

Protective Peptides that are Orally Active and Mechanistically Non-Chiral

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## Running Title Page

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**Abstract:** Previous reports identified two peptides that mimic the action of neuroprotective proteins derived from astrocytes. These peptides, NAPVSIPQ and SALLRSIPA prevent neuronal cell death produced by electrical blockade, N-methyl-D-aspartate and beta amyloid peptide (25-35). In the present study, all D-amino acid peptides of NAPVSIPQ and SALLRSIPA were synthesized and compared respectively to the corresponding all L-amino acid peptides. In rat cerebral cortical test cultures co-treated with 1  $\mu$ M tetrodotoxin, the D-amino acid peptides produced similar potency and efficacy for neuroprotection as that observed for their respective L-amino acid peptides. Since all these peptides tested individually exhibited attenuation of efficacy at concentrations > 10 pM, combinations of these peptides were tested for possible synergies. Equimolar D-NAPVSIPQ and D-SALLRSIPA combination treatment produced potent ( $EC_{50}$ : 0.03 fM) neuroprotection that did not attenuate with increasing concentrations. Similarly, the combination of L-NAPVSIPQ and D-SALLRSIPA also had high potency ( $EC_{50}$ : 0.07 fM) without attenuation of efficacy. Combined administration of peptides was tested in a model of fetal alcohol syndrome and in a model of learning impairment: apolipoprotein E knockout mice. Intraperitoneal administration of D-NAPVSIPQ plus D-SALLRSIPA to pregnant mice (E8) attenuated fetal demise after treatment with an acute high dose of alcohol. Furthermore, oral administration of D-NAPVSIPQ plus D-SALLRSIPA significantly increased fetal survival after maternal alcohol treatment. Apolipoprotein E knockout mice injected with D-NAPVSIPQ plus D-SALLRSIPA showed improved performance in the Morris water maze. These studies suggest therapeutic potential for the combined administration of neuroprotective peptides that can act through a mechanism independent of chiral recognition.

The chirality requirements for the neuroprotective action of two peptides have been examined: NAPVSIPQ and SALLRSIPA. These peptides were discovered while investigating two glial proteins: activity dependent neuroprotective protein (ADNP) (Bassan et al., 1999) and activity dependent neurotrophic factor (ADNF) (Brenneman and Gozes, 1996). Both of these glial proteins are neuroprotective and regulated by vasoactive intestinal peptide (VIP), a polypeptide shown to have growth-promoting and neurotrophic properties *in vitro* and *in vivo* (Brenneman and Eiden, 1986; Gressens et al., 1993). NAPVSIPQ and SALLRSIPA are synthesized peptides that exhibited neuroprotection at femtomolar concentrations (Gozes and Brenneman, 2000). Previous structure activity-studies with either an alanine scan (Wilkemeyer et al., 2003) or conservative substitutions (Brenneman et al., 1998) have shown complete loss of neuroprotective activity with replacement of single amino acids. In cerebral cortical cell cultures, these peptides have been shown to prevent neuronal cell death associated with tetrodotoxin (electrical blockade), the beta-amyloid peptide (the Alzheimer's disease neurotoxin), N-methyl-D-aspartate (excitotoxicity) and gp120, the human immune deficiency virus envelope protein (Brenneman et al., 1998; Bassan et al., 1999). In addition, NAPVSIPQ and SALLRSIPA have been shown to provide protection from oxidative stress in pheochromocytoma and neuroblastoma cell lines (Steingart et al., 2000; Offen et al., 2000). Recent studies have shown that these peptides can be protective as antagonists of ethanol inhibition of L1-mediated cell-cell adhesion (Wilkemeyer et al., 2002). Furthermore, these peptides have been shown to be protective *in vivo*. For example, daily injections of SALLRSIPA or NAPVSIPQ to newborn apolipoprotein E-deficient mice accelerated the acquisition of developmental

reflexes and, in the case of NAPVSIPQ, prevented short-term memory deficits (Bassan et al., 1999). Intranasal administration of NAPVSIPQ or SALLRSIPA promoted learning and memory (Gozes et al., 2000 and 2002), subcutaneous injection of NAPVSIPQ protected against edema formation, behavioral deficits and death in a model of closed head injury (Beni-Adani et al., 2001). Intravenous injection of NAPVSIPQ after mid cerebral artery occlusion (a model for stroke in rodents), resulted in protection against apoptosis, smaller infarct size and behavioral improvements (Leker et al., 2002).

Furthermore, pretreatment with an equimolar mixture of SALLRSIPA and NAPVSIPQ resulted in protection against fetal demise and growth retardation associated with fetal alcohol syndrome (Spong et al., 2001). In the present study, the neuroprotective action of NAPVSIPQ and SALLRSIPA in either the D or the L-enantiomer conformation was investigated in cerebral cortical cultures and in various models of neurodegeneration and growth retardation. The results suggest that D- enantiomers of NAPVSIPQ and SALLRSIPA maintain femtomolar-acting neuroprotective activity *in vitro* and further show that combined treatment with these peptides *in vivo* has significant protective actions against severe oxidative stress and neurodegeneration. Because NAPVSIPQ and SALLRSIPA maintain their biological efficacy in the D-form, studies were performed that demonstrate for the first time that these peptides can elicit protection after oral administration.

The current studies have first utilized neuroprotection against tetrodotoxin as an *in vitro* screen to compare the D- and L- forms of NAPVSIPQ and SALLRSIPA. Electrical blockade was chosen as the model system to make these comparisons because of its relevance to activity-dependent mechanism of development as well as its first use in

the detecting and characterizing the action of both activity dependent neurotrophic factor and activity dependent neuroprotective protein, the parent proteins for SALLRSIPA and NAPSVIPQ, respectively. The choices of in vivo models was on the basis of precedent with previous studies that utilized a mouse model of fetal alcohol syndrome (Spong et al., 2001) and the apo lipoprotein E knockout mice (Bassan et al., 1999) that had been shown to be responsive to the L-forms of at least one of these peptides. Therefore the choice of experimental systems was based primarily on preceding studies; however, both the in vitro and in vivo systems share a common link with induced apoptosis. Combinations of the peptides are emphasized in the present study because the previous work with the fetal alcohol model indicated that both L-peptides were required for maximum protection from both fetal demise and growth restriction.

## Methods

### Peptide preparation

Peptides were obtained from SynPep (Dublin, CA). All peptides were > 97 % pure as determined by reverse phase chromatography and capillary electrophoresis. Peptide mass was confirmed by mass spectrometry. The primary sequence of the ADNP-derived NAPVSIPQ is: Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln or an all D-amino acid derivative (D-NAPVSIPQ). The ADNF-like peptide was Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (L-SALLRSIPA), or the all D-amino acids peptide (D-SALLRSIPA).

### Cell culture

Rat cerebral cortical cell cultures were prepared by previously described methods (Hill et al., 1993). Briefly, dissociated cerebral cortical tissue was seeded on a confluent layer of astrocytes previously prepared from newborn rat brain cerebral cortex. Twenty-four hours later, fluorodeoxyuridine (15µg/ml) was added to the cultures. Cultures were maintained in 5% horse serum in MEM supplemented with defined media components as previously described (Romijn et al., 1984). After four days growth *in vitro*, the cultures were given a complete change of medium and treated once with peptide dissolved in phosphate buffered saline. The duration of the treatment period was four days. To assess neuroprotection, cultures were co-treated with 1 µM tetrodotoxin (TTX), a model of apoptotic death that is relevant to neurons dependent on electrical activity for their survival (Brenneman et al., 1983). To terminate, the cells were fixed in 2.5% paraformaldehyde for immunocytochemistry and neurons identified with antibodies against neuron specific enolase by previously described methods (Schmechel et al.,

1978). Neuronal cell counts were performed on 30 fields (200 X magnification) without knowledge of treatment.

### **Animal models:**

#### **Fetal alcohol syndrome (FAS) in mice**

A well-established model for FAS was used to test the efficacy of the peptides in mice (Webster et al., 1980). This paradigm is a test for efficacy against severe oxidative stress produced from alcohol administration (Spong et al., 2001). The model was chosen in that it allowed for a rapid and relevant evaluation of agents efficacious against severe oxidative stress as well as its relevancy to FAS. To assess the protective effects of the peptides, the number of fetal demises was determined. The animals were sacrificed at E18 (10 days after peptide/alcohol treatment). The number of living fetuses, fetal demises and total litter size (living fetuses + demises) was determined. This allowed for the documentation that all litters began with the same number. In the surviving E18 fetuses, fetal and fetal brain weights were evaluated to assess the protective effects of the peptides. C57-B16J female mice were kept under a 12h light, 12h dark regimen with food and water available at all times. The mice received humane animal care in compliance with the "Guideline for Care and Use of Experimental Animals." Six-week-old females (21-24 grams) were mated with C57-B16J males for 4h. The presence of a vaginal plug was considered day 0 of pregnancy. Mice were intraperitoneally injected with 25% ethyl alcohol in saline (v/v) or vehicle alone at 0.03 ml/kg maternal body weight at 0900 on E8. Pretreatment with the study peptides was given 30 minutes prior to alcohol as described. D-NAPVSIPQ or L-NAPVSIPQ (0.5mg) was dissolved in 50  $\mu$ l DMSO and diluted with filtered Dulbecco's phosphate buffered saline (DPBS) to a final volume of 5 ml (concentration 100 $\mu$ g/ml); of this 0.2 ml (200 $\mu$ l) was given per animal. D-SALLRSIPA (1.0mg) was dissolved in 1 ml of DPBS and diluted in filtered DPBS to a final volume of



10 ml (concentration 100 $\mu$ g/ml); of this solution, 0.2 ml (200 $\mu$ l) was administered for the 20 $\mu$ g dose. This solution was further diluted 1:10 in DPBS for the 2 $\mu$ g dose.

Dosages of the peptides were D-NAPVSIPQ (20 $\mu$ g), D-SALLRSIPA (20 $\mu$ g or 2 $\mu$ g), NAPVSIPQ (20 $\mu$ g) + D-SALLRSIPA (20 $\mu$ g). Since the animals receiving alcohol were incapacitated for approximately 6h following injection, food and water were withheld from all groups for the initial 6 hours after injection, to allow accurate assessment of fetal weights. The mean pup weight for each litter was calculated, with the litter mean used for all statistical analysis. Percent demises were calculated by dividing the number of demises by the total number of fetuses (live plus demises). Statistical analysis included ANOVA for continuous variables, Mann-Whitney U for nonparametric data, Chi-square for categorical variables or Fisher's exact test where appropriate with  $p < 0.05$  considered significant.

#### Apolipoprotein E knockout mice: behavior assay

Inheritance of apolipoprotein E4 is a major risk factor in Alzheimer's disease (Corder et al., 1993). These studies, along with the investigations of apolipoprotein E-deficient animals indicated that an apolipoprotein E functioning system is required for repair functions (Ignatius et al., 1986). Memory deficits and cholinergic impairments have been described in adult apolipoprotein E -deficient mice. Furthermore, developing apolipoprotein E -deficient mice have been shown to be cognitively impaired (Gordon et al., 1995; Gozes et al., 1997). Peptides (0.5 mg each) were dissolved in 0.01M acetic acid (30 microliters) and further diluted with saline. 0.5 microgram of each of the test drugs (D-NAPVSIPQ or D-SALLRSIPA) were daily injected in 20 microliters (subcutaneous)/mouse. This protocol was used for the first 4 days of life. From day 5-10, the amount of the peptides and the solution volume was doubled. From day 11-14, the

amount of peptide injected was 2  $\mu\text{g}$  each in 80  $\mu\text{l}$  saline/mouse. A week after cessation of peptide injection, the animals were subjected to the Morris water maze as previously described (Bassan et al., 1999). In brief, mice were subjected to two daily tests in a water maze, including a hidden platform. The experiment was conducted over a four-day period. Every day for the first test, both the platform and the animal were situated in a new location with regards to the pool (with the pool being immobile). The experiment was performed as follows: the animal was positioned on the platform for 0.5 min and then placed in the water and allowed to find the platform for 90 seconds. After finding the platform or being placed on the platform for an additional 0.5 min, the animal was placed back in the water (in the previous position) for a second daily test and searched again for the hidden platform (retained in the previous position). The time required to reach the platform in the second trial was recorded, indicative of short-term (working) memory. Significance was assessed by one way ANOVA with Student Neuman-Kuels multiple comparison of means test.

## Results

### In vitro neuroprotection

As shown in figure 1, the D- and L-forms of SALLRSIPA were similar in both potency and efficacy in preventing neuronal cell death associated with electrical blockade with TTX. The  $EC_{50}$  for L-SALLRSIPA was 0.03 fM and that of D-SALLRSIPA 0.07 fM. The maximum efficacies produced by each peptide were not significantly different from each other. The survival-promoting activity of each peptide attenuated significantly at concentration  $\geq 10$  pM ( $p < 0.05$ ). Likewise, the D- and L-forms of NAPVSIPQ were very similar, each exhibiting a complex dose response with two apparent maxima (figure 2). The  $EC_{50}$ 's for L-NAPVSIPQ were 0.01 fM and 2 pM and for D-NAPVSIPQ: 0.07 fM and 2 pM. Efficacies at the higher potency peak were not significantly different between the peptides, and the maximum efficacy of the D-peptides differed by only 10%.

Combinations of peptides were also tested. For these experiments, the two peptides were given in equimolar amounts of each peptide at the amount indicated in each figure. In figure 3, the effect of D-NAPVSIPQ plus D-SALLRSIPA was shown to produce a different dose response from that observed with either agent alone. Significantly, there was no attenuation of the survival-promoting activity at higher concentrations of the combined D-peptides. The  $EC_{50}$  was very potent at 0.03 fM and the maximal efficacy was evident for 7 orders of magnitude. The number of surviving neurons after D-NAPVSIPQ + D-SALLRSIPA treatment ( $\geq 0.1$  fM) was not different from that of control cultures. Thus, a broader therapeutic range of effective concentrations was apparent when the D- peptides were used in combination. Similar

experiments conducted with both L-SALLRSIPA and L-NAPVSIPQ resulted in >60% loss of maximum efficacy with no change in potency in comparison to that observed after treatment with D-NAPVSIPQ + D-SALLRSIPA.

Another series of experiments was conducted to show the effect of combining L- and D- forms of NAPVSIPQ and SALLRSIPA. As shown in figure 4, the treatment with L-NAPVSIPQ and D-SALLRSIPA resulted full efficacy and high potency ( $EC_{50}$ : 0.07 fM) in preventing apoptotic death of neurons treated with TTX. There was no apparent attenuation of the protective activity at high concentrations (> 1 pM) of peptide; i.e., synergy was again evident. In contrast, treatment with D-NAPVSIPQ and L-SALLRSIPA resulted in full efficacy but attenuation of the survival-promoting activity at concentrations  $\geq 0.1$  pM. These data indicate that D-SALLRSIPA is the only peptide among the L- and D-peptides studied that produced no attenuation of survival-promoting activity when used in combination with any of the other peptides.

### ***In vivo efficacy***

The studies of the protective properties of D- and L- peptides of NAPVSIPQ and SALLRSIPA were extended to a mouse model of fetal alcohol syndrome (FAS) and to apolipoprotein E -deficient mice, a model of cognitive impairment and neurodegeneration. In the FAS model, the efficacy of the peptides was assessed by counting the number of surviving fetuses. The effects of peptide treatments administered intraperitoneally are presented in figure 5. Treatment with alcohol resulted in 37% fetal demise in comparison to 6% in controls. A 30 min pretreatment with either D-NAPVSIPQ or D-SALLRSIPA significantly reduced fetal demise in comparison to those

treated with alcohol alone ( $p < 0.03$ ). A combination of D-NAPVSIPQ and D-SALLRSIPA was also protective, however a clear dose-dependency was observed, with 20  $\mu\text{g}$  of each being effective and 2  $\mu\text{g}$  of each not significantly increasing fetal survival in comparison to ethanol-treated controls. L-NAPVSIPQ and D-SALLRSIPA, a combination shown to be protective in the in vitro testing, also produced protection in the FAS model.

To extend the studies in the FAS model, higher doses were used to test if the D-peptides were effective after oral administration (figure 6). After oral gavage, 40  $\mu\text{g}$  of D-NAPVSIPQ was not effective. However, treatment with a combination of D-NAPVSIPQ and D-SALLRSIPA significantly increased the number of surviving fetuses in comparison to the group treated with ethanol alone. The body weights were also measured in the same experiment as shown in figure 7. Significant decreases in body weight were observed after the alcohol treatment in comparison to the control group. However, oral treatment with the D-peptides did not significantly increase the body weight in comparison to the fetuses from the mothers injected with alcohol alone. Treatment with D-NAPVSIPQ and D-SALLRSIPA alone had no effect on body weight in comparison to controls.

Performance of ApoE deficient mice was tested in the Morris water maze to assess the effects of the D-peptides on short-term spatial memory. Increases in performance as measured by decreases in latencies to find a hidden platform were observed a week after cessation of the 2-week daily D-SALLRSIPA plus D-NAPVSIPQ injections, i. e. in 21-day-old mice exposed to a 4-day training protocol (figure 8). Short-term memory processes were examined by performance in the water maze, measuring the

time required to find the hidden platform in the second of two daily trials. The platform location and the starting point in which the animal was placed in the water were held constant within each pair of daily trials, but both locations were changed every day. All groups of tested mice learned to find the hidden platform after 4 testing days as assessed by comparing the latencies to find the hidden platform on the first and the last trial days ( $p < 0.01$ ). However, differences were observed already on the first day of testing, with apolipoprotein E -deficient mice treated with the peptide mixture finding the platform faster than saline-treated apolipoprotein E -deficient mice ( $p < 0.05$ ). Further significant differences were observed on the second day of testing between these two groups ( $p < 0.03$ ).

## Discussion

The original intent of the present study was to demonstrate that D-enantiomers of SALLRSIPA and NAPVSIPQ had no neuroprotective properties thereby addressing the stereoselective specificity of these protective peptides for their receptors/binding sites. However, these experiments did not produce the expected effect as the D-isomers of both peptides had the full efficacy and potency of their respective L-isomer peptides in the models tested. Thus, the action of these two peptides was concluded to be independent of classical stereoisomeric ligand-receptor interaction. Furthermore, the non-chiral nature of the peptide-mediated protection was utilized to test for the potential of these peptides as orally active agents. Low doses of these peptides were shown to protect mouse embryos from death in a model of FAS. Furthermore, the impairment of learning and memory performance of apolipoprotein E gene knockout mice was attenuated with D-peptide treatment. The efficacy of the D-peptides in the present study is in contrast to those observed in a model of stroke (Leker et al., 2002). In the mid-cerebral artery occlusion paradigm, L-NAPVSIPQ was found to provide protection while D-NAPVSIPQ (tested at a single dose) was not effective. These studies indicate that the protective actions of D-NAPVSIPQ are dose-dependent and may not be completely predictive of L-NAPVSIPQ (Leker et al., 2002).

Although unusual, other examples of biologically active peptides have been reported to be equally efficacious and potent in both the all D- and all L-conformation. However, to our knowledge, the present study is the first report of neuroprotective action that does not exhibit a stereoselectivity. A number of the previous studies of non-chiral effects of peptides suggested membrane phenomena, for example through the formation

of pores, as in the case of the antibiotic magainin and cecropin A. (Wade et al., 1990). The D-enantiomeric peptides of both of these peptides exhibit similar antibiotic and single channel conductance as their respective L-enantiomers. Another example of peptides that produced biological actions equally well in the D- or L- form is amyloidogenic peptide (A $\beta$ <sub>1-42</sub>) derived from the amyloid precursor protein that is believe to have a causative role in the neurodegeneration observed in Alzheimer's disease (Cribbs et al., 1997). Although it is not clear if the toxic A $\beta$  peptide forms pores, there is a report of A $\beta$ <sub>1-40</sub> forming a cation channel in model membranes (Arispe et al., 1993). In the case of A $\beta$ <sub>1-42</sub>, the toxicity associated with this peptide may be mediated by cross-linking of receptors through fibrillary forms of this peptide. The formation of protofibrils and fibrills may not be dependent on chirality. A recent report indicates that L-NAPVSIPQ does bind to and inhibit A $\beta$  aggregation, suggesting structural interaction between these two peptides (Ashur-Fabian et al., 2003).

D-peptides have been shown to interact with chaperone systems. For example, D-peptides compete with L-peptides for the same binding site in DnaJ and this interaction can inhibit refolding functions of this chaperone system (Bischofberger et al., 2003). In addition, all-D and all-L conformers of the functional element of alphaA-crystallin do not show marked differences in their chaperone-like activity (Bhattacharyya and Sharma, 2001). To speculate, a chaperone-like function may also be affected by these peptides. The latter is of particular interest in that SALLRSIPA is very similar to CALLRCIPA, a sequence found in the chaperone heat shock protein 60 (hsp60) (Gozes and Brenneman, 1996).



Recent studies indicate that D-peptides derived from chemokines can interact with the CXCR4 receptor (Zhou et al., 2002). Remarkably, the D-peptides act as antagonists to this receptor, resulting in inhibition of CXCR4-dependent HIV-1. In the case of the chemokine related peptides, the D-peptides exhibited either equivalent or in greater potency than L-peptide counterparts. However, these actions were confined to an antagonist action. It is not clear if NAPVSIPQ and SALLRSIPA are acting to stimulate a neuroprotective process or providing an inhibition of a function associated with neuronal cell death. If the model of the chemokine receptor were relevant to the present study, our results could be interpreted as antagonizing a pro-apoptotic pathway. There is no previous observation of a non-stereoselective agonist action that is receptor-mediated.

The fundamental finding of the present study is that two neuroprotective and structurally related peptides do not exhibit stereoselective constraints in order to produce their biological activity at extraordinarily high potencies. Whether a similar recognition site or a common mode of entry account for the initial events of cellular interaction remain to be established. Previous studies have not provided a systematic comparison of actions between the two peptides, yet a diverse set of actions have been observed in central nervous system-related preparations. Neuroprotective actions of SALLRSIPA have been associated with regulation of calcium homeostasis (Glazner et al., 2000) and transcription factors (Glazner et al., 1998; White et al., 2000). A slightly longer form of SALLRSIPA (ADNF14) has been shown to be associated with stimulation of the MAPK and protein kinase C pathways (Gressens et al., 1999). NAPVSIPQ neuroprotection has been associated with stimulation of cGMP and nitric oxide (Ashur-Fabian et al., 2001), with the effects on cGMP being more relevant pharmacologically to the neuroprotective

actions. Thus, the L-peptides of NAPVSIPQ and SALLRSIPA have multiple pharmacological effects on neuroprotection with a unifying mechanism not yet apparent. Supportive of distinctive mechanisms is contrasting concentration –effect curves of the two peptides. While both peptides exhibit extraordinary potencies at femtomolar concentrations on neurons in culture, NAPVSIPQ clearly has two peaks of neuroprotective activity while SALLRSIPA has one. Interestingly, in enriched neuronal cultures (sparse number of glia), both peptides show less potent neuroprotective effects, suggesting a glial involvement in the full potency neuroprotective effect (Brenneman et al., 1998; Zemlyak et al., 2000).

All of the peptides investigated in the current study have exhibited attenuating biological responses with either a single or double inverted U-shaped dose response. Although characteristic of many peptide responses, the mechanism(s) of these effects remains unclear. Alternative explanations of these attenuating responses with increasing concentration of peptide include: receptor desensitization, ligand-ligand interactions, or activation of different and opposing mechanisms that are dose-dependent.

Exploration of the effects of each of the peptides alone and in combination has concentration-effect profiles that suggest synergistic rather than additive actions. Whereas it is clear that the L- forms of both NAPVSIPQ and SALLRSIPA behave similarly to their respective D-form *in vitro*, the combinations resulted in synergies with respect to the inverted-U shaped dose response; i.e., the attenuation of neuroprotective activity with increasing concentrations. It is apparent that all combinations involving D-SALLRSIPA resulted in clear plateauing effect with no loss of protective response with increasing concentrations. The broadening of the effective concentrations of these

agents increases the probability of their potential clinical success by obviating the confounding property of concentration-dependent attenuation of protection. Regardless of mechanism, our view is that the combined administration of these peptides has distinct pharmacological advantages including neuroprotection elicited at high potency that is insensitive to the use of the D-isomeric form of NAPVSIPQ and SALLRSIPA. However, evidence of similar synergies from combined peptide treatment *in vivo* remains to be demonstrated.

The discovery of the activity of the D-peptides along with the demonstration that these peptides are orally active opens up a new arena for peptidic therapeutic agents. Although larger amounts of peptide are required for biological activity through the oral route of administration in comparison to *i.p.* or intranasal routes, the advantages for drug applications are significant. As shown in figure 6, only oral treatment with D-SALLRSIPA (40  $\mu$ g) alone prevented fetal demise whereas the same amount of D-NAPVSIPQ did not significantly reduce the amount of fetal death in comparison to the alcohol group. Importantly, when given intraperitoneally both D-peptides produced effective protection from fetal death in comparison to the alcohol group. These data indicate that the route of administration is an important variable in evaluating the action of the D-peptides and further suggest that both the individual and combination of peptides should be tested, as they differ from the protective activity observed with the L-peptides given intraperitoneally. As shown previously, both L-peptides also prevented growth restriction, while the individual L-peptides had no effect on fetal weight (Spong et al., 2001). In the present study, the D-peptides at the same dose did not prevent the loss of fetal weight (see figure 7). Furthermore, previous studies with the same model

indicated that at the same dosage (20  $\mu$ g) as employed for the individual D-peptides in the present study, L-NAPVSIPQ was effective in preventing fetal death but L-SALLRSIPA was not (Spong et al., 2001). Treatment with each of the D-peptides produced a significant increase in fetal survival as compared to the alcohol-treated group. The pharmacodynamic/pharmacokinetic responses that contribute to the protective actions of the D-peptides will be important next steps in characterizing their action. These initial studies did not reveal any gross toxic responses to the D-peptides, but systematic studies of toxicity, metabolism and biodistribution from the D-peptides will also be necessary next steps for the advancement of these agents. The current studies are an important first step in our evaluation of neuroprotective D-peptides as therapeutic agents.

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## Footnotes

a) Financial support provided by the Intramural Research Program of NICHD and the US-Israel Binational Science Foundation.

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## Legends for Figures

Figure 1. Survival-promoting effects of L-SALLRSIPA and D-SALLRSIPA on TTX-treated cerebral cortical cultures. Treatment duration was 4 days and cultures were assayed by neuronal cell counts without knowledge of the treatment group. Significant increases in neuronal cell counts were observed at concentrations from 0.01 fM to 0.1 nM from both peptides ( $p < 0.05$ ) in comparison to cultures treated with 1  $\mu$ M TTX alone. Each value is the mean of 3-4 determinations  $\pm$  the standard error. Control cultures had cell counts that were  $307 \pm 4$ .

Figure 2. Comparison of the survival-promoting activity of L-NAPVSIPQ and D-NAPVSIPQ on TTX-treated cerebral cortical cultures. The treatment paradigm and cultures conditions were as described for figure 1. In comparison to cultures treated with TTX alone, significant increases in neuronal cell counts were observed at concentrations of peptide ranging from 0.01 fM to 1 nM for both peptides ( $p < 0.05$ ). Each value is the mean of 3-4 determinations  $\pm$  the standard error.

Figure 3. Comparison of neuroprotective activity of combined peptide treatment (D-NAPVSIPQ + D-SALLRSIPA vs L-NAPVSIPQ + L-SALLRSIPA) in TTX-treated cerebral cortical cultures. Peptides were all tested in equimolar amounts for 4 days. Significant increases in neuronal cell counts in comparison to cultures treated with TTX alone were observed at peptide concentrations  $> 0.01$  fM to 1 nM ( $p < 0.05$ ). Treatment with the combination of D-NAPVSIPQ + D-SALLRSIPA in the cultures co-treated with

TTX resulted in cell counts that were not significantly different from control from 0.1 fM to 1 nM. In contrast, treatment with the combination of L-NAPVSIPQ + L-SALLRSIPA in cultures co-treated with TTX resulted in cell counts that were less than controls at all concentrations tested. Each value is the mean of 3-4 determinations  $\pm$  the standard error.

Figure 4. Comparison of neuroprotective activity from combined peptide treatment (D-NAPVSIPQ + L-SALLRSIPA vs L-NAPVSIPQ + D-SALLRSIPA) in TTX-treated cerebral cortical cultures. Equimolar amounts of peptide were used at each treatment concentration. For treatment with D-NAPVSIPQ + L-SALLRSIPA, significant increases in neuronal cell counts in comparison to cultures treated with TTX alone were observed at peptide concentrations  $> 0.01$  fM to 0.1 nM ( $p < 0.05$ ). Treatment with L-NAPVSIPQ + D-SALLRSIPA also resulted in significant increases in neuronal cell counts in comparison to cultures treated with TTX alone were observed at peptide concentrations  $> 0.01$  fM to 1 nM ( $p < 0.05$ ), although the shape of the concentration-effect curve was different than that of D-NAPVSIPQ + L-SALLRSIPA. Attenuation of the survival-promoting activity of D-NAPVSIPQ + L-SALLRSIPA was observed at concentrations  $\geq 0.1$  pM in contrast to L-NAPVSIPQ + D-SALLRSIPA, which produced no attenuation from 0.01 pM to 1 nM. Each value is the mean of 3-4 determinations  $\pm$  the standard error. These data were obtained within the same experiments as that described in figure 3.

Figure 5. Effect of peptide treatment on fetal survival in a model of fetal alcohol syndrome. A single i.p. injection of 25% ethyl alcohol in saline was given at 0.03 ml/g body weight to pregnant mice at embryonic day 8. Peptides were injected 30 min. prior to the administration of alcohol. Fetal survival was assessed on embryonic day 18. The asterisk indicates significant decrease in fetal death/litter in comparison to pregnant mice treated with alcohol alone ( $p < 0.001$ ). Treatment with the combination of D-peptides (20  $\mu$ g) versus treatment with the individual peptide produced no significant difference for the intraperitoneally administered D-peptides. The number of litters was  $\geq 8$  for each treatment group. Each litter was used as the basis for statistical analysis and 8-10 fetuses/ litter were observed.

Figure 6. Oral administration of D-peptides prevented fetal death in model of fetal alcohol syndrome. D-NAPVSIPQ (D-NAP) and D-SALLRSIPA (D-SAL) were given by gavage to pregnant mice at embryonic day 8. The peptides were administered 30 min prior to the i.p. injection of alcohol. Significant increases in fetal survival were observed in the groups given D-NAPVSIPQ and D-SALLRSIPA at both 40 and 100  $\mu$ g ( $p < 0.01$ ). Oral administration with D-NAPVSIPQ alone had no significant effect in preventing fetal demise after alcohol treatment. In contrast, oral treatment with D-SALLRSIPA significantly decreased fetal death in comparison to the alcohol-treated group ( $p < 0.01$ ). The number of litters was  $> 12$  for each treatment group. Each litter was used as the basis for statistical comparison and there were 8-10 fetuses/ litter.

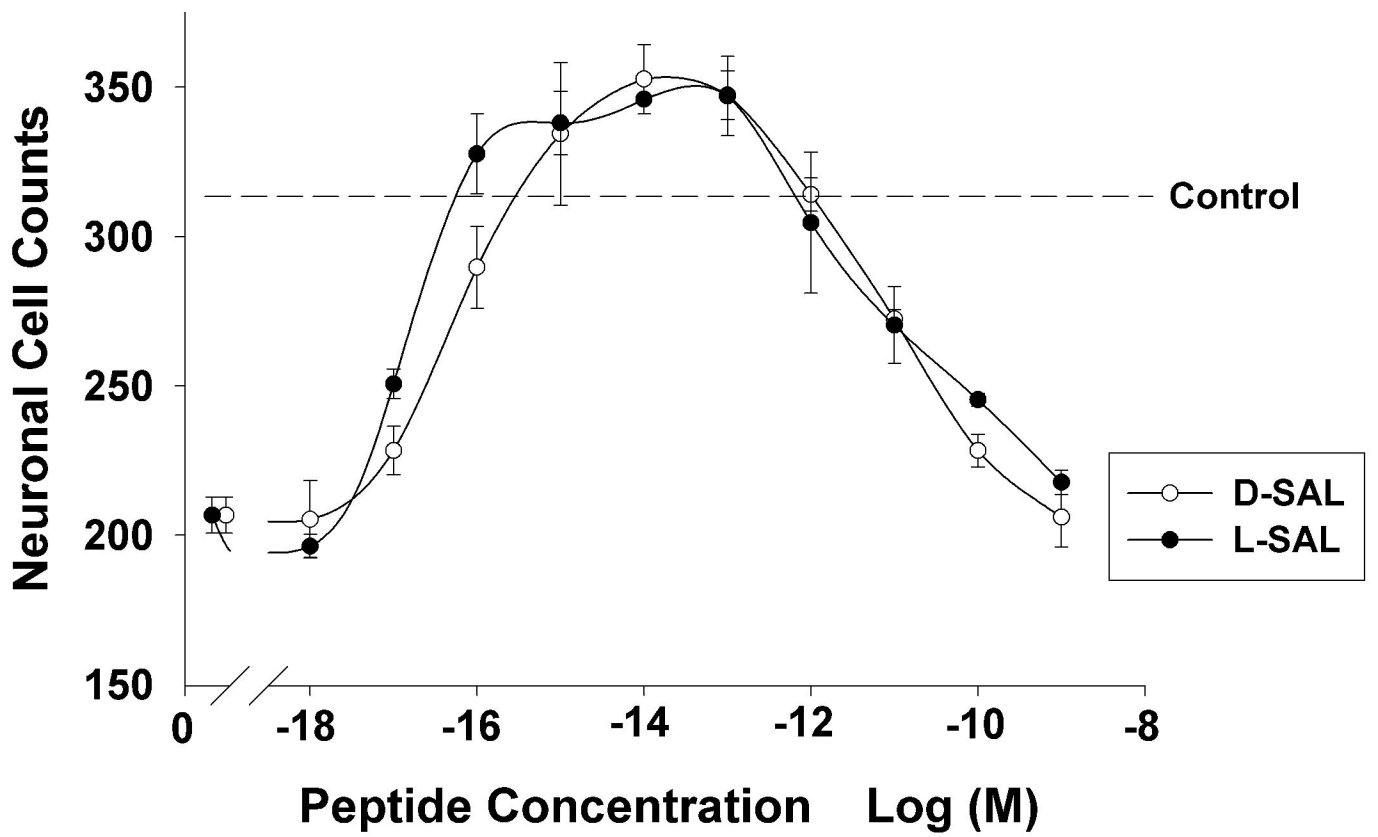
Figure 7. Oral administration of D-peptides did not prevent growth restriction in a model of fetal alcohol syndrome. The fetal weights of the surviving animals corresponding to the experiment described in figure 6 are shown. Oral treatment with the D-peptides had no detectable effect on fetal weight in comparison to fetuses from mothers given the alcohol treatment alone. The D-peptides given alone had no effect on fetal weight in comparison to controls.

Figure 8. D-NAPVSIPQ + D-SALLRSIPA injection results in improved performance in the water maze. Experiments were performed with ApoE-deficient mice following chronic subcutaneous injections of a mixture of D-NAPVSIPQ + D-SALLRSIPA (as described in the methods section). Latencies to reach the hidden platform in the water maze are shown. Groups tested were apolipoprotein E -deficient (n=40); apolipoprotein E -deficient treated with D-NAPVSIPQ+D-SALLRSIPA (n=25), control (n=25). Mice from all groups tested learned to find the hidden platform after 4 testing days. On test day 1, the apolipoprotein E-deficient mice + D-peptides exhibited a significantly reduced latency in finding the platform than the apolipoprotein E-deficient group \* $p < 0.05$ . Similarly, on day 2 of testing, the D-peptide treated group also had significantly less latency as compared to the apolipoprotein E -deficient mice (\*\* $p < 0.03$ ).

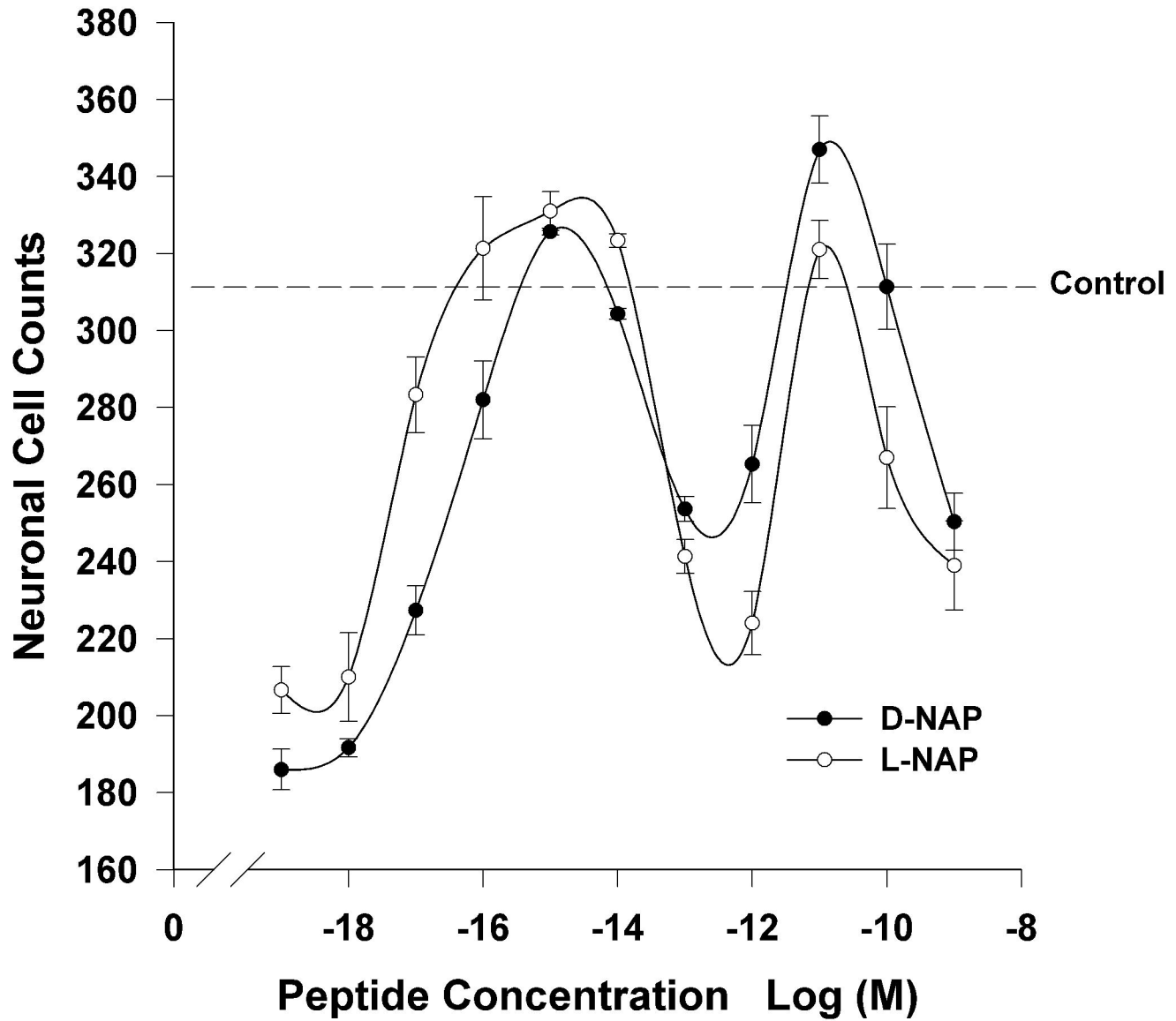
## Figures



**Figure 1**



**Figure 2**



**Figure 3**

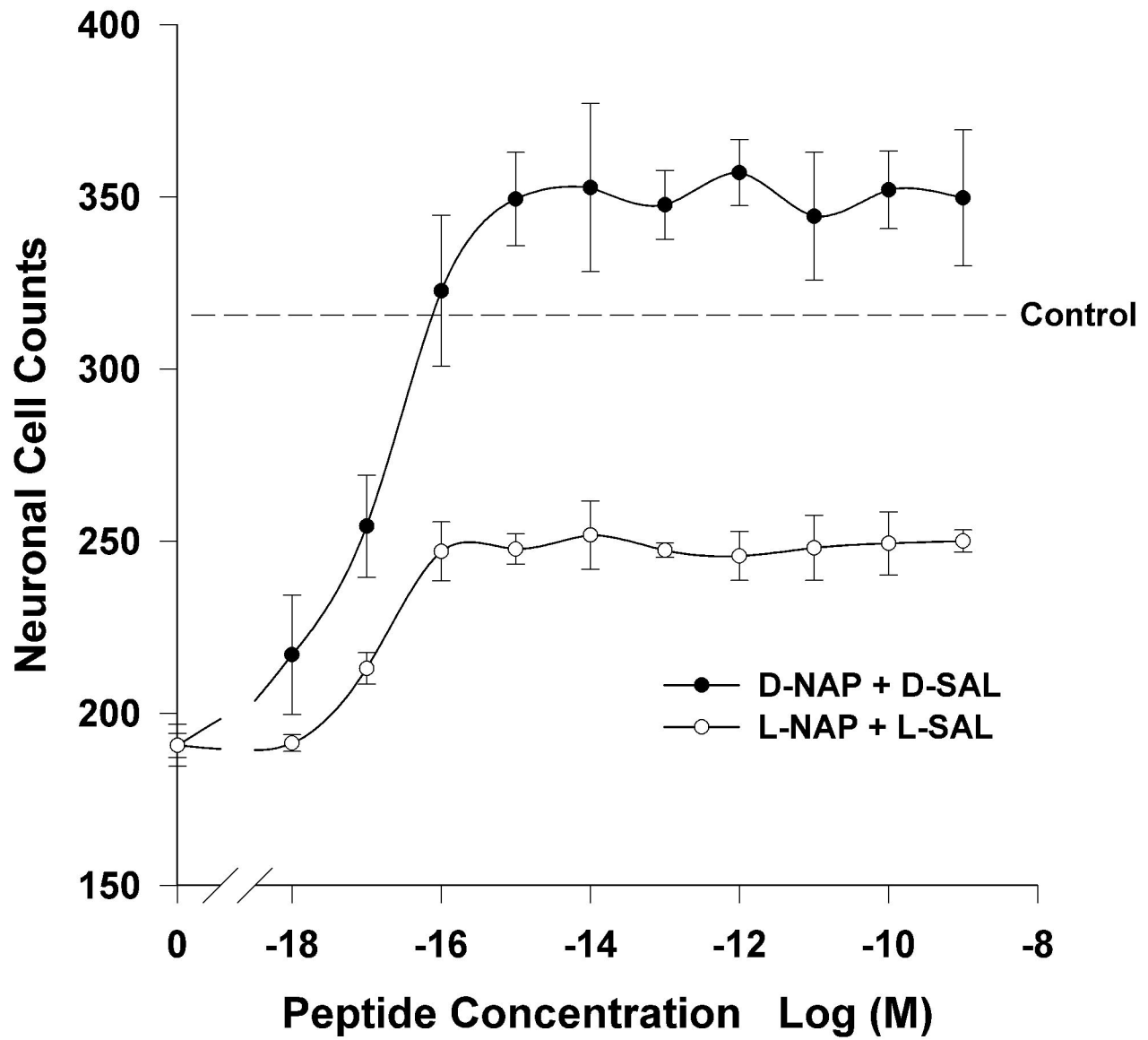
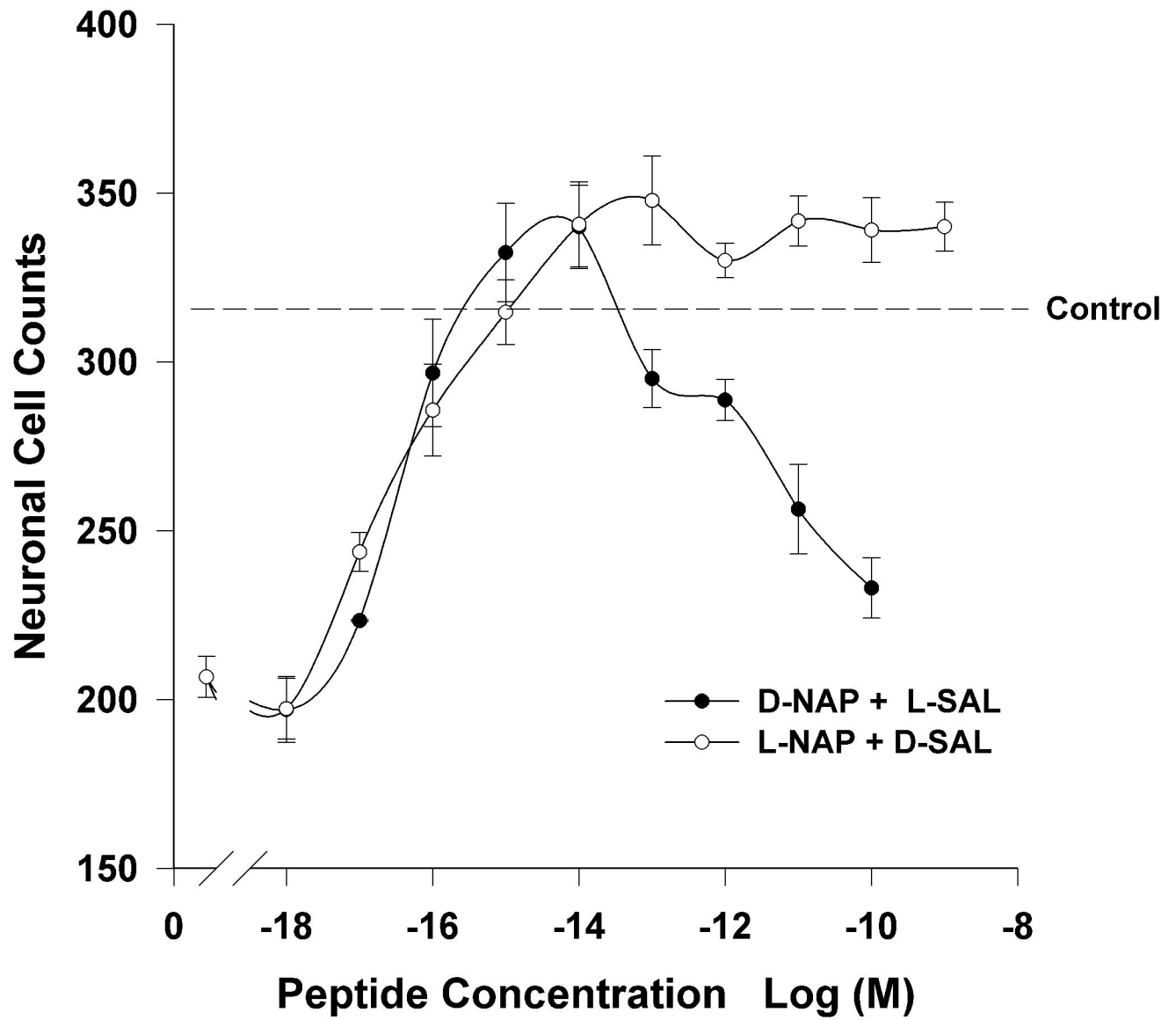
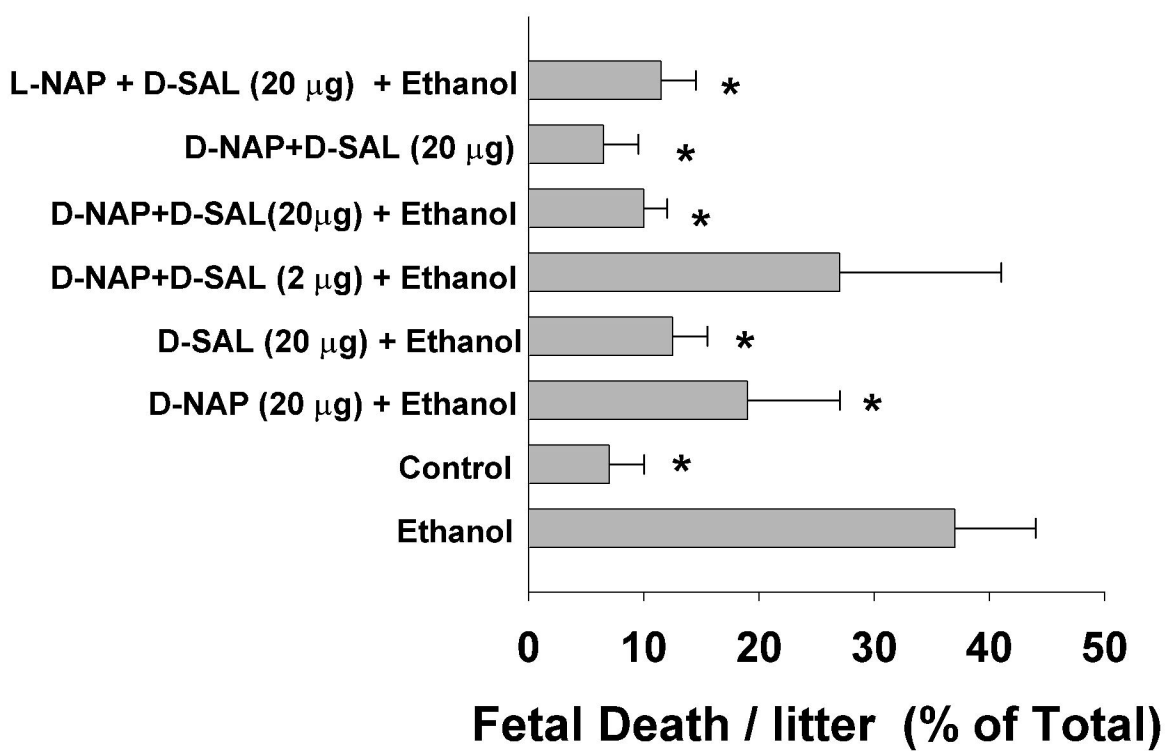


Figure 4



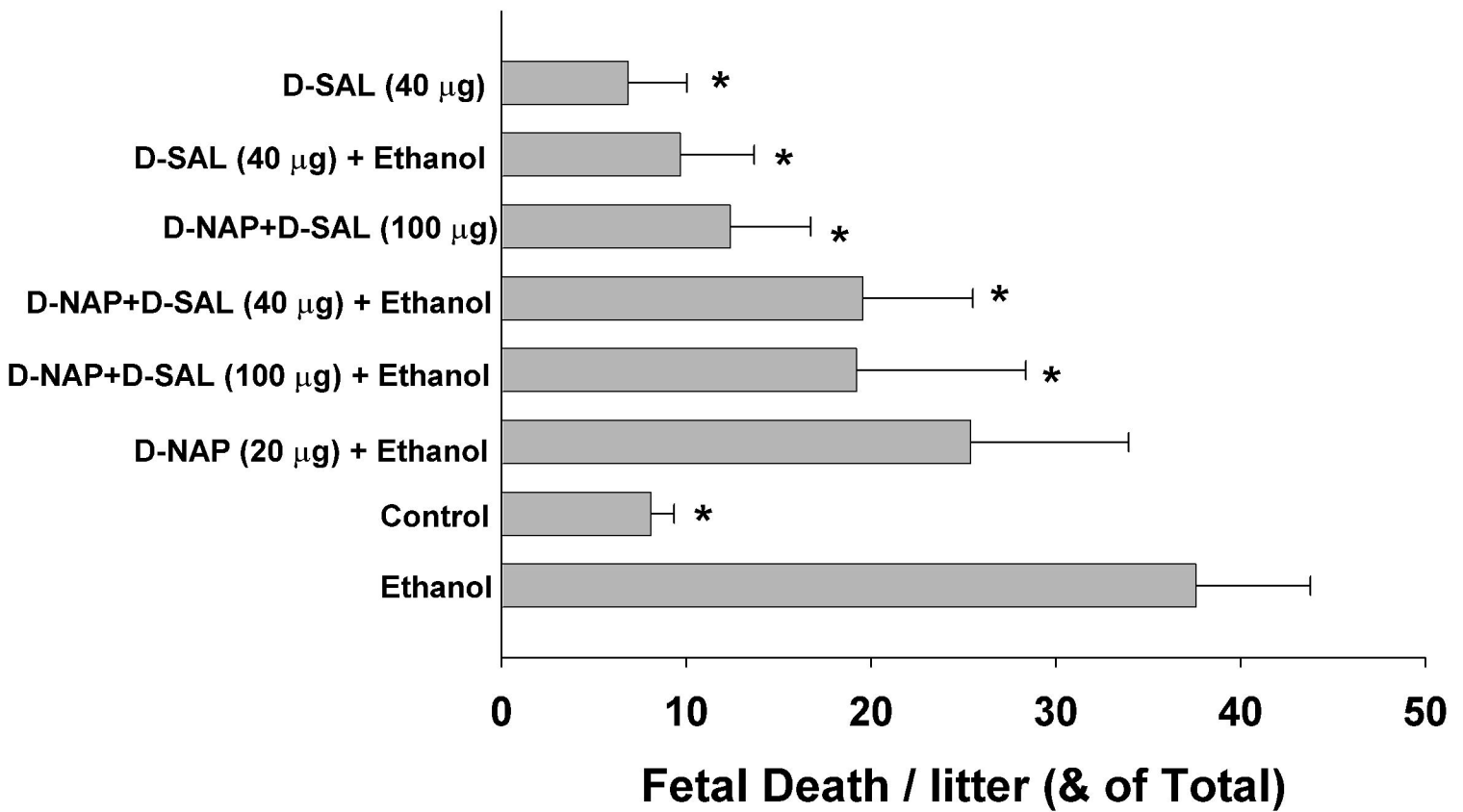
**Figure 5**

## Intraperitoneal Administration of Peptides



**Figure 6**

## Oral Administration of Peptides



**Figure 7**

## Oral Administration of Peptides

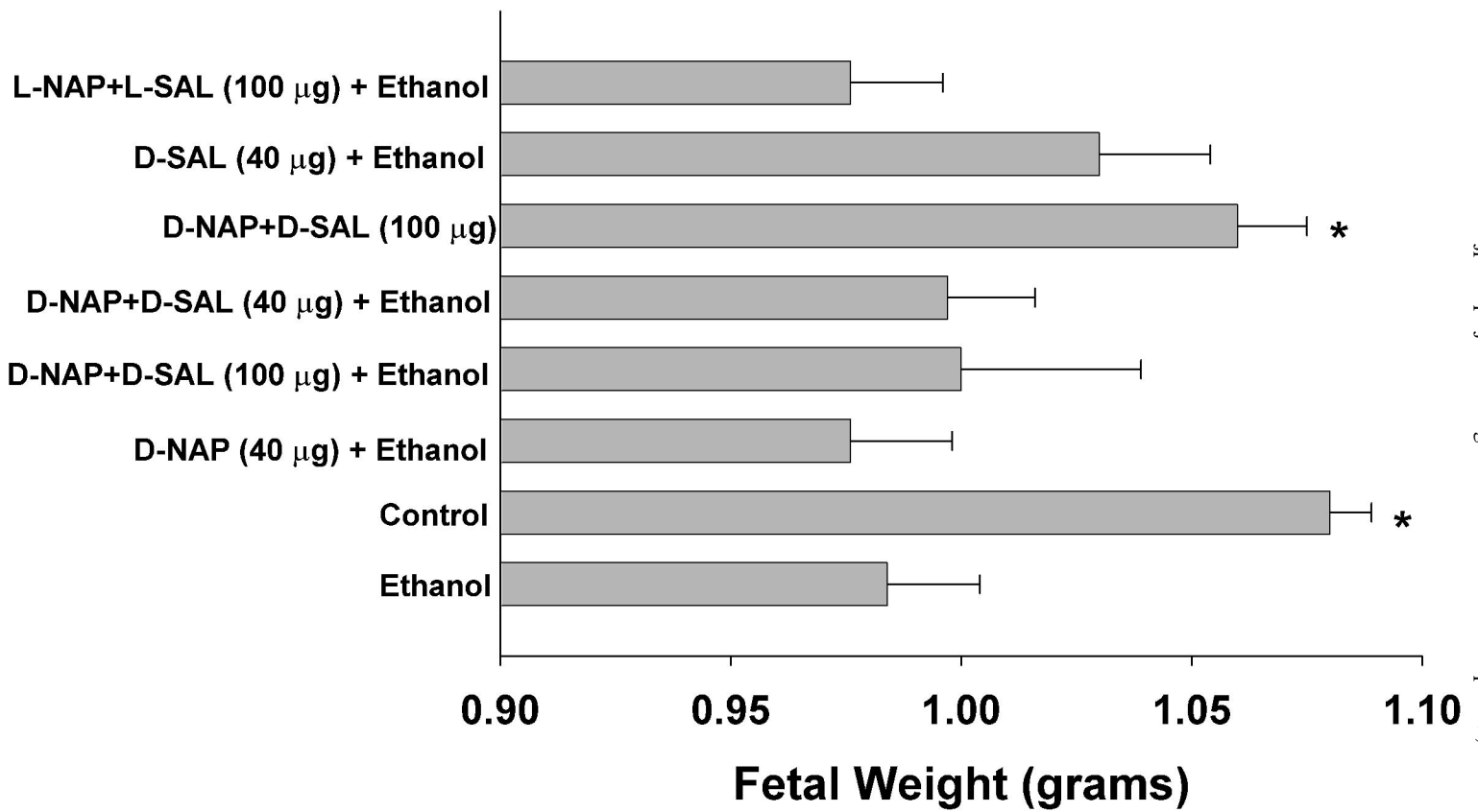


Figure 8

