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N-Acetylserotonin, Melatonin and Their Derivatives Improve Cognition and Protect against p-Amyloid-Induced Neurotoxicity

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ABSTRACT: **After a single injection of cholinergic neurotoxin ethylcholine aziridinium (AF64A, 3 nmol intracerebroventricularly** (i.c.v.)), **rats failed to perform the tasks in the active avoidance (learning and retention paradigms) and water maze tests. N-Acetylserotonin** (NAS), **melatonin and their newly synthesized derivatives, CA-15 and CA-18, (0.3-3.0 mg/kg daily for 12-14 days) reversed the effect of AF64A in a dose-dependent manner with CA-18 being the most active. Melatonin and NAS caused sedation absent in CA-18-treated rats. The studied compounds (25-500 μM for 72 hr) protected against β-amyloid peptide (βAP) fragment 25-35-induced neurotoxicity in cerebellar granule cell culture. Our results suggest that neuroprotecting properties of these compounds might mediate their cognition-enhancing effects. The results obtained warrant the further search for the novel types of safe neuroprotectors among the synthetic NAS/melatonin derivatives.**

INTRODUCTION

It is known that dysfunction of circadian rhythms in Alzheimer's disease (AD) can be compensated by exogenous melatonin.[1] It was also suggested that the positive effect of chronic prophylactic administration of melatonin as gerontoprotector is based on its antioxidative properties.[2] Numerous studies indicate that melatonin as free radical scavenger displays pronounced neuroprotective effects against neurotoxic action of the excitatory amino acids (excitotoxicity) and toxic effect of beta-amyloid peptide (βAP)—one of the specific hallmarks of AD.[3] Recently neuroprotective activity was revealed for the melatonin precusor N-acetylserotonin (NAS).[4] Since neurodegenerative processes in AD are associated with the decreased cognitive functions, it was reasonable to study the effect of melatonin, NAS and their newly synthesized derivatives on cognitive functions in animal models of AD-type neurodegeneration.

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In the present study both *in vivo* and *in vitro* models were used. The *in vivo* study involved the neurotoxin-induced animal model of AD, based on observation that intracerebroventricular (i.c.v.) administration of ethylcholine aziridinium ion (AF64A) produced chronic cholinergic hypofunction and learning and memory impairment in rat analogous to that observed in AD.[5] Currently this model is also used for screening of the compounds for their potential cognition-enhancing properties.[6,7] At the cellular level *(in vitro)* the adequate model of AD-type degeneration is believed to be the neuronal cell culture degeneration induced by βAP fragment 25-35.[8] In the present study we examined cognition-enhancing and neuroprotective properties of melatonin, NAS and their newly synthesized derivatives CA-15 and CA-18 in the above-mentioned animal and cell models of AD-type neurodegeneration.

MATERIALS AND METHODS

Chemicals

NAS and melatonin derivatives C A-15 and C A-18 were identified by HI-nuclear magnetic resonance (NMR) and elemental analysis. pAP fragment 25-35 was purchased from Bachem; AF64 was purchased from RBI. All other reagents were purchased from Sigma. Melatonin, NAS and C A-15 and C A-18 were preliminarily dissolved in dimethyl sulfoxide (DMSO) and diluted by water each day prior to use.

Animals

Male Wistar rats (12-16 weeks old, 280-450 g) were used in behavioral experiments. Rats were kept at 12 hr light: 12 hr dark schedule (lights on/off at 4:00/16:00 hr) with free access to water and food.

Rats were anesthetized with ether and placed into a stereotaxic frame before the surgery. Freshly prepared from AF64 solutions of AF64A (3 nmol/3 μl) or vehicle (cerebrospinal fluid, CSF) were injected into each of lateral cerebral ventricles. After surgery, rats were given a recovery period (12 days) before being tested in behavioral experiment. Melatonin, NAS and their derivatives were administrated orally (in starch solutions) once a day around the time of circadian light-off during the whole recovery period.

Behavioral Studies

Active Avoidance Test

Training was conducted in a two-chamber shuttle box according to the procedure described earlier.[7] The conditioned stimulus was a 5-sec light followed by the unconditioned stimulus, a 1-mA shock, which was delivered to the grid in the lit chamber. The rat avoided the shock by crossing through to the other (dark) chamber. The avoidance during the conditioned stimulus was considered as a correct response. Training procedure consisted of 35 trials (learning test). Fifteen further trials were given 24 hr later (retention test).

TABLE 1. The influence of melatonin, NAS, and its o-benzil homologs on active avoidance performance of the AF64A-treated rats

No.	Compounds	mg/kg	Rats	Correct Responses, %	
				Learning Test	Retention Test
1	Control (i.c.v. CSF)	solvent	33	79.3 ±5.4*	76.3 + 6.3*
2	AF64A alone	solvent	31	37.4 ±9.8	43.8 + 8.1
3	Melatonin	3	9	75.4 ±7.0**	72.2 ±5. 8*
	Melatonin	0.3	11	79.0 ±6.0*	83.0 ±3.2*
4	NAS	1	10	65.4 ±8.8*	56.0 ±9.2
5	C A- 15	3	9	61.4 + 7.4	68.0 ±8.4**
6	C A- 18	1	10	50.0 ±8.8	48.1 ±9.8
	CA-18	0.3	11	84.0 ±5.4*	82.7 ± 5.0*

NOTE: Rats were treated i.c.v. AF64A (3 nmol/ 3µl) or CSF (control groups) and were given a recovery period (12-14 days) before being tested in the behavioral experiment. The experimental rats were orally given the studied compounds during the recovery period. Shuttle-box avoidance performance: *Learning test.* Following 20 acquisition trials, rats were given 3 blocks of 5 trials. *Retention test.* Two blocks of 5 trials were given 24 hr after the learning trial. Data are presented as the mean + SEM percentage of correct responses summarized over each block and were analyzed by a statistical test (ANOVA); **p* <0.001, ***p* <0.05 vs the AF64A-treated group.

Statistical Treatment

Each experimental group, i.e., control, AF64A-treated groups and groups for each concentration of the tested compounds, contained 9-11 rats. Data of the number of correct responses from the last 15 trials of the first 35 trials (learning test) or first 15 trials of the retention test were collected for each rat. The mean ± SEM of correct responses was calculated for total number of rats in groups. Data were analyzed as the mean ± SEM in percentage of the maximum possible number of correct responses (=100%) by analysis of variance (ANOVA) followed by post hoc comparisons.

Morris Water Maze Test

The test was started 2 days after the last injection of tested compounds and was performed daily for the period from 3 to 9 days. Round swimming pool (1.8 m diameter and 0.45 m high) with 22°C water was placed in the center of the room. The platform was located 1 cm below the surface of the water. Starting points for the swims were at the cardinal compass points (N, S, E, W), which were selected in a semirandom fashion for each rat on each trial.

Statistical Treatment

Each experimental group, i.e., control, AF64A-treated groups and groups for each concentration of the tested compounds, contained 9-11 rats. Results were estimated as time required for a rat having fallen into the water pool to reach the plat-

form (a sum of 2 trials from a different position every day). Data were analyzed as the mean ± SEM (ANOVA followed by post hoc comparisons).

Neuronal Cell Culture

Cerebellar granule cells (CGC) were prepared from the postnatal rats (7-8 days old) by the following procedure based on the generally accepted methods.[9] The pieces of cerebellum were digested with 0.25 mg/ml trypsin for 25 min at 37°C and incubated for 5 min in 0.1% soybean trypsin inhibitor. After washing, cells were dissociated by triturating. Following 2 centrifugation-resuspension steps, the cells were plated at a density of 2.5-5 x 10 cells per ml on polylysine-coated 24-well plates (Corning) and maintained at 37°C in a humidified incubator with 5% CO_2/95% room air. The medium was composed of Eagle's minimum essential medium and fetal calf serum (10%) supplemented with 20 mM potassium chloride, 10 mM glucose, 2 mM glutamine, and 50 μg/ml gentamycin sulfate. Cytosine arabinoside (10 μM) was added 24-48 hr later to prevent the replication of nonneuronal cells.

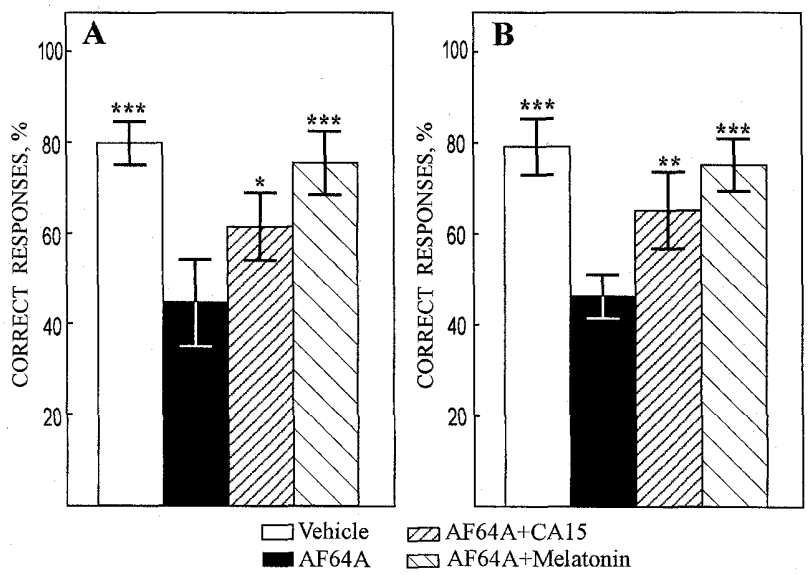

FIGURE 1. Cognition-enhancing effect of melatonin and CA15. Shuttle-box avoidance performance of vehicle-treated rats *(n* = 11), AF64A-treated rats and AF64A-treated rats receiving melatonin or CA-15 (3 mg/kg, once daily, 12-14 days, orally; *n* = 10), where *n* is the number of rats in each group. (A) Learning test. Following 20 acquisition trials, rats were given 3 blocks of 5 trials. (B) Retention test. Two blocks of 5 trials were given 24 hr after the learning trial. Data are presented as mean ± SEM percentage of correct responses summarized over each block; $^{**}p < 0.05$, $^{***}p < 0.001$ vs AF64A-treated group, where *p* is the significance level; *not significant (post hoc ANOVA).

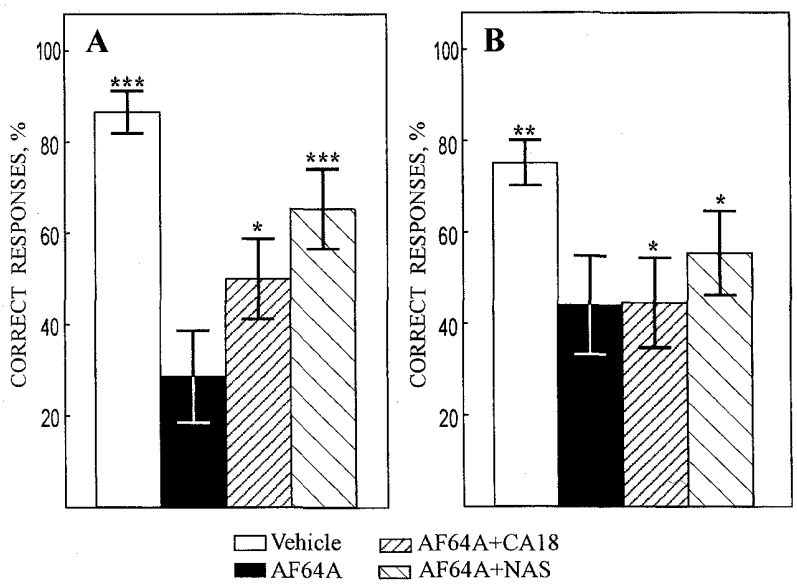

FIGURE 2. Cognition-enhancing effect of CA18 and NAS. Shuttle-box avoidance performance of vehicle-treated rats (n = 11), AF64A-treated rats and AF64A-treated rats receiving NAS or CA-18 (1 mg/kg, once daily, 12-14 days, orally; n = 10), where n is the number of rats in each group. (A) Learning test. Following 20 acquisition trials, rats were given 3 blocks of 5 trials. (B) Retention test. Two blocks of 5 trials were given 24 hr after learning trial. Data are presented as mean + SEM percentage of correct responses summarized over each block; $^{**}p < 0.05$, $^{***}p < 0.001$ vs AF64A-treated group, where p is the significance level; *not significant (post hoc ANOVA).

Toxic Assays

The neurotoxic and neuroprotective effects of NAS, melatonin and their derivatives were tested in mature cultures at 7-8 days *in vitro* (7-8 DIV) after changing of medium to a fresh medium without serum with Supplement N1 (Sigma). The βAP 25-35 (Bachem) was dissolved by sonication in sterilized distilled water at a concentration of 1 mM. Solutions of all reagents were added to the wells with cultures at 25 μM, and the effect was observed during the next days by microscopy.

Quantitative Assessment

Neuronal viability was evaluated by morphometric cell counting using the presence of neurites and smooth, round cell bodies as criteria of survival. The cells were examined under the phase-contrast microscope Axiovert 25C with videocamera and Software miroMedia PCTV VideoCap program for image scanning and photography. Cell survival was quantified by counting the number of viable neurons in pre-marked microscope fields prior to, and 4 days after the exposure. The difference in numbers of living neurons before and after 4 days of treatment was determined.

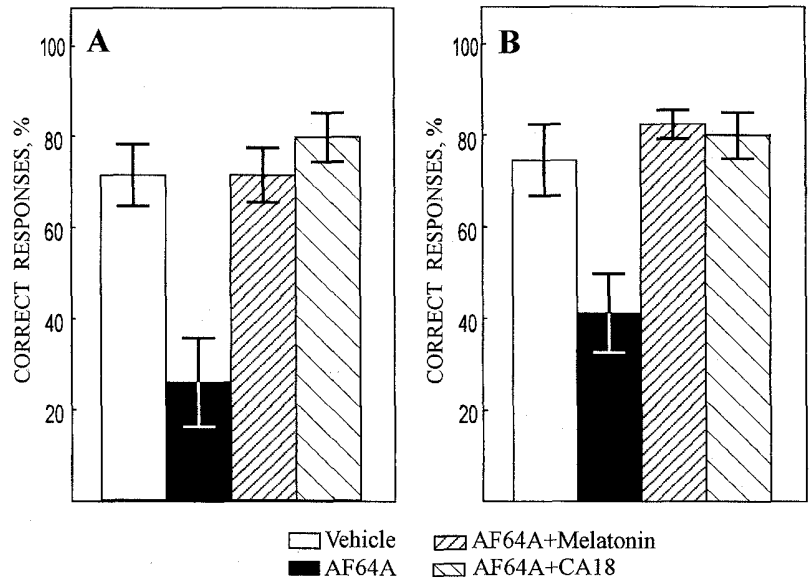

FIGURE 3. Cognition-enhancing effect of melatonin and CA18. Shuttle-box avoidance performance of vehicle-treated rats (n = 11), AF64A-treated rats (n = 10) and AF64A-treated rats receiving melatonin or CA-18 (0.3 mg/kg, once daily, 12-14 days, orally; n = 11), where n is the number of rats in each group. (A) Learning test. Following 20 acquisition trials, rats were given 3 blocks of 5 trials. (B) Retention test. Two blocks of 5 trials were given 24 hr after learning trial. Data are presented as the mean ± SEM percentage of correct responses summarized over each block; p <0.001, where p is the significance level. The values of p illustrate that there was a significant difference in avoidance performance vs AF64A-treated group (post hoc ANOVA).

Statistical Analysis

For each experiment, we used 4-6 separate wells of a 24-well multiwell plate for control (with 0.05% DMSO) and for each concentration of compound or composition of compound with PAP. Each well equaled one observation (average of 25 cells per microscope field). Experiments are repeated 2-3 times (« = 12-18). For graphical presentation, average data from representative experiments were expressed as a percentage of survival cells in comparison with control ± SEM and analyzed by ANOVA and Student t-test.

RESULTS

Active Avoidance Test

AF64A (3 nmol/3 µl i.c.v.) dramatically decreased rats performance in learning and retention paradigms of the active avoidance test (Figs. 1-3, TABLE 1).

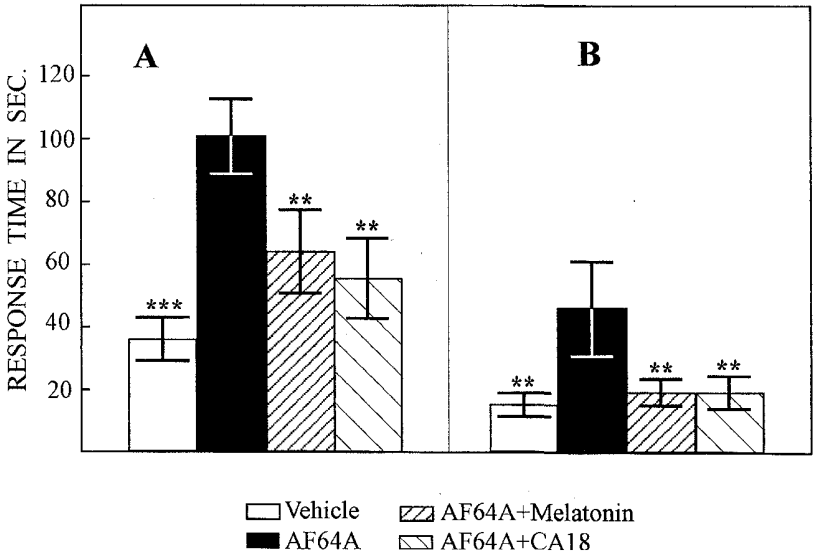

FIGURE 4. Average swim latency made by rats, when trained (A) 1 and (B) 5 days to find a platform in the Morris water maze test. Performance of vehicle-treated rats (n = 11), AF64A-treated rats (n = 10) and AF64A-treated rats receiving melatonin or CA-18 (0.3 mg/kg, once daily, 12-14 days, orally; n = 11) was started 3 days after the last injection of compound and was performed daily during the period from the 3rd to the 7th day (n is the number of rats in each group). Results were estimated as time required for rat having fallen into the water pool to reach a platform; $^{**}p < 0.05$, $^{***}p < 0.001$ vs the AF64A-treated group, where p is the significance level (post hoc ANOVA).

NAS (1 mg/kg per os (p.o.) daily) improved the performance of AF64A-treated rats in learning (Fig. 2A) but not in retention (Fig. 2B) paradigms of the active avoidance test.

The effect of melatonin was studied in 2 doses: 0.3 and 3 mg/kg (p.o. daily). The lower dose of melatonin (0.3 mg/kg) completely restored rats performance in the learning (Fig. 3A) and retention (Fig. 3B) paradigms of the active avoidance test. The cognitive-enhancing effect of the higher dose of melatonin (3 mg/kg) was significant but somewhat less pronounced than the effect of the lower dose (0.3 mg/kg) (Fig. l A and B).

CA-15 (3 mg/kg p.o. daily) improved the performance of AF64A-treated rats in retention paradigm of the active avoidance test (Fig. 1B). CA-15 demonstrated a strong tendency (although not reaching the level of statistical significancy) towards improvement of the learning ability of the AE64A-treated rats (Fig. 1 A).

The effect of CA-18 was studied in 2 doses: 0.3 and 1 mg/kg (p.o. daily). The lower dose of CA-18 (0.3 mg/kg) completely restored rats' performance in the learning (Fig. 3A) and retention (Fig. 3B) paradigms of the active avoidance test. The higher dose of CA-18 (1 mg/kg) demonstrated a strong tendency (although not

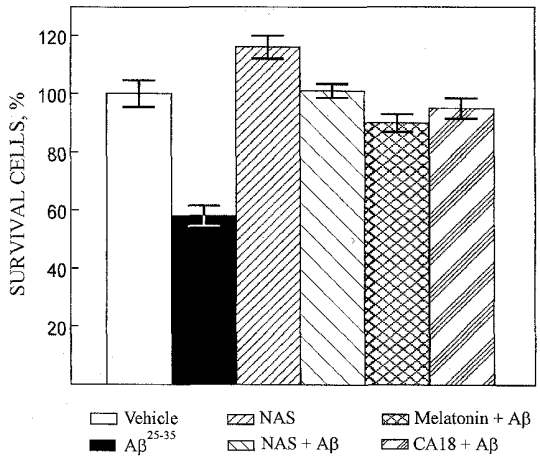

FIGURE 5. Protective effect of N-acetylserotonin (NAS), melatonin and CA-15 against the toxicity of (βAP 25-35 in mature cultures of cerebellar granule cells. Cultures 8 DIV were treated with vehicle, 25 mM βAP, 25 mM βAP and 25 mM NAS, or 25 mM melatonin, or 25 mM CA-15 for 4 days. The difference in numbers of living neurons before and after 4 days of treatment were determined. The amount of viable neurons is expressed as mean (%) above viable in preliminary photography of the same place. Experiments were repeated 2-3 times (n = 12-18). For graphical presentation, average data from representative experiments are converted to percentages of control group's viability. Results are expressed as a percentage of survival cells to compare with control ± SEM and were analyzed by a statistical test (ANOVA).

reaching the level of statistical significance) towards the improvement of the learning (Fig. 2A) but not in the retention (Fig. 2B) ability of the AF64A-treated rats.

Although we did not plan to perform the special evaluation of the general locomotor activity, we did notice the unspecified sedative effect in rats treated with melatonin (3 mg/kg) starting from the third to fourth day of treatment and in rats treated with NAS (1 mg/kg) starting from the sixth day of treatment.

Melatonin (0.3 mg/kg) and CA-18 (0.3 and 1 mg/kg) did not induce sedation in rats.

Morris Water Maze Test

AF64A significantly increased the response time in comparison to rats treated with the vehicle (CSF) (Fig. 4A and B). Melatonin and CA-18 (0.3 mg/kg daily) decreased the response time of AF64A-treated rats. Only the results observed on day 1 (Fig. 4A) and day 5 (Fig. 4B) are presented.

Neuronal Cell Culture

Earlier it was shown that exposure of mature cultures of CGC against the fragment of PAP reduced the cell viability in a dose-dependent manner (IC_{50} = 25 μM).

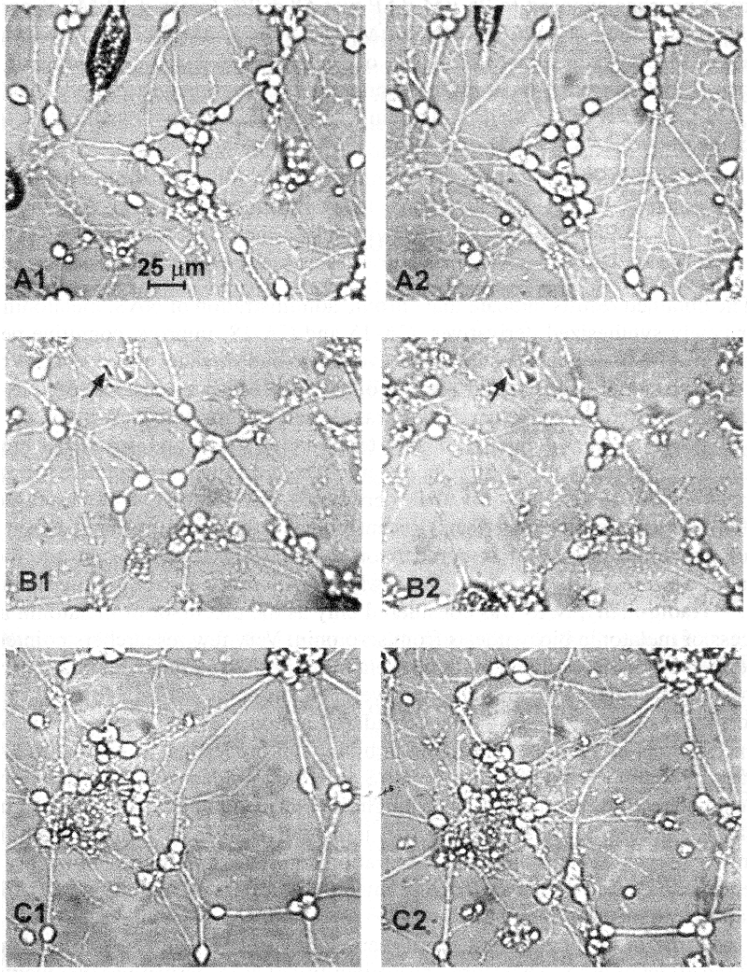

FIGURE 6. Protective effect of N-acetylserotonin (NAS) against the toxicity of pAP 25-35 in mature cultures of cerebellar granule cells. Cultures 8 DIV were treated with vehicle (**A1**), 25 mM βA (**B1**) or 25 mM PA and 25 mM NAS (**C1**) for 4 days, at which time photographs of the same part of the microscope fields were taken (**A2**), (**B2**) and (**C2**), respectively. Living cultures, "darkfield" method *(arrowhead* shows the mark on a plate).

Morphological changes (shrinkage of the body and fragmentation of neurites) were observed only when cells were exposed to PAP for 3 days or longer.[10] In the present study we used βAP in concentration of 25 μM to evaluate the ability of the compounds to protect neurons against β-amyloid (βA) neurotoxicity. Incubation of cultures only with NAS or melatonin in the concentration range 25-200 μM increased the amount of living CGCs by 16-28% or 3-10% of control, respectively. The via-

bility of neurons exposed to 25 μM (βAP was 58 ± 7% (mean ± SEM) of control, while coincubation of cultures with 25 μM NAS and 25 mM βAP resulted in an increase of viable neurons up to 101 ± 5% of control (Figs. 5 and 6) in the same conditions. Melatonin and CA-18 at 25 μM protected neurons against 25 μM βAP (90% and 93%, respectively) as well. Melatonin was less effective in preventing PA toxicity compared to NAS (Fig. 5).

DISCUSSION

The obtained results indicate that chronic administration of NAS, melatonin, and their newly synthesized derivatives, CA-15 and CA-18, improve cognitive performance of AF64A-treated rats in active avoidance and in water-maze tests. What is more, these compounds exerted the neuroprotective effect against the βA (25-35)-induced neurotoxicity in the cerebellar granule cell culture. It is known that the neurotoxic action of AF64A related, in part, to oxidative stress, and its indexes are persisting up to 4 months.[11] In this vein, one might suggest that antioxidative properties of melatonin, NAS and, possibly, their derivatives, CA-15 and CA-18, are responsible for their neuroprotective effects. Recent studies indicate that the antioxidant ability of melatonin is inferior in comparison to NAS,[24] and, at least in one model, melatonin exerted a prooxidant effect, while NAS exerted strong antioxidant action.[12] Traditionally, NAS was considered only as the precursor of melatonin in the process of melatonin biosynthesis from serotonin. Very few researchers pointed out the effects of NAS independent from melatonin, i.e., its memory facilitating,[13] hy-pothermic,[14] analgesic,[15] antihypertensive,[16-18] antidepressant[19] and antioxidative action.[20] NAS, therefore, might be considered not only as melatonin precursor but as endogenous indolamine with its own biological properties. Since about 30% of melatonin is demethylated back into NAS,[21] the antioxidant effect of supraphysio-logical concentrations of melatonin[22]might be ascribed to NAS formed from melatonin. It is noteworthy that the very first indication of the NAS involvement in the cognitive processes came from the observation that scotophobin A, the memory neu-ropeptide, increased dark avoidance behavior in goldfish via inhibition of NAS me-thylation into melatonin.[13]

Under the *in vivo* conditions rapid methylation of NAS into melatonin[23] might limit the effect of NAS. Therefore, the availability of NAS derivatives that would not undergo the *in vivo* transformation into melatonin might be of therapeutic advantage. Our preliminary experiments indicated that systemic administration of CA-15 and CA-18 did not change the rat pineal levels of NAS and melatonin (Oxenkrug & Requintina, unpublished data). Although all studied compounds attenuated the AF64A-induced cognitive impairment, there were noticeable differences between the effects of NAS, melatonin, and their derivatives (TABLE 1). NAS was apparently the weakest among the studied compounds in the active avoidance test, while it was the strongest in the attenuating of βA-induced neurotoxicity. Since NAS is rapidly converted into melatonin in rats,[23] the effect of NAS in our *in vivo* experiments could not be attributed only to NAS but rather to the mixture of NAS and melatonin.

The occurrence of the sedative effect in rats treated with NAS and melatonin, and somewhat delayed appearance of sedation in NAS- than in melatonin-treated rats

suggest that melatonin but not NAS is responsible for the sedative action. The absence of the sedative effect in rats treated with C A-18 might be of therapeutic advantage of the synthetic NAS/melatonin derivatives.

We have found that C A-15 and C A-18 in addition to their positive effect on cognition exerted antihypertensive and antidepressant-like effects.[24] The antidepres-sant-like activity (decreasing the duration of immobility in the mouse tail suspension test) was more pronounced in CA-18- than in CA-15-treated rats. The combination of cognition-enhancing and antidepressant effect in the one and the same compound might be of additional therapeutic advantage.

The results of our studies warrant the further search of the novel types of safe neuroprotectors among the synthetic NAS/melatonin derivatives.

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