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Research Report

Neuroprotective effect of the peptides ADNF-9 and NAP on hypoxic–ischemic brain injury in neonatal rats

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ABSTRACT

Perinatal asphyxia is an important cause of neonatal mortality and subsequent serious sequelae such as motor and cognitive deficits and seizures. Recent studies have demonstrated that short peptides derived from activity-dependent neurotrophic factor (ADNF) and activity-dependent neuroprotective protein (ADNP) are neuroprotective at femtomolar concentrations. However, the effect of these peptides on the hypoxic–ischemic brain injury model is unknown. The aim of this study is to investigate the effects of the peptides ADNF-9 and NAP on neurodegeneration and cerebral nitric oxide (NO) production in a neonatal rat model of hypoxic–ischemic brain injury. Seven-day-old Wistar Albino rat pups have been used in the study ($n=42$). Experimental groups in the study were: sham-operated group, ADNF-9-treated hypoxia–ischemia group, NAP-treated hypoxia–ischemia group, ADNF-9+NAP-treated hypoxia–ischemia group, and vehicle-treated group. In hypoxia–ischemia groups, left common carotid artery was ligated permanently on the seventh postnatal day. Two hours after the procedure, hypoxia (92% nitrogen and 8% oxygen) was applied for 2.5 h. ADNF-9, NAP, and ADNF-9+NAP were injected (intraperitoneally; i.p.) as a single dose immediately after the hypoxia period. Brain nitrite levels, neuronal cell death, and apoptosis were evaluated in both hemispheres (carotid ligated or nonligated) 72 h after the hypoxic–ischemic insult. Histopathological evaluation demonstrated that ADNF-9 and NAP significantly diminished number of "apoptotic cells" in the hippocampal CA1, CA2, CA3, and gyrus dentatus regions in both hemispheres (ligated and nonligated). When compared with vehicle-treated group, combination treatment with ADNF-9+NAP did not significantly reduce "apoptotic cell death" in any of the hemispheres. ADNF-9 and NAP, when administered separately, significantly preserved the number of neurons CA1, CA2, CA3, and dentate gyrus regions of the hippocampus, when compared with vehicle-treated group. The density of the CA1, CA2, and dentate gyrus neurons was significantly higher when combination therapy with ADNF-9+NAP was used in the carotid ligated hemispheres. In the nonligated hemispheres, combination therapy preserved the number of neurons only in the CA1 and dentate gyrus regions. Brain nitrite levels were evaluated by Griess reagent and showed that hypoxic–ischemic injury caused a significant increase in NO production. Brain nitrite levels in ADNF-9+NAP-treated animals were not

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different in carotid ligated or nonligated hemispheres. The peptides ADNF-9 and NAP significantly decreased NO overproduction in the hypoxic–ischemic hemisphere, whereas no significant change appeared in hypoxia alone and also in the sham-operated group. These results suggest the beneficial neuroprotective effect of ADNF-9 and NAP in this model of neonatal hypoxic–ischemic brain injury. To our knowledge, this is the first study that demonstrates a protective effect of these peptides against hypoxia–ischemia in the developing brain.

1. Introduction

Cerebral hypoxia–ischemia is a major cause of perinatal brain injury and is an important cause of neonatal mortality and leading ultimately by neurological sequelae such as cerebral palsy, mental retardation, learning disability, and epilepsy (Berger and Garnier, 1999; Tan et al., 1998; Tomfighi et al., 1997). Despite the fact that perinatal asphyxia closely corresponds to experimental models of cerebral hypoxia–ischemia, where successful neuroprotective interventions were introduced, currently no agent has been proven valuable to improve the chronic sequelae of perinatal asphyxia in the clinical setting (Tomfighi et al., 1997; Vannucci and Perlman, 1997). It is obvious that destructive processes such as glutamate and nitric oxide (NO) neurotoxicity, free radical formation, intracellular calcium accumulation, and immune/inflammatory activation continue to damage the brain for many hours after oxygenation and circulation have been restored (Delivoria-Papadopoulos and Mishra, 1998; Fellman and Raivio, 1997; Higuchi et al., 1998; Tan et al., 1998). Pharmacological agents may provide neuroprotection in this condition interfering with any of these processes.

The most accepted and widely used animal model of perinatal asphyxia is a modification of the Levine preparation described by Rice et al. (Vannucci, 2000), which includes combination of ischemia, obtained by unilateral occlusion of carotid artery, followed by exposure to hypoxia in 7-day-old rats. Neurodevelopmental stage of 7-day-old rats corresponds to that of newborn infants (Romijn et al., 1991; Tomfighi et al., 1997). This animal model represents a useful tool for studying potential neuroprotective strategies capable of preventing or limiting the perinatal hypoxic–ischemic injury in humans.

Two peptides NAP and ADNF-9 are associated with novel glial proteins regulated by vasoactive intestinal peptide and may provide protective intervention in a model of hypoxic–ischemic brain injury in newborn rats. Neuropeptides exhibit multiple roles in the maintenance of homeostasis. They were found to exert neurohormonal and neurotransmitter effects in the central and peripheral nervous system. They also act as regulators of cell division, differentiation, and survival (Gozes et al., 1999; Said, 1996b). VIP is extensively distributed in the nervous system (Gozes et al., 1987). Its neuroprotective properties were first described in spinal cord cell cultures with a maximum effect at concentrations as low as 0.1 nM (Brenneman and Eiden, 1986). It has been shown that some of the neuroprotective action of vasoactive intestinal peptide (VIP) is mediated by two different glial derived proteins: activity-dependent neurotrophic factor (ADNF) (Brenneman and Gozes, 1996) and activity-dependent neuroprotective protein (ADNP) (Bassan et al., 1999). Their active sites have been identified and synthesized as short peptides ADNF-9 (16), a 9-amino acid peptide, and NAP, an 8-amino acid peptide (Bassan et al., 1999). Both ADNF-9 and NAP are protective at femtomolar concentrations in vitro against the neural toxicity of a wide range of compounds and cellular insults (Brenneman et al., 1998; Divinski et al., 2004; Glazner et al., 1999; Gozes and Divinski, 2004; Holtser-Cochav et al., 2006; Zamostiano et al., 1999). The peptides are also neuroprotective in vivo against diverse neuronal insults, including excitotoxicity (Gressens et al., 1997), closed head injury (Beni-Adani et al., 2001), ischemic brain injury (Leker et al., 2002), apoplipoprotein E deficiency (Bassan et al., 1999), exposure to the cholinotoxin ethylcholine aziridium (Gozes et al., 2000), and prenatal ethanol exposure (Spong et al., 2001). Therefore, the aim of this study was to investigate the possible neuroprotective and ameliorating effect of ADNF-9 and NAP treatment immediately after hypoxic–ischemic injury induced neuronal cell death, apoptosis, and NO formation in a neonatal rat model.

2. Results

2.1. *Effects of ADNF-9 and NAP treatment on neuronal density*

Seventy-two hours after hypoxic–ischemic brain injury, treatment with ADNF-9 and NAP significantly preserved the number of neurons CA1, CA2, and CA3 regions of hippocampus and dentate gyrus in the right and left hemispheres when compared with vehicle-treated group ($p<0.05$) (Tables 1–2). The densities of the CA1, CA2, and dentate gyrus neurons were significantly higher in the ADNF-9+NAP combination treatment group when compared with vehicle-treated group ($p<0.05$) in the left hemispheres. The neuronal densities of CA1 and gyrus dentatus were significantly higher than vehicle-treated groups in the right hemispheres in the brains of these animals (Table 3). The neuronal densities of CA3 region in the left hemispheres and CA2 and CA3 regions of the right hemispheres were not significantly different between ADNF-9+NAP and vehicle-treated groups ($p>0.05$) (Table 3) (Fig. 1).

2.2. *Effects of ADNF-9 and NAP treatment on apoptosis*

TUNEL-positive cells showed the typical morphological features of apoptosis such as the chromatin condensation, cytoplasmic budding, and apoptotic bodies. Seventy-two hours after hypoxic–ischemic brain injury, increased number

Table 1 – The effect of systemic ADNF-9 treatment on neuronal density of the hippocampus and apoptotic cell and nitrite levels of newborn rats with hypoxic–ischemic brain injury in the left (ligated) and the right (nonligated) hemispheres

	CA1[a]	CA2[a]	CA3[a]	GD[a]	Apoptotic cell (%)	Nitrite values (μM)[a]
Neuronal density of left hemispheres						
1—Sham ($n=7$)	58.05±1.71	38.21±0.58	26.61±2.00	68.09±1.9	1.14±0.17	7.91±0.99
2—ADNF-9 ($n=7$)	57.64±1.15	36.43±1.87	25.57±1.13	65.52±3.57	1.62±0.22	7.78±0.77
3—Vehicle ($n=7$)	40.57±3.9	31.00±0.81	20.94±1.08	57.14±1.77	9.85±1.05	12.94±4.09
p values						
1 vs 3	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
2 vs 3	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Neuronal density of right hemispheres						
1—Sham ($n=7$)	59.12±0.69	38.74±0.37	27.82±1.04	67.60±1.10	0.90±0.41	7.54±1.03
2—ADNF-9 ($n=7$)	58.85±2.11	38.25±1.89	25.22±0.66	66.28±2.13	1.62±0.16	10.5±3.28
3—Vehicle ($n=7$)	49.10±2.13	34.03±0.65	22.92±0.53	59.57±1.81	3.47±0.49	12.71±4.3
p values						
1 vs 3	0.0001	0.0001	n.s.	0.0001	0.0001	n.s.
2 vs 3	0.0001	0.0001	0.0001	0.0001	0.0001	n.s.

n.s. = not significant.
[a] The values are presented as mean ± SD (min–max).

of cells with fragmented DNA labeled by TUNEL assay were found in the hippocampus (CA1, CA2, CA3, and dentate gyrus) of the vehicle-treated groups, when compared with sham-operated group ($p<0.05$). ADNF-9 and NAP treatments significantly reduced the number of TUNEL-positive neurons in the CA1, CA2, CA3, and dentate gyrus regions of the hippocampus, as compared with the vehicle-treated groups in both hemispheres ($p<0.05$) (Tables 1–2). Combination of the ADNF-9+NAP did not significantly affect apoptotic cell death when compared with vehicle-treated groups in the left and right hemispheres (Table 3). For quantitative measurement of the number of cells that underwent apoptosis, 100 cells were randomly counted in these different areas and the percentage of the apoptotic cells was calculated (Tables 1–3) (Fig. 2).

2.3. *Effects of ADNF-9 and NAP treatment on nitrite levels*

In sham-operated groups, as expected, the mean nitrite levels were not significantly different between the right and left hemispheres in the brains of these animals. ADNF-9 and NAP treatment significantly decreased the mean nitrite level on the ligated side when compared ($p<0.05$) with the mean value of vehicle-treated animals, whereas no significant NO change was recorded in the nonligated hemisphere (Tables 1–2), consistent with our previous report (Kumral et al., 2004a).

Table 2 – The effect of systemic NAP treatment on neuronal density of the hippocampus and apoptotic cell and nitrite levels of newborn rats with hypoxic–ischemic brain injury in the left (ligated) and the right (nonligated) hemispheres

	CA1[a]	CA2[a]	CA3[a]	GD[a]	Apoptotic cell (%)	Nitrite values (μM)[a]
Neuronal density of left hemispheres						
1—Sham ($n=7$)	58.05±1.71	38.21±0.58	26.61±2.00	68.09±1.9	1.14±0.17	7.91±0.99
2—NAP ($n=7$)	57.14±0.37	37.50±1.32	26.14±1.06	64.85±3.28	1.37±0.52	9.11±1.57
3—Vehicle ($n=7$)	41.00±0.95	32.35±0.73	19.82±0.98	54.28±1.25	7.09±0.31	17.91±3.50
p values						
1 vs 3	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
2 vs 3	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Neuronal density of right hemispheres						
1—Sham ($n=7$)	59.12±0.69	38.74±0.37	27.82±1.04	67.60±1.10	0.90±0.41	7.54±1.03
2—NAP ($n=7$)	58.96±1.25	36.85±1.77	27.78±1.95	67.50±1.65	1.13±0.76	17.22±3.29
3—Vehicle ($n=7$)	48.00±0.95	34.00±0.70	22.65±0.42	57.78±1.04	3.40±0.60	17.84±3.06
p values						
1 vs 3	0.0001	0.0001	n.s.	0.0001	0.0001	0.0001
2 vs 3	0.0001	0.006	0.0001	0.0001	0.0001	n.s.

n.s. = not significant.
[a] The values are presented as mean ± SD (min–max).

Table 3 – The effect of systemic ADNF-9+NAP treatment on neuronal density of the hippocampus and apoptotic cell and nitrite levels of newborn rats with hypoxic–ischemic brain injury in the left (ligated) and the right (nonligated) hemispheres

	CA1[a]	CA2[a]	CA3[a]	GD[a]	Apoptotic cell (%)	Nitrite values (μM)[a]
Neuronal density of left hemispheres						
1—Sham ($n=7$)	58.05±1.71	38.21±0.58	26.61±2.00	68.09±1.9	1.14±0.17	7.91±0.99
2—ADNF-9 + NAP ($n=7$)	45.42±2.29	34.57±0.78	21.85±1.90	62.42±0.78	6.90±1.09	16.04±1.59
3—Vehicle ($n=7$)	41.00±0.95	32.35±0.73	19.82±0.98	54.28±1.25	7.09±0.31	17.91±3.5
p values						
1 vs 3	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
2 vs 3	0.006	0.008	n.s.	0.0001	n.s.	n.s.
Neuronal density of right hemispheres						
1—Sham ($n=7$)	59.12±0.69	38.74±0.37	27.82±1.04	67.60±1.10	0.90±0.41	7.54±1.03
2—ADNF-9+NAP ($n=7$)	51.71±2.69	35.70±1.96	23.78±1.14	64.98±0.58	3.94±0.98	16.88±2.88
3—Vehicle ($n=7$)	48.00±0.95	34.00±0.70	22.65±0.42	57.78±1.04	3.40±0.60	17.84±3.06
p values						
1 vs 3	0.0001	0.0001	n.s.	0.0001	0.0001	0.0001
2 vs 3	0.0001	n.s.	n.s.	0.001	n.s	n.s.

n.s. = not significant.
[a] The values are presented as mean±SD (min–max).

In contrast, the mean nitrite levels were not significantly different when compared with vehicle-treated groups values ($p>0.05$) on the ligated and nonligated side of the combination of ADNF-9+NAP.

3. Discussion

In this study, we focused on systemic administration of two peptides, ADNF-9 and NAP, and demonstrated that these two peptides can prevent neuronal cell death, apoptosis, and cerebral NO production in a newborn rat model of hypoxic–ischemic brain injury. The present data indicate that these peptides are effective neuroprotective agents in this particular animal model when administrated consequently after exposure to hypoxic–ischemic insult. The neuroprotection shown in this model indicates possible therapeutic implications in humans. Since the treatment of human perinatal asphyxia should be performed immediately after the insult, we investigated the neuroprotective potency of two peptides, ADNF-9 and NAP, in rat pups with hypoxic–ischemic brain injury. This study, for the first time, shows that a single dose of ADNF-9 and NAP administration provides neuroprotection in the neonatal rat brain immediately after the hypoxic–ischemic brain injury. Furthermore, interestingly we demonstrated that combination therapy with NAP and ADNF-9 was less effective than single therapies of NAP or ADNF-9.

Vasoactive intestinal peptide (VIP) is a major brain neuropeptide, and original studies have demonstrated that it is associated with brain development in mice and rats and with the maintenance of neurons (Sari and Gozes, 2006). The neuroprotective action of VIP is mediated through two different glial derived proteins: ADNF (Glazner et al., 1999) and ADNP (Bassan et al., 1999). Their active sites have been identified and synthesized as the short peptides ADNF-9 (Brenneman et al., 1998), a 9-amino acid peptide, and NAP, an 8-amino acid peptide (Bassan et al., 1999). Remarkably, both peptides act at concentrations in the femtomolar range (Glazner et al., 1999; Offen et al., 2000). The protective effects of ADNF-9 and NAP had not been investigated in the neonatal rat model of hypoxic–ischemic brain injury yet.

We have previously shown that neonatal hypoxic–ischemic insult caused hippocampal CA1 neuronal injury, spatial memory deficit, NO overproduction and apoptosis (Beni-Adani et al., 2001; Kumral et al., 2004a,b). Hypoxic–ischemic brain injury is a serious cause of death and disability in the human newborn. The developmental stage of the brain of the 7-day-old rat pup resembles that of newborn humans (Palmer et al., 1990). The Rice–Vannucci–Brierley rat pup hypoxia–ischemia (HI) model (Rice et al., 1981) therefore may best match the injury caused by birth asphyxia in full-term human infants. The neonatal rat HI model has been well characterized and extensively used to assess synthetic neuroprotective agents (Hagberg et al., 1994; Kumral et al., 2003; Palmer et al., 1990). In the present study, ADNF-9 and NAP treatment rescued neurons in the CA1, CA2, CA3, and gyrus dentatus regions in this model of hypoxic–ischemic brain injury. And combination therapy significantly increased neuronal density in only CA1, CA2, and gyrus dentatus regions in the carotid ligated hemispheres and CA1 and dentate gyrus regions in the nonligated hemispheres. Previously, both NAP and ADNF-9 have been shown to promote axonal elongation in primary rat hippocampal and cortical cultures (Smith-Swintosky et al., 2005), and ADNF-9 was shown to promote synapse formation (Blondel et al., 2000). Interestingly, in this HI model, we showed that combined therapy of NAP and NADF-9 did not act synergistically. This result may be explained by distinct mechanisms of action in this animal model of NAP and ADNF-9 through complementary, but different mechanisms. In addition, pharmacological distinction between these two peptides may lower this synergy. Furthermore, the dosage of these two peptides in this study may eliminate the synergy and lower doses may be effective.

Apoptosis contributes significantly to cerebral damage in the perinatal period. Infants who die after intrauterine insults have a significant number of cells in the brain with the

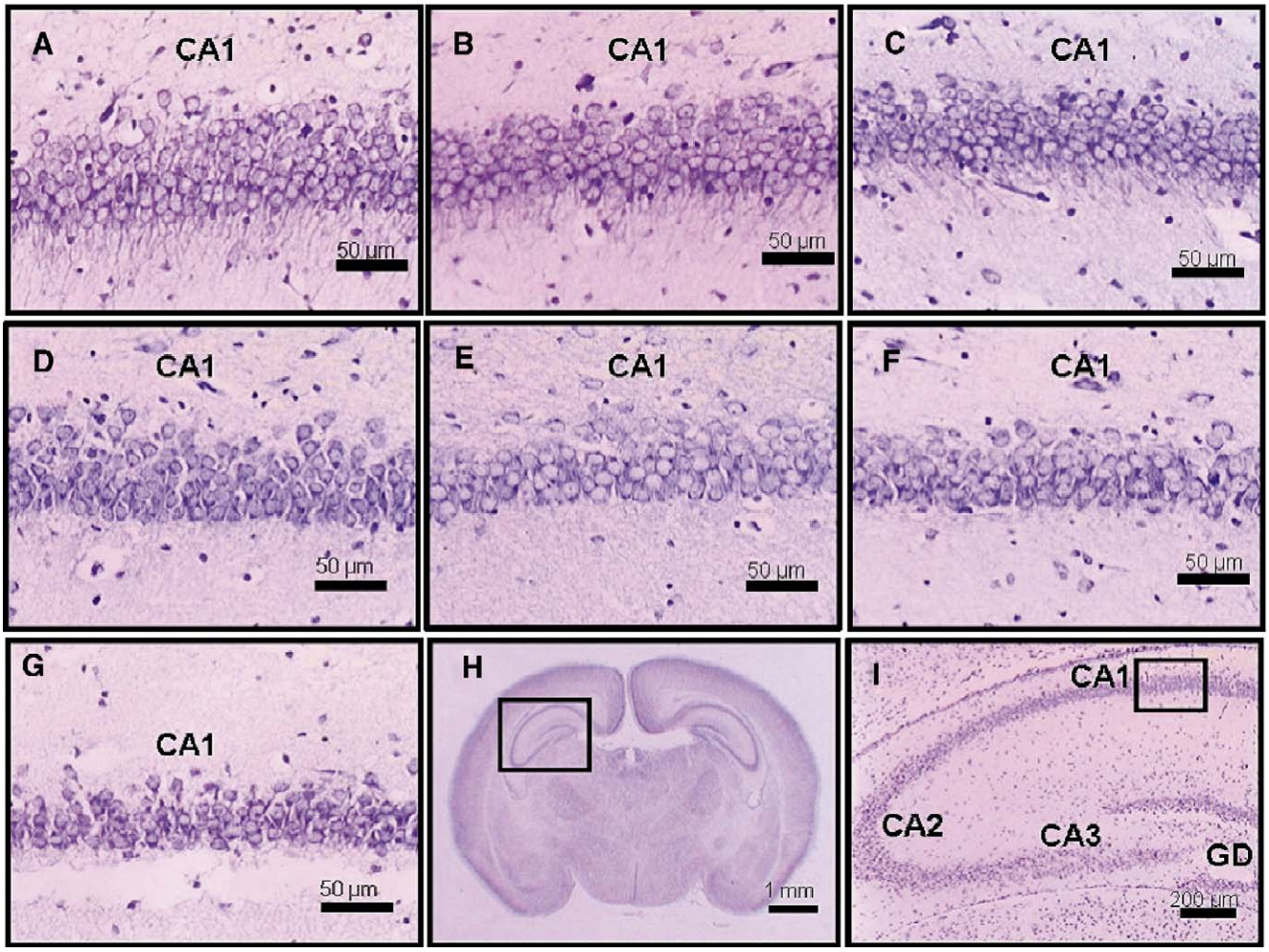

Fig. 1 – Effect of ADNF-9 and NAP treatment on the neuronal density in the CA1 region of hippocampus. Representative pictures obtained by cresyl violet staining. Lower (H) and higher magnifications (I) of coronal section from CA1 region of hippocampus of neonatal rat brain tissues from sham-operated group. The ADNF-9 (B), NAP (C), and combination of ADNF-9 + NAP (F) groups significantly preserved number of neurons of hippocampal CA1 region as compared with vehicle-treated rat pups; ADNF-9 (D), NAP (E), and combination of ADNF-9 + NAP (G). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

morphologic characteristics of apoptosis (Edwards et al., 1997). In the neonatal hypoxic–ischemic model, there is evidence that cerebral ischemia leads to delayed cell death with DNA damage (Pulera et al., 1998). We investigated DNA fragmentation in this rat model of HI brain damage using TUNEL staining by randomly selected cells. Only few cells with fragmented DNA labeled by TUNEL assay were found in CA1, CA2, CA3, and gyrus dentatus of the sham-operated rat pups. Seventy-two hours after hypoxic–ischemic brain injury, an increase in the number of TUNEL-positive cells was observed. In the brains of ADNF-9 and NAP-treated rat pups, apoptotic cell counts significantly decreased. The precise mechanism by which NAP and ADNF-9 produce neuroprotection is not clear, but some relations with antiapoptotic pathways have been described. ADNF-9 increases the levels of hsp60 in rat cortical neurons (Zamostiano et al., 1999). It enhances nuclear factor κB-DNA binding (Glazner et al., 2000) and ADNF-14, a larger variant of ADNF-9, activates protein kinase C and mitogen-activated protein kinase (Gressens et al., 1999). NAP protects against apoptosis in vivo in a rat model of stroke as measured by the number of caspase 3-positive cells and fragmented DNA staining (Leker et al., 2002). NAP has been further shown to act as a peptide chaperone protecting against toxic protein aggregation (Ashur-Fabian et al., 2003). NAP furthermore reduces the level of p53, a key regulator of cellular apoptosis (Gozes et al., 2004). ADNP has been found to be essential for brain formation during embryogenesis as ADNP-knockout mice exhibited defective neuronal tube closure and a lack of PAX6-gene expression (Pinhasov et al., 2003). Our results showed that the combination of ADNF-9 and NAP did not significantly reduce apoptotic cell death in both hemispheres. There may be three possibilities: this result may be related with (1) the dosages of two peptides, (2) the elimination of effectiveness on the apoptotic mechanisms when using these agents together, or (3) this experimental model is not a proper model for determination the effectiveness of the combination

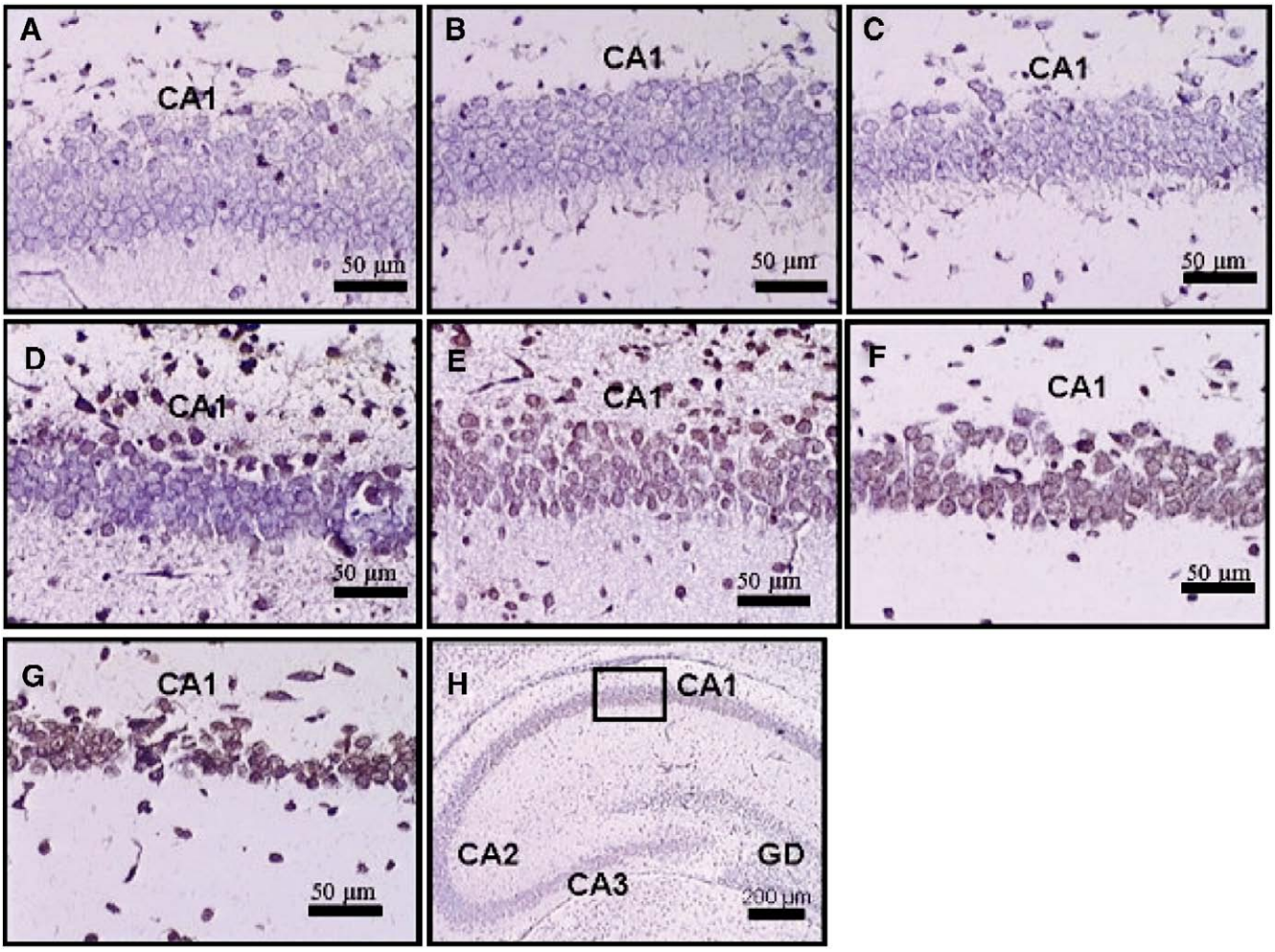

Fig. 2 – Effect of ADNF-9 and NAP treatment on the TUNEL immunoreactivity in hippocampus. Lower (H) and higher magnifications (A) of coronal section from CA1 region of hippocampus of neonatal rat brain tissues from sham-operated group, stained with cresyl violet. Panels D, E, and G represent nuclear fragmentation which is characteristic for apoptosis from control group. TUNEL-positive cells were significantly decreased treatment with ADNF-9 (B), NAP (C), when compared with control groups; ADNF-9 vehicle (D), NAP vehicle (E). Combination of ADNF-9+NAP (F) groups not significantly decreased apoptosis compared with combination of ADNF-9+NAP vehicle (G). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

therapy taking into consideration that cell density did not lead to these results.

In the present study, ADNF-9 and NAP treatment significantly reduced NO formation particularly in the "hypoxic–ischemic" hemisphere of the brain in neonatal rats. The increase in NO formation has been implicated in the pathogenesis of hypoxic–ischemic brain damage in neonatal rats (Higuchi et al., 1998; Kumral et al., 2004a; Tsuji et al., 2000). NO synthase (NOS) inhibitors and some agents that inhibit NO formation in vivo have been shown to be useful in the treatment of hypoxic and ischemic brain injury (Higuchi et al., 1998; Ishida et al., 2001; Tsuji et al., 2000). VIP-associated activities are mediated, in part, by increased or decreased nitric oxide (NO) production (Said, 1996a). NO has an important role in neurotransmission and neurotoxicity (Kriegsfeld et al., 1999; Kugler and Drenckhahn, 1996; Leker et al., 2002; Lipton, 1999). The results of the present study indicated that the two neuropeptides were able to influence NO synthesis, probably by reducing the increase arising from the inducible form of NOS (iNOS) produced following ischemia (Gozes et al., 2000; Kumral et al., 2004a). Two peaks of NO production have been observed on the damaged side of the cortex in the neonatal rat, one during hypoxic exposure and the other during the late period beginning after 6 h (Tsuji et al., 2000). The second peak in NO metabolites is thought to be introduced mainly by iNOS as supported by the finding that selective inhibitor of iNOS suppresses this prolonged peak (Tsuji et al., 2000). The time point that was selected in the present study (72 h after insult) suggests that ADNF-9 and NAP inhibit NO production that is mediated by iNOS. According to the results of our experiments, the two neuropeptides had no inhibitory effect on NO response against hypoxia alone, whereas it had a significant inhibitory effect on NO response under hypoxic–ischemic conditions. In our study, we found that combination of two peptides was not more effective than vehicle.

This is also the first in vivo demonstration of the selective inhibitory effect of ADNF-9 and NAP on NO overproduction in hypoxic–ischemic brain injury of newborn rats. We expect to execute further studies to explore the possible effects of ADNF-9 and NAP on NO in hypoxia–ischemia.

In conclusion, hypoxic–ischemic injury to the CNS can have devastating lifelong effects to the developing fetus or to the neonate. our results suggest that the treatment with ADNF-9 and NAP is associated with neuronal density of hippocampus, decreased apoptotic cell index, and decreased NO overproduction in this animal model. To our knowledge, this study is the first in vivo demonstration that ADNF-9 and NAP can protect the developing brain against the deleterious effects of a hypoxic–ischemic insult, when using alone rather than their combination. Further studies investigating the antiapoptotic and antioxidant mechanisms of ADNF-9 and NAP clinical trials in patients with hypoxic–ischemic brain injury will provide additional data about the potential use of the two neuropeptides in prevention of this disease.

4. Experimental procedures

4.1. *Animals*

This study was performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylul University, School of Medicine. Wistar rats with dated pregnancies were maintained at the same center and housed in individual cages with free access to water and laboratory chow. Forty-nine offspring delivered spontaneously were reared with their dams until the time of initial experimentation at 7 days of postnatal age (P7; day of birth is day 1).

4.2. *Induction of hypoxic–ischemic brain injury and treatment groups*

A modification of Levine preparation was used as a model for perinatal hypoxic–ischemic brain injury as previously described (Kumral et al., 2003; Rice et al., 1981; Vannucci and Vannucci, 1997). Forty-two pups underwent permanent unilateral carotid ligation. The midline of the neck was incised at the longitudinal plane under halothane anesthesia. The left carotid artery was permanently ligated with 5-0 surgical silk. Total time of surgery never exceeded 5 min. Animals were excluded from the study if there was bleeding during ligation or respiratory arrest resulting from anesthesia. Following a 2 h period of recovery and feeding, the animals were exposed to a 2.5 h period of hypoxia (92% N_2, 8% O_2) by placing them in airtight containers partially submerged in a 37 °C water bath to maintain a constant thermal environment.

The peptides ADNF-9 and NAP were purchased from Peptide Technologies Corporation (Gaithersburg, MD) and Sigma Genosys (Woodlands, TX). Purity (>95%) and identity were assessed by the company using high-performance liquid chromatography and mass spectrometry analyses. ADNF-9 was dissolved and diluted in filtered DPBS. NAP was dissolved in 10% dimethyl sulfoxide in phosphate-buffered saline (PBS; 0.13 M NaCl, 0.003 M KCI, 0.01 M Na_2HPO_4, and 0.002 M KH_2PO_4) and stored as 1 mM aliquots. NAP was stable in solution and could be aliquoted and frozen for later use without loss of activity (Wilkemeyer et al., 2003). Final peptide solution was freshly prepared by stock peptide solution diluted 100-fold in distilled sterile phosphate-buffered saline followed by further serial dilution in distilled sterile phosphate-buffered saline (Zusev and Gozes, 2004). The rat pups were received an intraperitoneal injection of peptides (NAP, ADNF-9, or NAP + ADNF-9, $n=7$, each groups) at a dose of 0.3 μ/g of body weight (0.1 ml) just after the retrieval from the hypoxia chamber. Sham-operated pups underwent the same surgical procedure with neither ligation of the carotid artery nor hypoxia (sham-operated group, $n=7$). After this procedure, the pups were returned to their dam for 72 h until sacrifice.

4.3. *Histopathological evaluation*

All histopathological analyses described below were performed by an investigator blind to rat's treatment. For histological examination, 72 hours after hypoxia the animals in each group were perfused by 10% formalin. Brain tissues were removed and fixed in 10% formalin in phosphate buffer for 3 days. The brains were sectioned coronally into sequential 6 μm slices using a rat brain slicer. Brain sections were taken from each subject according to the Paxinos and Watson coordinates at the coronal plate (Paxinos and Watson, 2002). For each brain, samples were taken from areas far from bregma −2.2 mm, −2.84 mm, and −3.43 mm that were seen at hippocampus CA1, CA2, CA3, and GD regions.

4.4. *Estimation of hippocampus neuron density*

Each sample was subjected to the estimation of hippocampus CA1, CA2, CA3, and dentate gyrus neuron density. Three sections on average from each brain were selected according to the regions spanning from bregma −2.2 mm, −2.84 mm, and −3.43 mm that were seen at hippocampus CA1, CA2, CA3, and GD regions. The images were analyzed by using a computer-assisted image analyzer system consisting of a microscope (Olympus BH-2 Tokyo, Japan) equipped with high-resolution video camera (JVC TK 890E, Japan). The numbers of CA1, CA2, CA3, and dentate gyrus were counted by the help of a 15,000 μm^2 counting frame viewed through a 20× Nikon lens at the monitor. The counting frame was placed randomly five times on the image analyzer system monitor, and the neuron numbers of CA1, CA2, CA3, and dentate gyrus regions of hippocampus were counted (UTHSCA Image Tool for windows version 3.0 software) and the average was taken. All counting and measurement procedures were performed blindly. The neuron numbers of CA1, CA2, and CA3 and gyrus dentatus regions of the hippocampus were calculated separately for the right and left hemispheres (Kumral et al., 2004b).

4.5. *In situ cell death detection*

To detect DNA fragmentation in cell nuclei, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction was applied to the paraffin sections by

using a commercial kit (Promega, USA). After deparaffinization, the sections were treated with 20 μg/ml proteinase K for 10 min, with 0. 3% H_2O_2 in methanol for 10 min and 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Then, the sections were incubated with TUNEL reaction mixture for 60 min at 37 °C. Further incubation with peroxidase-conjugated antibody was performed for 30 min at 37 °C. The sections were stained with diaminobenzidine solution for 10 min at room temperature and then counterstained with mayers hematoxylin. For quantitative analysis of TUNEL-positive cells in hippocampus, cells exhibiting apoptotic features (condensed cytoplasm and chromatin, intense TUNEL reactivity and a rounded cell body) were counted in CA1, CA2, CA3, and dentate gyrus regions using a Olympus BH-2 Tokyo microscope at ×200 magnification connected to a 14-in. monitor. For establishing apoptotic index, 1000 cells were counted in CA1, CA2, CA3, and dentate gyrus regions of hippocampus. Cells showing apoptotic morphology were given as percentile.

4.6. *Measurement of nitrite levels*

The nitrite levels of the brain samples were measured by previously described methods (Calapai et al., 2000; Higuchi et al., 1998). Briefly, following sacrifice of the animals, the brains were dissected into right and left hemispheres and transported to the laboratory on an ice-cooled plate. After addition of buffer (0.1 M, pH 7.5, potassium phosphate buffer (PBS), 20 mM EDTA (1:10; w/v)), the brain hemispheres were homogenized by an ultrasound homogenizer (Bandelin Electronics, Berlin, Germany) on ice. Then, left and right hemisphere supernatants were obtained through centrifugation (14,000 rpm for 10 min, then 20 min at +4 °C). Following centrifugation, the supernatants were assayed for nitrite contents. Nitrite is generated by the rapid oxidation of NO. To assay nitrite, we used a modification of a previously published method (Green et al., 1982; Kumral et al., 2004b; Baskin et al., 1997). Right (hypoxic-nonligated; $n=7$) and left (hypoxic-ligated; $n=7$) hemisphere samples and control samples ($n=7$) were analyzed. Aliquots of 100 ml were mixed with 100 ml of equal volumes of a Griess reagent (A: naphthylethylenediamine dihydrochloride; B: sulfanilamide in o-phosphoric acid) mixture in a 96-well microtiter plate (Maxisorb Immunoplate, NUNC). After 10 min of incubation at room temperature, the absorbance at a wavelength of 540 nm was measured in a microplate reader (Model 230S; Organon Technica). A range of 2-fold dilutions of sodium nitrite (0–128 μM) in PBS were run in each assay to generate a standard curve.

4.7. *Statistical analysis*

All data regarding the NO levels are expressed as the mean ± standard deviation (SD). Statistical comparison between treated, sham, and vehicle groups (neuron density, mean NO levels, and apoptotic index from the right and left hemispheres of the animals in all experimental groups) were performed by one-way ANOVA and Tukey's test to determine significant differences between the groups (SPSS 11.0, Chicago, IL, USA). A probability level (p) of 0.05 or less was chosen to represent statistical significance.

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