Study of the cytotoxicity and antioxidant capacity of N/OFQ(1–13)NH₂ and its structural analogues

Margarita Kirkova, Rositsa Zamfirova, Monika Leśkiewicz¹, Marta Kubera¹, Władysław Lasoń¹, Simeon Todorov

Institute of Neurobiology, Bulgarian Academy of Sciences, Acad. G. Bonchev 23, 1113 Sofia, Bulgaria

¹Institute of Pharmacology, Polish Academy of Sciences, Smętna 12, PL 31-343 Kraków, Poland

Correspondence: Simeon Todorov, e-mail: simeon@bio.bas.bg

Abstract:

The effect of side-chain shortening of N/OFQ(1–13)NH₂ at position 9 ([Orn⁹]N/OFQ(1–13)NH₂, [Dab⁹]N/OFQ(1–13)NH₂, [Dap⁹]N/OFQ(1–13)NH₂) was studied regarding potential toxicity and antioxidant capacity. Staurosporine- and H₂O₂-induced damage of SH-SY5Y neuroblastoma cells was not changed in the presence of N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂, but was strongly enhanced in the presence of [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂. Moreover, treatment of cells with the latter two analogues alone led to cell injury. Neuropeptide-dependent differences in the viability of SH-SY5Y cells were also observed, i.e., a cytoprotective effect was observed only in the presence of N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂. Compared to [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂, the effects of N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂ were more beneficial in systems generating free oxygen radicals (O₂^{•−} and •OH), as well as on the antioxidant status of rat brain and liver. Taken together, our findings show that N/OFQ(1–13)NH₂ and its structural analogue [Orn⁹]N/OFQ(1–13)NH₂ possess more favorable profiles than the other two nociceptin (N/OFQ) analogues. The present results suggest that shortening of the side-chain of N/OFQ(1–13)NH₂ might increase cell damage and reduce the viability of SH-SY5Y neuroblastoma cells. Moreover, such alterations may lead to changes in free-oxygen generating systems and in antioxidant status in animal tissues.

Key words:

N/OFQ(1–13)NH₂, [Orn⁹]N/OFQ(1–13)NH₂, [Dab⁹]N/OFQ(1–13)NH₂, [Dap⁹]N/OFQ(1–13)NH₂, SH-SY5Y neuroblastoma cells, lipid peroxidation, glutathione; antioxidant enzyme activities

Abbreviations: Glu-6-P-DH – glucose-6-phosphate dehydrogenase, GSH – glutathione, GSH-Px – glutathione peroxidase, GSSG-Red – glutathione reductase, LP – lipid peroxidation, MDA – malondialdehyde, N/OFQ – nociceptin, OS – oxidative stress, ROS – reactive oxygen species, SOD – superoxide dismutase, TBARs – thiobarbituric acid reactive substances

Introduction

Nociceptin (N/OFQ) is a potent endogenous agonist for the opioid-like receptor, nociceptin/orphanin peptide receptor (NOP receptor). The NOP receptor is

a G-protein-coupled receptor with a high degree of structural homology to classical opioid receptors [11, 30]. Despite the high degree of sequence similarity between N/OFQ and opioid peptides (as dynorphin A), N/OFQ does not interact with μ -, δ - or κ -opioid receptors, and opioid peptides do not interact with the NOP receptor [5]. Different mechanisms of action are reported for each ligand. For example, the vasodilator responses to endomorphins are mediated, in large part, by the release of nitric oxide (NO), whereas the vasodilator responses to N/OFQ are mediated by an N (G)-nitro-L-arginine methyl ester (L-NAME)-insensitive mechanism [9].

It is well known that N/OFQ regulates many central functions, such as pain transmission, learning and memory, fear and anxiety, as well as feeding and locomotor activity. Its hyperalgesic and anti-opioid effects, and its effects on neuropathic pain, are well studied [10, 18, 26, 28, 29, 34]. However, data regarding the toxicity and pro- or anti-oxidant ability of N/OFQ are very scarce, especially when compared to information for opioid peptides. The inhibition of cell growth and the induction of apoptosis is suggested to be one of the mechanisms of opioid neurotoxicity [44].

Excitotoxicity and oxidative stress (OS) are considered to be the major mechanisms of neuronal cell death in neurodegenerative disorders such as Parkinson's and Alzheimer's diseases. Reactive oxygen species (ROS), generated extracellularly or intracellularly by various mechanisms, are risk factors that initiate and promote neurodegeneration [16, 38, 39].

The formation of superoxide and other ROS (or oxygen-derived free radicals), which may be generated by the release of endogenous opioid peptides (EOP) or by EOP/EOP receptor interactions, is suggested to be one of the major arrhythmogenic factors in ischemic hearts [42]. It has been found that ROS induce the expression of two neuropeptide genes: the opioid proenkephalin and the opioid-related proorphantin (also known as pronociceptin), suggesting a role for nociceptin in injury and stress responses of the central nervous system and in neuropathophysiological conditions [35]. A dramatic increase in N/OFQ gene expression has also been demonstrated in astrocytes, in response to inflammatory mediators and OS [4]. It has been reported that N/OFQ, in concentrations that are observed in cerebrospinal fluid after fluid percussion brain injury (FPI), increases superoxide (O_2^-) production in a cyclooxygenase-dependent manner [2, 22]; it also increases pyruvate kinase C (PKC)-dependent O_2^- production after insult [1]. According to Kulkarni and Armstead [22], superoxide generation links N/OFQ release to impaired N-methyl-D-aspartate cerebral vasodilation after brain injury. Using a battery of behavioral tests, Marti et al. [27] showed that co-administration of the NOP receptor antagonist J-113397 and L-DOPA to 6-hydroxydopamine hemilesioned rats produces an additive attenuation of Parkinson's symptoms. Based on these results, the authors suggest that the beneficial/protective effect of NOP receptor ligands may be due, at least in part, to antioxidant mechanisms.

It remains unknown whether N/OFQ can alter endogenous cell antioxidant systems (enzyme and non-enzyme) and lipid peroxidation. Many novel NOP-receptor ligands have been obtained by N/OFQ-sequence modifications and have been synthesized in the past few years [7, 12, 13, 32]. In a previous investigation [31], we tested the effects of N/OFQ(1–13)NH₂ and its structural analogues [Orn⁹]N/OFQ(1–13)NH₂, [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂ on rat vas-deferens (r.v.d.) preparations. The basic amino acid Lys in N/OFQ(1–13)NH₂ was replaced by Orn (ornitin), Dab (diaminobutanoic acid) or Dap (diaminopropanoic acid), respectively. We showed that side-chain shortening at position 9 increases ([Orn⁹]N/OFQ(1–13)NH₂) or decreases ([Dab⁹]N/OFQ(1–13)NH₂, [Dap⁹]N/OFQ(1–13)NH₂) the peptide's affinity to NOP receptors.

The aim of the current study was to investigate the possible correlation between the affinity, antioxidant activity (in free radical generating systems and in brain and liver) and putative toxicity (damage and viability) of the previously synthesized peptides in a neuroblastoma cell-line SH-SY5Y, expressing "orphan" opioid receptor [6, 40]).

Materials and Methods

Materials

Neuroblastoma cell-line SH-SY5Y was obtained from the American Type Culture Corporation (ATCC). NADP⁺, NADPH, reduced and oxidized glutathione, 2-thiobarbituric acid and riboflavin were obtained from Sigma-Aldrich (Germany). All other reagents used were analytical grade. N/OFQ(1–13)NH₂ and its analogues [Orn⁹]N/OFQ(1–13)NH₂, [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂ were synthesized in the Department of Organic Chemistry, University of Chemical Technology and Metallurgy (Sofia, Bulgaria). The solutions of the neuropeptides were freshly prepared in saline before each experiment. All solutions, used in the study, were prepared with over-glass re-distilled water.

Animals

The experiments were performed in male Wistar rats, each weighing 180–200 g, and housed at 22–25°C. The animals were allowed an adaptation period, with free access to food and water, and with a natural day/night light cycle. Prior to the experiments, the animals were fasted for 24 h with ad-libitum water-access. The animals were sacrificed under light ether anesthesia. The experiments have been performed according to the “Principles of laboratory animal care” (NIH publication No. 85–23, revised 1985), and the rules of the Ethics Committee of the Institute of Physiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

Preparations

SH-SY5Y neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Germany), supplemented with 10% fetal bovine serum (Gibco-BRL, Germany), 100 units/ml of penicillin and 100 µg/ml streptomycin (Sigma Co, USA), and kept in humidified atmosphere of 5% CO₂/95% O₂ at 37°C. After reaching 80% confluence, the cells were seeded onto appropriate multi-well plates at a density of 4×10^4 cells per 0.2 ml.

Rat brains were carefully removed, washed with cold 0.15 M KCl, homogenized in cold 0.15 M KCl–10 mM potassium phosphate buffer (pH 7.4) and centrifuged for 10 min at 3,000 rpm to obtain the “post-nuclear” homogenate.

Liver “post-nuclear” homogenate was also obtained after liver perfusion with cold 0.15 M KCl, followed by homogenization and centrifugation. Brain and liver homogenates were used for quantitative measurement of glutathione levels and lipid peroxidation. Part of these “post-nuclear” homogenates was centrifuged at 12,000 rpm for 20 min and antioxidant enzyme activities were measured in the supernatants.

Experimental design

The effects of the neuropeptides on SH-SY5Y neuroblastoma cell damage and cell viability were tested, using lactate dehydrogenase (LDH) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromate (MTT) assays, respectively. One day before the LDH assay the culture medium was changed to me-

dium supplemented with 1 % FBS. One hour before the addition of 0.5 µM staurosporine (Sigma Co, USA) or 0.5 mM hydrogen peroxide, SH-SY5Y cells were pre-treated with increasing concentrations (0.01–10 µM) N/OFQ or its structural analogues in 96-well plates. SH-SY5Y damage and viability was measured 24 h latter.

The effects of neuropeptides (0.1–100 µM) were also tested in free radical-generating systems, as well as on the antioxidant status of rat brain and liver (homogenates and supernatants) after a 30-min incubation at 30°C.

Analytical methods

LDH activity was estimated by a colorimetric assay (Cytotoxicity Detection Kit; Roche Diagnostic GmbH) that measures the amount of colored hydrazone, formed in a reaction of pyruvic acid with 2,4-dinitrophenylhydrazine. The intensity of the red color formed in this assay was measured at 490 nm. The color intensity was proportional to LDH activity and to the number of damaged cells.

The MTT assay was based on the enzyme-dependent conversion of a yellow tetrazolium salt to a colored formazan product, the concentration of which is proportional to the number of viable cells [21]. Cells (1×10^4 cells/well) in 96-well plates were incubated with increasing doses of neuropeptides at 37°C in 5% CO₂ for 24 h. MTT was added to each well (at a final concentration of 0.15 mg/ml) and incubated for 1 h at 37°C, before solubilizing the dye with 0.1 N HCl in isopropanol. The absorbance of each sample was measured at 570 nm in a 96-well plate-reader (Multiscan, Thermo Labsystems, Finland). The optimal density of formazan formed in control (untreated) cells was taken as 100% viability.

The deoxyribose-test was carried out according to the method of Halliwell et al. [15]. Hydroxyl radicals were generated in the following medium: 0.1 mM FeCl₃, 0.1 mM EDTA, 0.1 mM ascorbic acid, 0.5 mM H₂O₂ and 3.4 mM deoxyribose (DR) in 20 mM potassium phosphate buffer, pH 7.2 and in the presence of increasing concentrations of neuropeptides. After incubation of the samples at 37°C for 30 min, the degradation of DR, a detector of ·OH radicals, was measured in terms of the formation of thiobarbituric acid reactive substances (TBARSs). The samples were heated at 100°C for 15 min to develop the color and (after cooling) the absorbance at 532 nm was read

against appropriate blanks. A_{600} was considered a non-specific baseline drift and was subtracted from A_{532} .

The reduction of nitro-blue tetrazolium (NBT) by photochemically produced O_2^- radicals, generated in a riboflavin/methionine-, was measured in a medium containing: 50mM potassium phosphate buffer, pH 7.8, 1.17×10^{-6} M riboflavin, 0.2 mM methionine, 2×10^{-5} M KCN, 5.6×10^{-5} M NBT in the absence or presence of different neuropeptide concentrations [3].

Protein content was measured by the method of Lowry et al. [25].

Lipid peroxidation (LP) was determined according to the method of Hunter et al. [17] by measuring the amount of TBARs, formed in fresh “post-nuclear” homogenates (mg protein/ml) after 60 min-incubation at 37°C, and expressed in nmoles malondialdehyde (MDA)/mg protein. The absorbance at 600 nm was considered a non-specific baseline and was subtracted from A_{532} .

Total glutathione (GSH) level, measured according to Tietze [41], was expressed as ng/mg protein using GSSG as a reference standard.

Glutathione peroxidase (GSH-Px) activity, measured by the method of Gunzler et al. [14], was expressed in nmoles NADPH oxidized per min per mg protein, using a molar extinction coefficient of 6.22×10^6 M/cm.

Glutathione reductase (GSSG-Red) activity measured by the method of Pinto and Bartley [33] was expressed in nmoles NADPH oxidized per min per mg

protein, using a molar extinction coefficient of 6.22×10^6 M/cm.

Glucose-6-phosphate dehydrogenase (Glu-6-PDH) activity, determined after Cartier et al. [8], was expressed in nmoles $NADP^+$ reduced per min per mg protein, using a molar extinction coefficient of 6.22×10^6 M/cm.

Superoxide dismutase (SOD) activity, determined according to Beauchamp and Fridovich [3], was expressed in U/mg protein (one unit of SOD activity is the amount of the enzyme, producing a 50%-inhibition of nitro-blue tetrazolium – reduction).

Statistical analysis

The data were statistically analyzed by one-way ANOVA (Dunnett post-test), with $p < 0.05$ established as the minimum level of statistical significance.

Results

The effects of increasing concentrations (0.01– 10 μ M) of N/OFQ(1–13) NH_2 and its structural analogues – [Orn⁹]N/OFQ(1–13) NH_2 , [Dab⁹]N/OFQ(1–13) NH_2 and [Dap⁹]N/OFQ(1–13) NH_2 , on staurosporine- and H_2O_2 -induced damage in SH-SY5Y cells are presented on Figure 1. In accordance with previous studies [19, 23, 24], the release of LDH was enhanced (by nearly

Fig. 1. Effects of N/OFQ(1–13) NH_2 and its structural analogues on staurosporine (A)- and H_2O_2 (B)-induced damage in SH-SY5Y neuroblastoma cells. The release of lactate dehydrogenase (LDH) was measured at 490 nm. Values represent the mean $\pm$ SEM of 8–10 individual samples. Statistically significant differences vs. controls at * $p < 0.05$

Fig. 2. Effects of N/OFQ(1–13)NH₂ and its structural analogues on the viability of SH-SY5Y neuroblastoma cells. Cell viability (using MTT test) was measured at 570 nm. Values represent the mean $\pm$ SEM of 8–10 separate samples. Statistically significant differences vs. controls at * $p < 0.05$

50%) after staurosporine- or H₂O₂-induced cell injury. N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂ (in all concentrations, used) had no effect on LDH release in staurosporine-treated SH-SY5Y cells (Fig. 1A). [Orn⁹]N/OFQ(1–13)NH₂ also did not change LDH release in H₂O₂-treated SH-SY5Y cells, while the other two analogues – [Dab⁹]N/OFQ (1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂ (in their higher concentrations) increased LDH release (Fig. 1B). One reason for this effect may be the additive effect of both H₂O₂ and peptide analogues, because 10 μ M [Dab⁹]N/OFQ (1–13)NH₂ or 10 μ M [Dap⁹]N/OFQ(1–13)NH₂ alone increased LDH release by about $28 \pm 6.9\%$ and $33 \pm 4.6\%$, respectively.

Using the MTT test, the effects of the above neuropeptides (0.01–10 μ M) on SH-SY5Y cell viability were also studied. As seen in Figure 2, N/OFQ(1–13)

NH₂ and [Orn⁹]N/OFQ(1–13)NH₂, but not [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂, provided a cytoprotective effect (Fig. 2); the latter was observed even at the lowest peptide concentration.

In the above concentrations (0.1–10 μ M), neither N/OFQ(1–13)NH₂ nor its structural analogues exerted effects in free oxygen radical (O₂^{•−} and $\cdot$ OH) generating systems (Fig. 3). However, the presence of 100 μ M peptide in the reaction mixture decreased O₂^{•−} production by more than 40% (Fig. 3A). A concentration of 100 μ M of the neuropeptides tended to decrease $\cdot$ OH production, but a considerable and more clearly expressed inhibitory effect was observed in the presence of 1 mM peptide (Fig. 3B).

The effects of N/OFQ(1–13)NH₂ and its structural analogues on the antioxidant status of the rat brain and liver were also studied in the presence of different

Fig. 3. Effects of N/OFQ(1–13)NH₂ and its structural analogues in (A) superoxide radical generating system; (B) hydroxyl radical generating system. O₂^{•−}-depending NBT (nitro-blue tetrazolin) reduction was measured at 560 nm; $\cdot$ OH-depending DR degradation was measured at 532 nm. Values represent the mean $\pm$ SEM of 7–9 separate experiments. Statistically significant differences vs. controls at * $p < 0.05$

Tab. 1A. *In-vitro* effects of N/OFQ(1–13)NH₂ and its structural analogues on antioxidant enzyme activities in rat brain. Antioxidant enzyme activities were measured after a 30 min incubation at 30 °C with different neuropeptide concentrations. Values represent the mean ± SEM of 5–6 animals. Statistically significant differences vs. controls at * p < 0.05

Enzymes	Neuropeptides	without peptide	1.10 ⁻⁷ M peptide	1.10 ⁻⁶ M peptide	1.10 ⁻⁵ M peptide	1.10 ⁻⁴ M peptide
GSH-Px (nmoles NADP(H)/min/mg protein)	N/OFQ(1–13)NH ₂	34.0 ± 3.12	30.9 ± 2.22	32.4 ± 2.58	35.3 ± 3.75	35.1 ± 2.97
	[Orn ⁹]N/OFQ(1–13)NH ₂		29.8 ± 1.54	29.6 ± 2.41	29.2 ± 3.13	32.9 ± 2.08
	[Dab ⁹]N/OFQ(1–13)NH ₂		–	–	30.0 ± 2.01	31.0 ± 1.21
	[Dap ⁹]N/OFQ(1–13)NH ₂		–	–	32.7 ± 1.51	34.0 ± 1.64
GSSG-Red (nmoles NADP(H)/min/mg protein)	N/OFQ(1–13)NH ₂	15.2 ± 0.62	14.1 ± 0.61	14.1 ± 0.54	14.6 ± 0.88	14.6 ± 0.90
	[Orn ⁹]N/OFQ(1–13)NH ₂		14.1 ± 0.63	13.6 ± 0.74	13.5 ± 0.71	13.9 ± 1.02
	[Dab ⁹]N/OFQ(1–13)NH ₂		–	–	15.3 ± 0.25	16.2 ± 0.68
	[Dap ⁹]N/OFQ(1–13)NH ₂		–	–	15.5 ± 0.38	15.6 ± 0.18
Glu-6-P-DH (nmoles NADP(H)/min/mg protein)	N/OFQ(1–13)NH ₂	20.1 ± 0.63	21.0 ± 0.53	19.8 ± 0.53	21.6 ± 0.77	20.6 ± 0.58
	[Orn ⁹]N/OFQ(1–13)NH ₂		20.9 ± 0.77	21.8 ± 0.82	19.2 ± 0.64	20.0 ± 0.90
	[Dab ⁹]N/OFQ(1–13)NH ₂		–	–	18.8 ± 1.06	19.5 ± 0.38
	[Dap ⁹]N/OFQ(1–13)NH ₂		–	–	19.5 ± 1.00	19.9 ± 1.00
SOD (U/mg protein)	N/OFQ(1–13)NH ₂	18.6 ± 0.49	20.2 ± 1.31	18.7 ± 0.66	19.6 ± 0.64	20.4 ± 0.68
	[Orn ⁹]N/OFQ(1–13)NH ₂		18.6 ± 0.60	18.9 ± 0.81	18.0 ± 0.96	20.7 ± 0.96
	[Dab ⁹]N/OFQ(1–13)NH ₂		–	–	19.6 ± 1.13	18.9 ± 0.99
	[Dap ⁹]N/OFQ(1–13)NH ₂		–	–	19.4 ± 0.97	19.5 ± 0.71

Tab. 1B. *In-vitro* effects of N/OFQ(1–13)NH₂ and its structural analogues on antioxidant enzyme activities in rat liver. Antioxidant enzyme activities were measured after a 30 min incubation at 30 °C with different neuropeptide concentrations. Values represent the mean ± SEM of 5–6 animals. Statistically significant differences vs. controls at * p < 0.05

Enzymes	Neuropeptides	Without peptide	1.10 ⁻⁷ M peptide	1.10 ⁻⁶ M peptide	1.10 ⁻⁵ M peptide	1.10 ⁻⁴ M peptide
GSH-Px (nmoles NADP(H)/min/mg protein)	N/OFQ(1–13)NH ₂	176 ± 10	180 ± 10	179 ± 9	183 ± 9	171 ± 9
	[Orn ⁹]N/OFQ(1–13)NH ₂		165 ± 12	159 ± 10	162 ± 5	162 ± 11
	[Dab ⁹]N/OFQ(1–13)NH ₂		–	–	182 ± 13	176 ± 12
	[Dap ⁹]N/OFQ(1–13)NH ₂		–	–	181 ± 14	178 ± 16
GSSG-Red (nmoles NADP(H)/min/mg protein)	N/OFQ(1–13)NH ₂	35.8 ± 2.06	35.6 ± 2.38	37.1 ± 1.23	37.3 ± 1.64	37.5 ± 1.84
	[Orn ⁹]N/OFQ(1–13)NH ₂		35.7 ± 2.05	35.9 ± 1.00	37.4 ± 1.41	35.9 ± 1.91
	[Dab ⁹]N/OFQ(1–13)NH ₂		–	–	29.5 ± 2.86	29.8 ± 3.69
	[Dap ⁹]N/OFQ(1–13)NH ₂		–	–	35.2 ± 1.58	36.7 ± 1.46
Glu-6-P-DH (nmoles NADP(H)/min/mg protein)	N/OFQ(1–13)NH ₂	24.8 ± 2.17	26.7 ± 2.61	26.0 ± 2.82	25.2 ± 2.57	25.2 ± 2.44
	[Orn ⁹]N/OFQ(1–13)NH ₂		25.4 ± 2.13	26.3 ± 2.54	27.6 ± 2.38	23.3 ± 2.53
	[Dab ⁹]N/OFQ(1–13)NH ₂		–	–	25.4 ± 2.08	22.6 ± 3.11
	[Dap ⁹]N/OFQ(1–13)NH ₂		–	–	23.1 ± 2.92	25.1 ± 3.28
SOD (U/mg protein)	N/OFQ(1–13)NH ₂	69.5 ± 3.06	70.9 ± 4.18	67.0 ± 3.25	69.8 ± 3.41	67.3 ± 3.21
	[Orn ⁹]N/OFQ(1–13)NH ₂		70.0 ± 6.19	74.8 ± 7.58	74.7 ± 7.03	67.7 ± 5.48
	[Dab ⁹]N/OFQ(1–13)NH ₂		–	–	62.5 ± 1.25	62.0 ± 1.46
	[Dap ⁹]N/OFQ(1–13)NH ₂		–	–	65.0 ± 2.05	60.0 ± 3.14

Fig. 4. Effects of N/OFQ(1–13)NH₂ and its structural analogues on spontaneous lipid peroxidation (A) and glutathione levels (B) in rat brain. Lipid peroxidation and glutathione levels were measured after a 30-min incubation at 30°C with different neuropeptide concentrations. Values represent the mean $\pm$ SEM of 5–6 animals. Statistically significant differences vs. controls at * $p < 0.05$

concentrations neuropeptides (0.1–100 μ M). The activities of the antioxidant enzymes were not changed by the neuropeptides tested (Tab. 1A, 1B). However, unlike N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂, 100 μ M of [Dab⁹]N/OFQ(1–13)NH₂ or [Dap⁹]N/OFQ(1–13)NH₂ increased spontaneous LP (Fig. 4A) and decreased GSH levels in the brain (Fig. 4B). In liver, LP was decreased in the presence only of N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂ (Fig. 5A), while GSH levels were not changed by all neuropeptides (Fig. 5B).

Discussion

The present study showed that the effects of N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂ on the damage and viability of SH-SY5Y cells were different from those of the other two N/OFQ analogues – [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂. Judging by the extent of LDH release (an index for cell damage), staurosporine- or H₂O₂-induced damage of SH-SY5Y cells was not altered in the presence of N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂, but was strongly enhanced in the presence of [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂. Moreover, the latter two analogues alone led to cell injury. These data are in agreement with the observed differences in the effects of the tested neuropeptides on the viability of SH-SY5Y cells – a cytoprotective effect was observed only in the presence of the natural

neuropeptide N/OFQ(1–13)NH₂ and its analogue [Orn⁹]N/OFQ(1–13)NH₂.

At concentrations of 0.1–10 μ M, N/OFQ(1–13)NH₂ and its structural analogues showed no effects in chemical systems, generating O₂^{•-} or $\cdot$ OH radicals. However, a decreased production of O₂^{•-} and $\cdot$ OH radicals was observed in the presence of higher neuropeptide concentrations (100 μ M and 1 mM). Moreover, the radical-scavenging activity of N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂ was stronger than that of [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂. Taken together, these findings suggest that N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂ promote more beneficial effects in cells, as compared to the other two N/OFQ analogues.

It is well known that enhanced ROS production and OS can lead to cell damage, including that induced by treatment with H₂O₂ or staurosporine. Staurosporine, a potent protein kinase C inhibitor with a broad spectrum of activity [36], is known to induce apoptosis [37, 43] and to cause OS through mitochondrially generated ROS in a variety of cells [20]. Under normal conditions, ROS-induced damage is kept in check by an efficient array of cell antioxidant systems, whereas under pathological conditions, the oxidant/antioxidant balance is altered, either primarily or secondarily. The disturbance of this balance is usually evaluated by changes in the levels of endogenous cell antioxidant systems (enzymatic and non-enzymatic), as well as by changes in LP levels. This is the reason why neuropeptide effects on different antioxidant indices in the rat brain and liver were also studied.

Fig. 5. Effects of N/OFQ(1–13)NH₂ and its structural analogues on spontaneous lipid peroxidation (A) and glutathione level (B) in rat liver. Lipid peroxidation and glutathione levels were measured after a 30-min incubation at 30 °C with different neuropeptide concentrations. Values represent the mean ± SEM of 5–6 animals. Statistically significant differences vs. controls at * $p < 0.05$

In these *in-vitro* experiments, the following findings were obtained: i) in the rat brain and liver, the peptides (0.1–100 μM) had no effects on antioxidant enzyme activities; ii) in the brain, 100 μM N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂ had no effects, while [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂ increased spontaneous LP and decreased GSH levels, thus manifesting a pro-oxidant activity; iii) in the liver, N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂ decreased LP, demonstrating an antioxidant effect, while [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂ did not induce any changes. It should be mentioned that these effects were established only in the presence of high (non-physiological) peptide concentrations. Nevertheless, the effects of N/OFQ(1–13)NH₂ and [Orn⁹]N/OFQ(1–13)NH₂ were again favorable, as compared to those of [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂.

The peptide analogues, tested in the present study, differed from N/OFQ(1–13)NH₂ in their structure, i.e. the Lys at position 9 was substituted with Orn, Dab or Dap. Orn is a structural analogue of Lys, with a side-chain that is shorter by one CH₂-group. This might be the reason for the observed similarities between the effects of [Orn⁹]N/OFQ(1–13)NH₂ and N/OFQ(1–13)NH₂ found in our previous [31] and present studies. According to Naydenova et al. [31], the strong inhibitory effect of [Orn⁹]N/OFQ(1–13)NH₂ on smooth-muscle activity is related to its increased affinity to the receptor. It is thought that the interaction with the receptor is facilitated as a result of a better steric location of the NH₂-group in the N/OFQ chain (position

9). The subsequent shortening of side-chain length (in the case of [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂) decreases the distance between the N/OFQ basic chain and the receptor active center and additionally reduces its inhibitory activity. In accordance with the above data, the present results suggest that the shortening of N/OFQ side-chain length might also contribute to increase cell damage and reduce the viability of SH-SY5Y neuroblastoma cells. Such effects may be mediated by a decrease in free-oxygen scavenging activity and changes in antioxidant status in animal tissues.

Acknowledgments:

This study was performed within the framework of the Agreement of Scientific Cooperation between the Bulgarian Academy of Science and the Polish Academy of Science. The research was supported by Grant L-1510/2005 of the National Research Fund, Sofia, Bulgaria. The authors wish to thank Prof. Vesnikov and Assoc. Prof. Naydenova (Department of Organic Chemistry, University of Chemical Technology and Metallurgy, Sofia, Bulgaria) for synthesis and the characterization of N/OFQ(1–13)NH₂ and its analogues [Orn⁹]N/OFQ(1–13)NH₂, [Dab⁹]N/OFQ(1–13)NH₂ and [Dap⁹]N/OFQ(1–13)NH₂.

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Received:

May 25, 2009; in revised form: November 16, 2009.