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The nootropic and neuroprotective proline-containing dipeptide noopept restores spatial memory and increases immunoreactivity to amyloid in an Alzheimer's disease model

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Abstract

The effects of the novel proline-containing nootropic and neuroprotective dipeptide, noopept (GVS-111, N-phenylacetyl-L-prolylglycine ethyl ester) were investigated in NMRI mice following olfactory bulbectomy. We have shown previously that these animals developed Alzheimer's disease (AD)-like behaviour, morphology and biochemistry including impairment of spatial memory, regional neuronal degeneration and elevated Aβ peptide brain levels. In the current investigation, spatial memory was assessed using the Morris water maze and serum antibodies to *in vitro* morphologically characterized amyloid structures of both $A\beta_{(25-35)}$ peptide and equine lysozyme, as well as to neurotrophic glial factor S100b, were analyzed by enzyme-linked immunosorbent assay (ELISA). Noopept (administered at a dose of 0.01 mg/kg for a period of 21 days and during a further 5 days training) restored spatial memory and increased serum antibody levels to oligomers of $A\beta_{(25-35)}$ peptide but not to equine lysozyme amyloid or S100b protein in bulbectomized animals. The positive immunotropic effect of noopept to $A\beta_{(25-35)}$ peptide prefibrillar aggregates was more marked in sham-operated compared to the bulbectomized subjects which were characterized by an overall suppression of immunoreactivity. Enhancement of the immune response to $A\beta_{(25-35)}$ peptide prefibrils caused by noopept may attenuate the neurotoxic consequences of amyloid fibrillization and also be associated with an improvement in spatial memory in bulbectomized mice. These actions of noopept, combined with it's previously reported neuroprotective and cholinomimetic properties, suggests that this dipeptide may well be useful for improving cognitive deficits induced by neurodegenerative diseases.

Keywords

noopept (GVS-111), memory improvement, olfactory bulbectomy, Alzheimer's disease, antibodies, $A\beta_{(25-35)}$ peptide, amyloid

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Introduction

AD is a prevalent neurodegenerative illness affecting millions of people worldwide (Maubach, 2003; Ross and Poirier, 2004). Currently, there is no effective therapy to prevent or halt the progression of the disease, although symptomatic treatments are available. Neurotransmitter-based therapy using acetyl cholinesterase inhibitors (Cacabelos *et al.*, 2000) and NMDA receptor antagonists (Desai and Grossberg, 2005) are in current use. Anti-inflammatory and anti-oxidative approaches, as well as compounds devised to block Aβ peptide aggregation are being tested in the clinic. Furthermore, β- and γ-secretase inhibitors designed to reduce generation of Aβ peptide are under investigation in addition to the development of a vaccine against β-amyloid (Nitsch, 2004). The efficacy of these treatments is relatively modest, while the incidence of withdrawals due to adverse events is moderately high (Evans *et al.*, 2004).

It is evident that AD has a complex multifactorial pathology, so it would be advantageous to explore novel therapeutic strategies (Vesey *et al.*, 2002) focused on diverse mechanistic targets (Sewell *et al.*, 2005). Nootropic drugs have received particular attention in this respect because they possess useful effects in the treatment of memory loss and age-related memory decline. Piracetam (Nootropil®) is the most well known first generation nootropic (Giurgea, 1972; Winblad, 2005), however, a significant proportion of AD patients appear to be unresponsive to it (Gualtieri *et al.*, 2002), prompting the search for agents which surpass piracetam both in cognitive improvement and neuroprotective activity. A novel dipeptide analogue of piracetam, (N-phenylacetyl-L-prolylglycine ethyl ester, GVS-111, designated as noopept, NP) was chosen from the series of acyl-proline-containing dipeptides (Seredenin *et al.*, 1995). It belongs to the latest generation of nootropics (Ostrovskaya *et al.*, 2002) with distinct neuroprotective properties (Pealsman *et al.*, 2003).

Noopept's characteristics were first demonstrated in three brain-damaged animal models, namely, frontal lobectomy, cortical compression and photochemically induced cortical thrombosis (Ostrovskaya *et al.*, 1999). These brain injuries are known to be accompanied by substantial glutamate release, increased intracellular potassium and calcium concentrations, plus free radical generation and accumulation of proinflammatory cytokines, all of which are common secondary outcomes of AD-like neurodegeneration (Kanazawa, 2001). It has been established that noopept not only attenuates the neurotoxic effects of glutamate on granular cerebellar neurones, but also prevents free radical oxidative damage and apoptosis in cultured cortical neurons from Down's syndrome foetus (Pealsman *et al.*, 2003). In addition, it blocks voltage dependent calcium channels and calcium dependent potassium channels (Bukanova *et al.*, 2002). Noopept exerts anti-inflammatory activity, it has a wide safety margin and its pharmacokinetics indicate specific bioavailability for the brain. In essence, noopept is between 200 to 50 000 times more potent than piracetam as a nootropic agent on a dose for dose basis (Ostrovskaya *et al.*, 2002).

In the current study, olfactory bulbectomized (OB) mice were chosen as a sporadic AD model which induces AD-like behavioural and neurochemical features including spatial memory impairment, regional loss of neurons, a cholinergic deficit and increased brain β-amyloid levels (Bobkova *et al.*, 2004). The relevance of the OB model to AD has been substantiated in parallel by Sohrabji *et al.* (2000) and by Hozumi *et al.* (2003). Recently, we have postulated that progressive autoimmunity accompanying AD can be considered as a potential drug target. The immune responses to amyloid structures not only of $A\beta_{(25–35)}$ peptide as a primary biomarker of AD, but also of the ubiquitous protein human lysozyme specifically diverge in the sera of AD patients with differing disease durations (Gruden *et al.*, 2004). The neurotrophic factor S100b and its antibodies (Abs) are also involved in AD neurodegeneration (Peskind *et al.*, 2001) and we have demonstrated that antibodies to this biomarker are elevated in shorter duration AD sufferers. The aim of this study was to evaluate the behavioural efficacy of noopept on spatial memory and autoimmune responses to *in vitro* morphologically characterized amyloid structures of both $A\beta_{(25–35)}$ peptide and equine lysozyme as well as to protein S100b in bulbectomized animals.

Methods and materials

Drugs and proteins

Noopept (N-phenylacetyl-L-prolylglycine ethyl ester, MW = 319) was designed and synthesized at the State V. V. Zakusov Institute of Pharmacology, RAMS (Seredenin et. al., 1995). $A\beta_{(25–35)}$ peptide was produced in the Pushchino Branch of academicians M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry RAS. Equine lysozyme was purified as described previously (Noppe *et al.*, 1996). S100b was purchased from Sigma, USA. In order to produce amyloid structures, $A\beta_{(25–35)}$ peptide (20 mg/ml) was incubated in 50 mM PBS, pH 8.0 for 16 h at 8 °C (Gruden *et al.*, 2004). Equine lysozyme (20 mg/ml) was incubated in 20 mM glycine buffer, pH 2.0 at 57 °C for 1 week and subsequently transferred to 2 mM sodium bicarbonate buffer, pH 8.0 (Mališauskas *et al.*, 2003).

Animals

NMRI male mice weighing 25 ± 0.6 g were supplied by Charles Rivers Laboratories, USA. This strain has previously been shown to exhibit good performance in the water maze (Klapdor and van de Straay, 1996) and also displays inherent immunosuppression (Freisleben *et al.*, 1994). Animals were allowed food and water *ad libitum* and housed in groups of eight in standard laboratory cages under 12 h: 12 h light–dark conditions (light from 8.00 AM) at 21–23 °C. All animal experiments were performed in accordance with the guidance of the National Institutes of Health for Care and Use of Laboratory Animals, NIH Publications No. 8023, revised 1978.

Olfactory bulbectomy procedure

Two groups of mice aged 10 weeks (n = 32) were employed in the experiments; one group underwent OB operation and the other

group was sham operated (SO). Mice were anaesthetized with isoflurane and 0.5% Novocaine used for local anaesthesia of the scalp. A single burr hole of 2 mm diameter (2 mm anterior to bregma, 0 mm laterally from the midline) was drilled over the olfactory bulbs. Both olfactory bulbs were carefully aspirated through a rounded needle attached to a water pump. The extent of the lesion was assessed both visually and histologically at the end of the experimental study. SO mice were treated similarly, except that the olfactory bulbs were not removed.

Drug treatment

Four weeks following OB or SO procedures, mice were randomly divided into four groups of eight animals. Two groups denoted as SO + NP and OB + NP were treated with noopept (0.01 mg/kg i.p.) daily for 21 days. In the remaining two groups denoted as SO + saline and OB + saline, the animals were administered with 0.9% saline (i.p.) for the same period. Either noopept (0.01 mg/kg) or saline was injected 1 h before training and memory testing (see Fig. 1). The 0.01 mg/kg dose of noopept was chosen from earlier studies using mice in cognitive tests (Seredenin *et al.*, 1995) and the 21-day treatment regime was based on the fact that clinical effects of nootropics achieve significance within 2–3 weeks of administration (Waegemans *et al.*, 2002).

Morris water maze paradigm

Experiments were performed in a test room with extra-maze cues to facilitate spatial learning. A circular swimming tank (80 cm diameter and 40 cm wall height with a hidden platform of 6 cm-diameter) – (State Institute of Biological Instrumentation, RAS, Russia) was filled to depth of 30 cm with water at 23 °C and rendered opaque by adding powdered milk. The dimensions of the tank were chosen according to a previous study by Klapdor and van de Staay (1996) who described good task acquisition with NMRI mice using these parameters. The tank was mentally divided into four quadrants: platform target quadrant (3), opposite quadrant (1), adjacent clockwise quadrant (4) and adjacent counterclockwise quadrant (2). The hidden platform was located in the middle of the third quadrant during training. It was submerged to a depth of 0.5 cm so as to be invisible to a swimming animal during the whole period of training. A video monitoring system (TSI, Germany) was used for recording the main behavioural parameters in the water-maze paradigm.

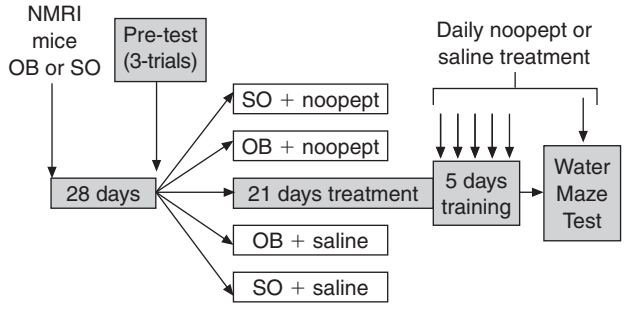

Figure 1 Noopept treatment protocol (0.01 mg/kg) daily for 21 days in OB or SO NMRI mice followed by noopept (0.01 mg/kg) administered daily, 1 h before training (5 days) then 1 h before testing on the final day in the Morris water maze

Fifty-four days after operation, learning ability and spatial memory were tested (Morris, 1984) as shown in Fig. 1. Initially, 34 OB and SO animals were assessed in the water maze to identify any inherent quadrant preference and those who showed some preference (2 mice) were eliminated from subsequent testing. Latency to reach the visible platform was then determined (three trials, 60 s each trial). Mice were then exposed to a total of 20 training trials over 5 days (i.e. four trials per day; 60 s each trial). During each trial, the latency to locate the hidden platform was evaluated up to a maximum of 60 s. Each animal was placed into the water facing the wall of the tank in one of three randomly selected quadrants other than that containing the hidden platform. If the animals failed to locate the platform within 60 s, they were placed on the platform for 10 s. Spatial memory was tested on the following day after completion of training with a single trial (60 s) in the absence of the hidden platform starting from a random position. During the test period, two parameters were recorded: quadrant occupancy time (s) spent in each quadrant and the number of entries in each quadrant expressed as a percentage of the total entries.

Atomic force microscopy (AFM)

The morphology of amyloid species of $A\beta_{(25–35)}$ peptide and equine lysozyme was examined using a PicoPlus (Molecular Imaging, USA) atomic force microscope using the imaging and sample preparation procedures as described previously (Mališauskas *et al.*, 2003; Mališauskas *et al.*, 2005). The dimensions of protein species were measured in cross-sections of AFM height images by using PicoPlus software (Molecular Imaging, USA).

ELISA assay

The level of serum Abs to amyloid species of both $A\beta_{(25–35)}$ peptide and equine lysozyme as well as to S100b protein in NMRI mice was quantified using solid phase ELISA as described previously (Gruden *et al.*, 2004). One day after assessment of spatial memory, blood samples from all groups were obtained by interorbital venipuncture under general anaesthesia. Sera for ELISA were produced by centrifugation of blood in a microcentrifuge (Eppendorf, USA), 3500 rpm at 4 °C for 15 min.

The titre of Abs to selected antigens in each serum sample was derived from the reciprocal of the greatest dilution at which the enzyme-substrate reaction gave an optical density value twice that of the mean optical density of control sera from naïve animals. The values of Abs titres at which we observed immunoreactivity to the antigens were expressed in relative dilution ratio units. Optical density was measured by using a microtitre plate reader (Flow Laboratory, USA). The mean serum level of Abs to amyloid species of both $A\beta_{(25–35)}$ peptide, equine lysozyme and S100b protein in NMRI naïve mice were determined as 8.2 ± 2.0, 8.0 ± 2.0 and 10.2 ± 2.4, respectively.

Statistical analysis

Statistical analysis of the spatial memory training and testing was carried out with ANOVA using the program Statistics 6 and ANOVA statistical package (CSS). The p values were reported for repeated measures. The preference for platform target quadrant in comparison with other indifferent quadrants was assessed by post hoc analysis using a multiple-range LSD test. The statistical significance of the difference of immunological parameters in the ELISA experiments was evaluated using two-tailed Student's t-test. All data were expressed as means ± sem.

Results

Behavioural experiments

In order to verify that there were no motor or visual impairments, latencies for all animals to locate a visible platform (in three trials) were screened in the Morris water maze before initiating the main spatial training schedule. Subsequent repeated measures ANOVA analysis between SO + saline, SO + NP, OB + saline and the OB + NP groups did not disclose any significant group effect ($F = 1.64$, $p = 0.22$).

During the succeeding 5-day course of water maze training, repeated measures ANOVA between all OB and SO groups indicated no significant group effect: $F(3.124) = 6173$, $p = 0.605$ but there was a significant outcome for the day of training: $F(4.496) = 47.118$, $p = 0.000001$. Hence, we observed a daily reduction in mean latency to locate the hidden platform in all OB, SO, saline and noopept treated groups. Post hoc examination revealed no statistical difference in learning abilities between any of the animal groups on each individual day of training as well as over the 5-day training course. This suggested that the speed of learning was comparable between all groups. In addition, noopept did not induce visual or motor deficits, neither did it modify water maze performance in OB or SO mice during training.

Spatial memory was tested on the day following completion of training. Two-way ANOVA was then performed in order to expose any possible link between group and quadrant as the main factors in the spatial memory test. This analysis of quadrant occupancy time revealed significant effects of quadrant ($F(3,112) = 34.2$; $p < 0.0001$), but not of group ($F(3,112) = 0.014$; $p > 0.05$) and there was a significant interaction between group and quadrant ($F(9,112) = 6.8$; $p < 0.0001$). A likewise analysis of number of entries also disclosed significant effects of quadrant, ($F(3,112) = 36.1$; $p < 0.0001$), no main effect of group ($F(3,112) = 2.3$; $p > 0.05$) and a significant interaction between group and quadrant ($F(9,112) = 9.4$; $p < 0.0001$). Consequently, a substantial link between quadrant and group in the water maze test was established using both quantified parameters of spatial memory (i.e. occupancy time and number of entries) in all groups of animals.

Group analyses of variance were performed to evaluate the differences in quadrant occupancy time and number of entries in each quadrant for each group, the results of which are presented in Table 1.

The data demonstrate that during spatial memory testing, when the platform was removed from the tank, a significant effect of quadrant as the main factor was verified both for quadrant occupancy time and number of quadrant entries in SO + saline, SO + NP and OB + NP groups. In contrast, the OB + saline animals did not exhibit preference for any of the four quadrants. Thus, bulbectomy impaired spatial memory in NMRI mice. Moreover, significant differences in quadrant preference were revealed in the OB + NP mice to both parameters (occupancy time, $F(3,28) = 7.48$; $p \leq 0.0008$) and (number of entries, $F(3,28) = 8.637$; $p \leq 0.000325$). High values of quadrant designation factor (for both occupancy time and number of entries) in the SO + NP group presumably reflects an increased homogeneity of the data under the influence of the dipeptide.

The results of post hoc analysis using LSD criteria are demonstrated in Fig. 2 (a and b). All experimental groups, except the OB + saline mice, searched the target platform quadrant (3) significantly more than other quadrants. This was true for both occupancy time and number of entries. In contrast, bulbectomized animals did not display any preference for the target quadrant, thus manifesting a spatial memory deficit. Noopept (0.01 mg/kg administered for 21 days) on the other hand did not modify either parameter of spatial memory (occupancy time and number of entries) in the SO group, but clearly restored spatial memory impaired by bulbectomy.

Table 1 Factor analysis of quadrant designation parameters in the water maze in OB and SO NMRI mice

Animal treatment groups	Quadrant designation factor, occupancy time		Quadrant designation factor, number of entries	
	F	p	F	p
SO + saline	$F(3,28) = 9.73$	0.0004*	$F(3,28) = 7.11$	0.0023*
SO + NP	$F(3,28) = 22.671$	0.00001*	$F(3,28) = 22.167$	0.00001*
OB + saline	$F(3,28) = 0.811$	0.503	$F(3,28) = 0.609$	0.616
OB + NP	$F(3,28) = 7.48$	0.0008*	$F(3,28) = 8.63$	0.000325*

* Denotes the significance of quadrant designation factor in water maze, $p < 0.001$.

The parameters of occupancy time and number of entries correspond to the time spent and entries into all quadrants by all animals of each group.

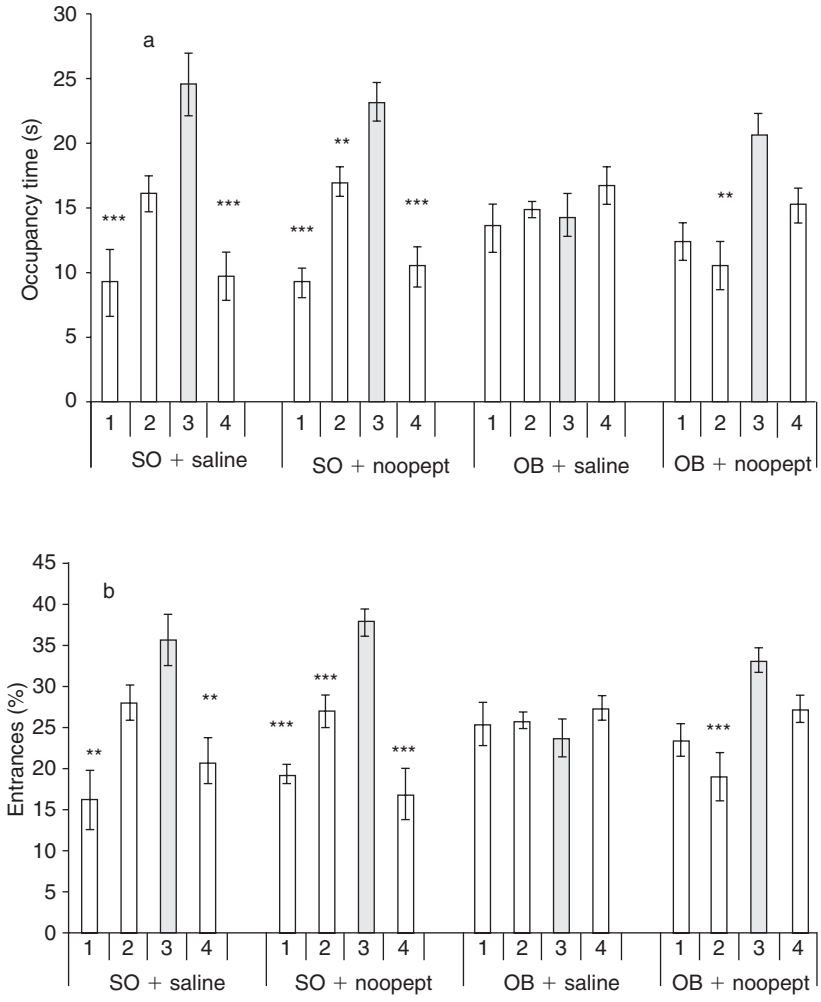

Figure 2 The effect of noopept on occupancy time (a) and entries in quadrants 1–4 (b) by OB and SO mice in the Morris water maze

** and *** denote the significance of quadrant designation factor in the water maze $p < 0.01$ and $p < 0.001$, respectively.

Atomic force microscopy imaging

The AFM analysis of amyloid species of $A\beta_{(25–35)}$ peptide and equine lysozyme were carried out before subjecting them to ELISA. This was particularly important since amyloid is characterized by an inherent diversity of species dependent on environmental conditions (Wang *et al.*, 2001; Malisauskas *et al.*, 2003). After incubation for 16 h in 50 mM PBS, pH 8.0 at 8 °C, $A\beta_{(25–35)}$ peptide formed round-shaped prefibrillar structures shown in Fig. 3a. They adhered to the mica surface as spherical-caps and their height estimated in cross-sections was equal to ca. 2 nm (Fig. 3c). The same images were acquired both in air and liquid environment indicating that the morphology and dimensions of the prefibrillar structures of $A\beta_{(25–35)}$ peptide were not affected by the mode and conditions of measurements.

Equine lysozyme was assembled into amyloid structures under the pH 2.0 and 57 °C conditions, transferred into ELISA 2 mM sodium bicarbonate buffer, pH 8.0 and subsequently assessed by AFM (Fig. 3b). The sample was a heterogeneous mixture of round-shaped oligomers of ca. 2 nm height measured in the cross-sections, linear and circular assemblies of oligomers and protofilaments of ca. 2 nm heights (Fig. 3d and e) with variable length from short stretches of ca. 20–40 nm to polymers of ca. 400 nm. The latter, nonetheless, did not proceed to thicker and longer mature fibrils. It is interesting to note, that in some linear and circular polymeric structures, the constituting ca. 2 nm height oligomers were still clearly identifiable (Fig. 3d and e).

Immunochemical experiments

The autoimmune response to amyloid species of $A\beta_{(25–35)}$ peptide and equine lysozyme as well as to S100b was measured by ELISA. The results for all groups of animals are presented in Table 2 as antibody titres.

Both SO and OB animals, injected with saline, possessed low serum antibody titres to all antigens tested in our experiments and there was no statistically significant difference between any of these antibody levels. However, sub chronic administration of noopept to OB mice caused an increase in the Abs titre to $A\beta_{(25–35)}$ peptide prefibrillar amyloid by a two-fold factor but not to equine

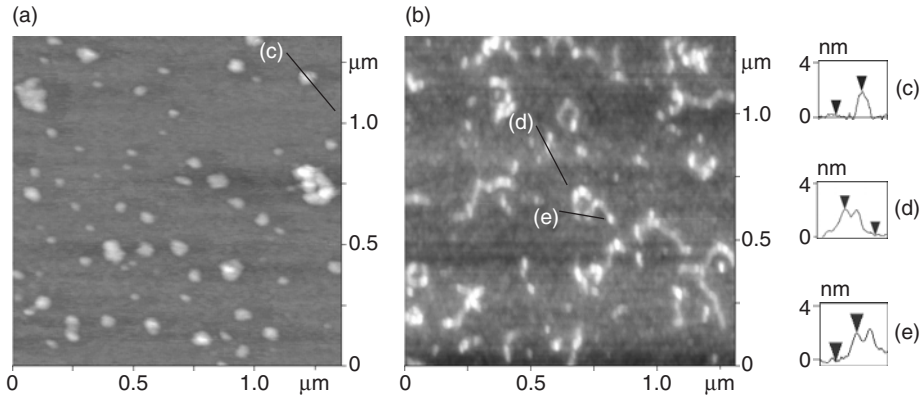

Figure 3 AFM images of amyloid species of $A\beta_{25-35}$ peptide (a) and equine lysozyme (b) subjected to ELISA assay. Cross-sectional height measurements of selected amyloid structures: (c) individual prefibrillar amyloid oligomer of $A\beta_{25-35}$ peptide, (d) linear and (e) annular amyloid structures of equine lysozyme. The cross-sections are indicated by black lines.

Table 2 Titres of serum antibodies to amyloid species of $A\beta_{(25-35)}$ peptide, equine lysozyme and S100b protein in OB and SO NMRI mice (mean ± sem)

Animal treatment groups	Titres of antibodies to selected antigens (mean ± sem relative dilution ratio units)		
	$A\beta_{(25-35)}$ peptide amyloid	Equine lysozyme amyloid	S100b
SO + saline	10.0 ± 2.0	10.4 ± 2.6	10.0 ± 2.0
SO + NP	52.4 ± 10.2*	18.6 ± 2.4	16.2 ± 2.4
OB + saline	12.2 ± 2.4	15.5 ± 2.6	10.0 ± 2.0
O + NP	26.8 ± 2.2*	10.0 ± 2.0	10.2 ± 2.2

*$p < 0.05$ – the level of antibodies in these groups of mice was compared to the antibody titres to $A\beta_{(25-35)}$ peptide amyloid in saline injected animals.

lysozyme amyloid species nor to S100b protein. An even more pronounced effect was observed following noopept administration to SO mice, whereby the antibody titre to $A\beta_{(25-35)}$ peptide amyloid was statistically elevated five-fold compared to saline treated SO animals (Table 2). Comparing the data obtained for each individual animal, we did not detect any direct correlation between the degree of memory deficit and the level of antibodies to the oligomers of $A\beta_{(25-35)}$ peptide.

Discussion

Restorative effect of noopept on spatial memory impairment in OB mice

The present data revealed that OB mice developed spatial memory impairment in the water maze model. Thus OB animals failed to show rescue platform quadrant preference during the learning procedure and this concurs with previously reported data (Hozumi, 2003; Bobkova, 2004). The main finding of the current study was that reestablishment of preference to a platform target quadrant occurred in OB mice treated with noopept, a dipeptidal analogue of vasopressin and piracetam (Seredenin *et al.*, 1995). Values of occupancy time in quadrant 3, which formerly contained the rescue platform, and the number of quadrant 3 entries in noopept treated OB animals were comparable to those of control SO mice injected with saline. It should be emphasized that bulbectomy itself did not change motor or visual functions in animals testifying to the specific action of bulbectomy on memory-related performance in the water maze paradigm. It should also be stressed that noopept did not affect the dynamics of learning in SO animals which supports the accepted view that nootropics are capable of restoring specific memory deficits without efficacy in the absence of mnestic disturbances (Giurgea, 1972).

Currently, there is a need for predictive AD animal models in the screening and development of new drugs to treat this disorder. Apart from a number of newly developed genetic models (van Dooren *et al.*, 2005), paradigms based on chemical, aspirative and electrolytic lesions of the brain remain in the focus of research endeavour (McDonald *et al.*, 1998). There are several reports that OB lesions induce multiple morphological, biochemical and behavioural features of AD such as deposition of Aβ peptide amyloid in the brain resulting in apoptotic neuronal cell death (Leung *et al.*, 2003), a deficit of BDNF, choline uptake into various brain structures (Sohrabji *et al.*, 2000), decreased stem cell production (Pagano *et al.*, 2000) and also multiple suppression of the immune system (Novoselova *et al.*, 2004). It is interesting to note, that clinical features of human AD also include the loss of olfactory discrimination and spatial disorientation (Kovacs *et al.*, 2001). Moreover, it has been established that cholinergic dysfunction is conducive to impaired cognition associated with neuropathological hallmarks of AD (fibrous aggregates consisting of

the Aβ peptide which develop into the amyloid plaques and paired helical filaments composed of hyperphosphorylated tau protein forming the neurofibrillary tangles) (Friedhoff *et al.*, 1999).

Furthermore, accumulation of senile plaques containing Aβ peptide and the formation of neurofibrillary tangles are commonly found in the bulbus olfactorius of AD patients (Esiri *et al.*, 1984). Cholinergic deficit is regarded as one of the main sources of OB-induced memory impairment (Hozumi *et al.*, 2003). This notion accords with the findings of Yamamoto (1997) who demonstrated the capability of acetylcholinesterase inhibitors to abolish memory disturbances caused by OB. Additional studies on noopept showed that it not only failed to inhibit cholinesterase activity *per se*, but that it also increased neuronal membrane sensitivity to acetylcholine applied microiontophoretically (10^{-9}–10^{-11} M) to isolated neurons of *Helix lucorum* (Ostrovskaya *et al.*, 2002). Specific mechanisms underlying this effect (such as a direct cholinomimetic action and/or enhancement of acetylcholine release or synthesis) have yet to be clarified. However, the structural similarity of noopept to known cholinergic receptor allosteric modulators and channel activators such as ABT-418 and ABT-089 (Decker *et al.*, 1997; Potter *et al.*, 1999; Reuter *et al.*, 2004) reinforce the possibility of a cholinomimetic mechanism in this dipeptide's mnemotropic profile. Another conceivable mechanism underlying noopept memory enhancing properties focuses on its metabolism. Thus, a pharmacokinetic study of noopept has demonstrated that the parent molecule gives rise to the formation of a stable metabolite (cyclo-prolylglycine), which is an endogenous mnemotropic cyclic dipeptide (Gudasheva *et al.*, 1996). These properties of noopept may well contribute towards restoration of spatial memory impaired by bulbectomy. Noopepts ability to attenuate the OB-induced deficit of spatial memory in the water maze is consistent with its positive effect on another model of contextual-dependant behaviour, conditioned freezing (Vysotskii *et al.*, 1999).

Immunological effect of noopept in OB mice

In ELISA experiments we have demonstrated that administration of noopept in bulbectomized mice increased antibody production to the specific marker of AD, prefibrillar amyloid of $A\beta_{(25–35)}$ peptide by a two-fold factor compared to controls. The effect of noopept in SO animals was however even more pronounced, the level of these antibodies being raised five-fold. NMRI mice are characterized by low immune reactivity (Freisleben *et al.*, 1994**)**, while OB induces additional immunosuppression (Novoselova *et al.*, 2004). This is consistent with a significant inhibition of immune reactions reported in advanced AD patients with severe cognitive impairment (Nitsch, 2004). It is noteworthy that using ELISA, we have analysed the prefibrillar amyloid species of $A\beta_{(25–35)}$ peptide, the morphology of which was evaluated by AFM. The morphology of the $A\beta_{(25–35)}$ peptide oligomers produced in our studies closely resemble that of $A\beta_{(1–40)}$ and $A\beta_{(1–42)}$ peptides (Klein *et al.*, 2002; Lashuel *et al.*, 2003; Stine *et al.*, 2003). It has also been demonstrated that the oligomers of both $A\beta_{(25–35)}$ and full-length Aβ peptides exert similar neurotoxic effects (Pike *et al.*, 1995; Kirkitadze *et al.*, 2002; Wu *et al.*, 2004). Aβ peptide prefibrillar aggregates have not only been found in soluble brain extracts from AD patients but also in transgenic mouse models displaying signs of neurotoxicity and memory impairment (Lesne *et al.*, 2006). They appear to disrupt synaptic signalling which induces an early-stage memory loss, leading subsequently to a broader scale effect including synaptic degeneration and nerve cell death (Wang *et al.*, 2001; Kirkitadze *et al.*, 2002). Immune-targeting of Aβ prefibrillar amyloid oligomers by vaccination has proved to be effective in neutralizing this type of neurotoxicity of amyloid oligomers in rabbits (Lambert *et al.*, 2001; Klein, 2002) and also in attenuating AD-like pathology in transgenic mice (Schenk *et al.*, 1999; Selkoe and Schenk, 2003; Buttini *et al.*, 2005). Thus, clearance of primary pathogenic Aβ peptide amyloid species would be a particularly important step in AD treatment.

Comparison of the behavioural and immunological data obtained for each individual animal in this study did not disclose any significant direct correlation between the degree of memory deficit and the level of antibodies to oligomers of $A\beta_{(25–35)}$ peptide. The data obtained probably testify that noopept possesses dual activity both as a cognitive enhancer and an immune stimulator, but the mechanisms underlying the mnemotropic and immunotropic effects may be different and independent of each other.

In our experiments, the immune response to lysozyme amyloid, which has been selected as an AD non-specific marker of protein aggregation (Gruden *et al.*, 2004), was not elevated either in OB or SO mice treated with noopept. Similar to $A\beta_{(25–35)}$ peptide, the amyloid sample of equine lysozyme contained only prefibrillar species including oligomers and protofilaments (Malisauskas *et al.*, 2003). Amongst them, the transient oligomers of equine lysozyme shared the cytotoxic activity of Aβ peptides (Malisauskas *et al.*, 2005). Thus, the immune reaction in both the AD and SO brain injury models was specific to $A\beta_{(25–35)}$ peptide prefibrillar species implicated in AD pathology but not to other amyloid aggregates.

We did not detect any significant changes in antibody level to S100b after noopept treatment either in OB or SO mice. However, S100b is associated with some features of AD where it is thought to play a role in neuritic pathology (Zimmer *et al.*, 2005). Indeed, the brain level of S100b is augmented during AD development possibly via raised secretion or release from damaged astrocytes, even though this neurotrophic glial factor is rapidly utilized by neurons (Petzold *et al.*, 2003; Rothermund *et al.*, 2003). We suggest that the lack of immunoreactivity towards S100b in any of our operated animals may have arisen from an overall suppression of the immune system (Novoselova *et al.*, 2004).

This study provides evidence that noopept restores spatial memory following bulbectomy and that this action is accompanied by a positive immunotropic effect with respect to Aβ peptide prefibrillar amyloid, known to play a causative role in the death of cholinergic neurons in AD development (Cacabelos *et al.*, 2000). Current results on neurotropic and immunotropic activity of noopept together with its cholinomimetic activity (Ostrovskaya *et al.*, 2002) and neuroprotective role against oxidative stress and Aβ peptide (Pealsman *et al.*, 2003) suggest that the dipeptide may have different underlying molecular mechanisms of memory

improvement. The findings of this study endorse the idea that small antiapoptotic molecules of this nature hold promise for the causal treatment of neurodegenerative diseases (Waldmeier, 2003) and this includes AD-associated cognitive impairment.

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