an adult or in absence of a perturbing agent.

Keywords

learning; NMDA; GABA; ADNF; ADNP

Background

Many investigations have focused on looking for strategies to prevent mental retardation and developmental disabilities in different pathologic conditions. In our previous work we have demonstrated that prenatal or postnatal treatment with two synthetic peptides derived from neuroprotective proteins can enhance learning in healthy adult and aged mice^{1,2}. These small 8 and 9 amino acid peptides, SAL (SALLRSIPA) from activity dependent neurotrophic factor

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(ADNF) and NAP (NAPVSIPQ) from activity dependent neuroprotective protein (ADNP) mimic the activity of their parent proteins. These parent proteins (ADNF and ADNP) are released by glial cells and are regulated by vasoactive intestinal peptide (VIP), a central nervous system neurotransmitter and neuromodulator with neurotrophic properties. .

Numerous studies have demonstrated the neurotrophic activity of the peptides NAP and SAL. Prenatal or postnatal administration of NAP&SAL prevented alcohol-induced learning deficit in a model for fetal alcohol syndrome (FAS)^{2,3}. In a model for FAS based on chronic alcohol exposure, treatment with SAL prevented alcohol-induced brain growth compromise and anatomic alterations⁴. Intranasal administration of NAP&SAL prevented learning deficit induced by a blocker of choline-uptake⁵. Daily injections of NAP+SAL to newborn Apo-lipoprotein E-deficient mice, a model for Alzheimer disease, accelerated the acquisition of developmental reflexes and long-term memory deficits^{6,7}. In a mouse model for Down syndrome, prenatal treatment with NAP&SAL prevented developmental deficits in newborns pups⁸.

Understanding the mechanism of action of these peptides is key in the development of a potential treatment for conditions that include developmental delay and learning deficits. In a model for FAS, the peptides have been shown to act through prevention of changes in the expression of NMDA and GABA receptors subunits^{9,10}. The GABA and the NMDA receptors for glutamate are pentameric and tetrameric ion channels respectively, whose function is determined by their subunit composition¹¹. These receptors are key components of a brain circuit that leads to learning¹². Specifically, the GABA receptor subunits GABA_Aβ3 and GABA_Aα5, and the NMDA receptor subunits NR2A and NR2B play an important role in the learning process.

Our objective was to see if learning enhancement of aged mice with NAP&SAL also acts through modification of the NMDA and GABA receptor subunits.

Material and Methods

Fourteen and a half month old C57Bl6/J male 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. Mice received humane animal care in compliance with the National Institutes of Health Guidelines for Care and Use of experimental animals. The protocol was approved by the National Institute of Child Health and Human Development Animal Care and Use Committee. Following previously published methods¹, mice were withheld food and water for 3 hours before treatment every day to avoid interference with drug absorption. For 10 consecutive days mice were treated via gavage with either D-NAP(20ug) +D-SAL (20 ug) (SynPep, Dublin, CA) [n=6 from 4 litters] or placebo [n=5 from 3 litters]. The all-D amino acid configurations of the peptides were used that allow for oral administration. D-NAP was diluted in 50μL of dimethyl sulfoxide and diluted in 2.5mL of filtered Dulbecco's phosphate-buffered saline solution; D-SAL was dissolved in the same solution. On the 11th day, brains from NAP+SAL and control groups were collected with microdissection and immediately frozen in dry ice.

RNA was isolated and cDNA synthesized with reverse transcriptase reaction, real time PCR was performed for gene expression and relative quantification was performed with calibrator-normalized data with efficiency correction, thus the data represent the delta-delta CT values; each sample was run in duplicate. Results are presented as the normalized ratio of NR2B, NR2A, GABA_Aα5 or GABA_Aβ3-to-GAPDH. Real-time PCR comparisons were made between the NAP+SAL and control groups. Statistical analysis included ANOVA, with P<0.05 considered significant (StatView v5.0.1, SAS, Cary, NC). A detailed description of the methods

are provided in the Appendix and is available online (Note to publisher-can you provide the link here?).

Results

Here we show that in the brain from aged male mice, a ten day treatment with the peptides D-NAP+D-SAL did not result in a modification in the expression of the NMDA receptor subunits NR2A and NR2B ($P>0.05$, Figure 1). Similarly, the GABA_A receptor subunits GABA_A α 5 and GABA_A β 3 expression was not modified by treatment with the peptides ($P>0.05$, Figure 2), Control $n=6$ from 4 litters and D-NAP+D-SAL $n=5$ from 3 litters. As previously reported, the animals treated with the peptides learned better in the Morris watermaze, a model of spatial learning¹.

Comment

Postnatal oral treatment with the peptides NAP+SAL resulted in learning enhancement in aged mice that is not mediated through the NMDA and GABA receptor subunits NR2A, NR2B, GABA_A α 5 and GABA_A β 3. This is in contrast to findings of administration of the peptides during development in a model for FAS, where alterations in these subunits were found in prevention of learning deficit^{9,10}.

Long term potentiation (LTP) is the electrophysiologic mechanism of learning¹³. NMDA activation has been shown to be the first step for the cell membrane depolarization and thus activation of LTP¹⁴. NMDA firing is regulated by the inhibitory tone of the neurotransmitter GABA towards the GABA_A receptor¹². The NR2B subunit has been shown to promote LTP and to be particularly abundant in states of high firing, as during development, whereas the NR2A subunit is known to reduce LTP and to be predominant with aging¹⁴. In a model for FAS we have shown that prenatal treatment with the peptides NAP&SAL resulted in an increase in NR2B and a decrease in NR2A after alcohol exposure, with respect to the alcohol group, which may explain the prevention of learning deficit in FAS. The subunits GABA_A α 5 and GABA_A β 3 also have been shown to be involved in learning^{15,16}. In a model for FAS, NAP&SAL administration after alcohol-exposure resulted in an over-expression of these subunits as compared to the controls, indicating that the peptides are able to modify the expression of these GABA_A subunits^{9,10}.

In this set of experiments we have shown that postnatal treatment with NAP&SAL in aged mice resulted in learning enhancement which is not mediated through the modification of these subunits expression. It may be that the peptides act through these receptors only in the presence of a perturbing agent or that the receptors are not modifiable beyond the development period. A previous study showed that LTP induction was not altered by NAP alone but only by the interaction of alcohol and NAP¹⁷. Another possibility is that different subunits in these receptors may have triggered the enhancement, as other subunits in the NMDA and GABA receptors have been targeted as important for LTP and learning^{14,18}.

Alternatively, it may be that in the postnatal period, when the synapses are already formed and the system is not perturbed by toxic agents (as alcohol), the peptides act through a different pathway. It has been shown that their action may reside in the potential to stabilize the cytoskeleton molecule tubulin after zinc intoxication¹⁹. Furthermore, ADNF increases the activity of the transcription factor CREB and of protein kinase A, thus contributing to regulation of transcription²⁰. All these studies were conducted in systems that were perturbed either by a toxic agent or a disease process, and the peptides have shown to prevent some of their sequelae. In addition, the peptides may work through inhibition of oxidative damage. The production of oxygen free radicals resulting in oxidative damage is a known mechanism of

aging neurodegeneration. Previously, the peptides have been shown to prevent alcohol induced alterations in oxidative stress²¹, as assessed by glutathione levels²². This may be a mechanism of the learning enhancement in the aged animals. We have shown that the neurotrophic properties of these peptides are also active in healthy subjects^{1,2} and further studies are needed to elucidate their mechanism of action in this population.

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Appendix to the Methods

For RNA extraction, samples were thawed and immediately homogenized with a sonicator (Janke & Kunkel, Wilmington, NC). The samples were processed with SV Total RNA Isolation System (Promega, Madison, WI). A 5-L aliquot was taken for spectro-photometric determination of RNA content. The remaining sample was stored at -80°C. With the use of 5µg of total RNA, the reverse transcriptase (RT) reaction was performed (Applied Biosystems, Foster City, CA) in a final volume of 150µL. For real-time polymerase chain reaction, the NR2A, NR2B, GABA_Aα5 and GABA_Aβ3 primer pair was designed and synthesized by TIB Molbiol (Adelphia, NJ). The receptor subunits primer sequences were GABA_Aβ3: 5'-CGA GTT GCC CTT GGG ATT AC-3' (sense) and 5'-GAT ATT CCC GTG AGC ATC CAC-3' (antisense); GABA_Aα5: 5'-GGC AGA CAG TAG GCA CTG AG-3' (sense) and 5'-GTC AGC ACT GTG GTC ACT CC-5' (antisense); NR2B: 5'-CCG CAG CAC TAT TAGA GAA CA-3' (sense) and 5'-ATC CAT GTG TAG CCG TAG CC-5' (antisense); NR2A: 5'-GGA AGT TGG ACG CTT TCA TC-3' (sense) and 5'-TCT TCC ATC TCA CCG TCA CC-3' (anti-sense). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard with the primer pair synthesized by Integrated DNA Technologies (Coralville, IA). GAPDH primer sequence was 5'-TGC ACC ACC AAC TGC TTA-3' (sense) and 5'-GGA TGC AGG GAT GAT GTT C-3' (antisense). With the use of the Fast Start DNA Master SYBR green 1 dye-base detection (Roche Diagnostics Corp, Indianapolis, IN), target genes and GAPDH expression were measured by real-time PCR with the Light Cycler with relative quantification software (Roche Diagnostics Corp.) and with melting point analysis to assess the specificity of the amplified genes. Optimization for NR2B, NR2A, GABA_Aα5 and GABA_Aβ3 and GAPDH-specific primers was performed with separate runs by varying magnesium chloride and primer concentrations, the amount of template, and annealing temperature, and optimized conditions were used as previously published^{9,10}.

The presence and purity of target gene sequence expression in the real-time PCR reaction were confirmed by gel electrophoresis. To further eliminate the risk of cross contaminations LightCycler Uracil-DNA Glycosylase (Roche Diagnostic Corp.) was added to the master mix in all the PCR experiments. The final volume of the RT PCR reaction was 20uL.

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Figure 1.

Postnatal treatment with the peptides D-NAP&D-SAL did not result in a significant difference in aged mice brains in NR2A and NR2B expression ($P>0.05$). The bar represents the ratio of NR2A-B/GAPDH mRNA expression $\pm$ SEM.

Figure 2.

Postnatal treatment with the peptides D-NAP&D-SAL did not result in a significant difference in aged mice brains in GABA_Aα5 and GABA_Aβ3 expression ($P>0.05$). The bar represents the ratio of NR2A-B/GAPDH mRNA expression $\pm$ SEM.