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Effects of chronic nimodipine on working memory of old rats in relation to defects in synaptosomal calcium homeostasis

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Abstract

The present study was designed to investigate whether chronic (from 12 to 23 months of age) dietary treatment with the L-type Ca^{2+} channel blocker nimodipine (30 mg/kg body weight) enhances the cognitive behavior of aged animals and whether such a treatment would have long-term effects on the mechanisms of Ca^{2+} regulation in synaptic terminals from the aged rat brain. Cognitive behavior was evaluated in an 8-arm radial maze in 6 test series comprising a total of 105 test sessions, with intervals of no training between series. Nimodipine-treated rats performed better than vehicle-treated, aged-matched controls in all the test series, making more correct choices every time a new series was initiated. However, differences between nimodipine- and vehicle-treated rats were most remarkable in the last three test series, when the rats were 19 to 22 months. In these series 74% of the nimodipine-treated rats were able to perform the task in 4 to 9 test sessions whereas only 12%, 14% or none of the control rats learned the task. To study Ca^{2+} regulation in synaptosomes derived from cerebral cortex and hippocampus, we analyzed $^{45}Ca^{2+}$ accumulation as well as the levels of the Ca^{2+}-binding proteins calbindin-D28K and calreticulin by Western blotting. Nimodipine administration had no effect on hippocampal synaptosomes but increased the levels of calbindin-D28K and calreticulin in cerebral cortex preparations. These results indicate that chronic nimodipine treatment from 12 to 23 months of age prevents age-induced learning deficits without showing any signs of toxicity, and that these effects are associated with a small increase in the levels of synaptosomal Ca^{2+}-binding proteins from cerebral cortex. The up-regulation of these proteins might provide a link between the long-term effects of nimodipine on gene expression and learning ability in old rats.

Keywords: Nimodipine; Old rat; Working memory; Eight-arm maze; Synaptosome; Cerebral cortex; Ca^{2+} homeostasis; $^{45}Ca^{2+}$ accumulation; Calbindin-D28K; Calreticulin

1. Introduction

Aging in rats induces deficits in the performance of tasks involving working and spatial memory. The mechanisms responsible for senescent memory loss are not well understood. Atrophy of cholinergic neurons in the medial septum and nucleus basalis appears to be involved, and treatments aimed at enhancing cholinergic function reverse learning and memory impairments (Everitt and Robbins, 1997; Martínez-Serrano and Björklund, 1997). Oxidative damage is considered a likely cause of age-related brain dysfunction because the brain has a high capacity to produce reactive oxygen species and a relatively low activity of the main antioxidative enzymes. Furthermore, the age-related loss of cognitive function in mice correlates with oxidative molecular damage in the cerebral cortex (Forster et al., 1996). One of the effects of the exposure of mammalian cells to oxidative stress is protein thiol modification associated with disruption of intracellular Ca^{2+} homeostasis (Orrenius et al., 1992).

The mechanisms that govern Ca^{2+} homeostasis in the cerebral cortex and the hippocampus are modified in old

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rats, and there is evidence of an increase in high-threshold Ca^{2+} currents in the hippocampus (Campbell et al., 1996; Thibault and Landfield, 1996), an increase in cytosolic Ca^{2+} levels in hippocampal cells and synaptosomes (Villalba et al., 1995), a decrease in Ca^{2+} extrusion across the plasma membrane (Michaelis et al., 1984; Martínez et al., 1987; Canzoniero et al., 1992), lower Ca^{2+} accumulation in intrasynaptosomal mitochondria (Peterson et al., 1985; Vitórica and Satrústegui, 1986; Martínez-Serrano et al., 1992), and a decrease in the levels of the cytosolic Ca^{2+}-binding protein calbindin-D28K in specific brain areas (Iacopino and Christakos, 1990; Villa et al., 1994; Amenta et al., 1995; De Jong et al., 1996; Krywkowski et al., 1996; Chuang-Kuo et al., 1997). There are also data indicating that these modifications may be involved in age-related learning and memory deficits. Thus, a low Ca^{2+} uptake capacity of synaptosomes and, particularly, of the mitochondrial $^{45}Ca^{2+}$ pool was found to correlate with poor working memory performance in rats ranging from 9 to 24 months of age (Huidobro et al., 1993). Thibault and Landfield (1996) found that learning in aged rats was inversely correlated with L-type Ca^{2+} channel density in the hippocampus. These and similar results obtained with synaptosomes (Blanco et al., 1994) suggest a clear deterioration of Ca^{2+}-buffering and -extrusion systems in the direction of increased $[Ca^{2+}]_i$ in the old rat brain, particularly when coping with high Ca^{2+} loads (Satrústegui et al., 1996). Furthermore, they support the notion that, Ca^{2+} inflow through voltage gated Ca^{2+} channels is inversely correlated with the behavior of old animals. This may explain the beneficial effects of organic Ca^{2+} channel blockers on behavioral performance in aged animals. Nimodipine, a 1,4-dihydropyridine that selectively blocks L-type Ca^{2+} channels and which has a greater ability to cross the blood–brain barrier than other Ca^{2+} entry blockers (Kazda et al., 1982), has been widely used in this regard (Deyo et al., 1989; Scribiane et al., 1989). Acute nimodipine treatment (1 mg/kg, daily dose, p.o.) has been shown to enhance the memory performance of 24-month old rats in the 8-arm radial maze task (Levere and Walker, 1991). Ingram et al. (1994) and Riekkinen et al. (1997) have reported that prolonged (3 months) nimodipine treatment has significant beneficial effects on maze learning (14-unit T maze or water-maze, respectively) in 24-month old rats.

Nimodipine facilitates learning in old rats and increases cerebral blood flow but has little effect on the peripheral circulation (Kazda et al., 1982). Whereas long-term effects of nimodipine on the cerebral microvasculature (De Jong et al., 1991, 1993) may be involved in the increase in cerebral blood flow, there is also evidence of direct neuronal effects of nimodipine which may be associated with its effects on learning. Thus, 5 months' administration of nimodipine to a senescent-prone animal, the aged, senescence-accelerated mouse (SAM), has been shown to attenuate neurochemical changes occurring in the brains of these animals, with increased choline acetyltransferase activity (Kabuto et al., 1995), nitric oxide synthase activity (Inada et al., 1997) and increased binding of Ca^{2+} channel agonists in the brain (Yamada et al., 1996).

In the present work we explored the possibility that chronic exposure to nimodipine could have also long-term effects on the mechanisms of Ca^{2+} homeostasis in the old rat brain. We first studied whether prolonged (12 months) administration of nimodipine, starting at 12 months of age, before the onset of the defect in Ca^{2+} accumulation in synaptosomes (Vitórica and Satrústegui, 1986), could enhance working memory performance in the old rats over the whole of the 12-month administration period. In addition, we investigated whether chronic nimodipine treatment could reverse the age-dependent deficits in Ca^{2+}-binding proteins and Ca^{2+} accumulation in hippocampal and cerebral cortex synaptosomes in old (24-month) animals. Since unimpaired handling of Ca^{2+} at pre- and postsynaptic sites is believed to be essential for the development of the plastic changes that underlie learning and memory (Izquierdo and Medina, 1997; Schurmans et al., 1997) and may be involved in age-dependent memory deficits (Lanahan et al., 1997), the effects of nimodipine on the mechanisms of Ca^{2+} regulation may provide a mechanistic basis to explain the behavioral improvement.

2. Materials and methods

2.1. Subjects

The animals used in this study were nineteen male Wistar rats (Interfauna, Barcelona, Spain) obtained as retired breeders at 10 months of age and housed from then onwards at the Center of Molecular Biology 'Severo Ochoa'. Rats were housed in groups of five animals per cage under a 12:12 h light–dark cycle and fed ad libitum (breeding stock from Interfauna) until the experiment began. At that time rats were 11 months old. The experiments reported in this study were performed following the recommendations included in the Guide for Care and Use of Laboratory Animals (US Department of Health and Human Services, National Institutes of Health) and the European regulations on the protection of animals.

2.2. Nimodipine treatment

Eight weeks prior to training on the maze and during behavioral testing, animals were housed individually and feeding was restricted so that a body weight of 80–85% of free-feeding levels was reached and was maintained throughout the experiment. Then, one month before training on the maze, the nimodipine treatment was started (see scheme in Fig. 1 for details). At that time, 10 animals were allocated to the nimodipine-treatment group and 9 animals were maintained as non-treated controls. The animals were

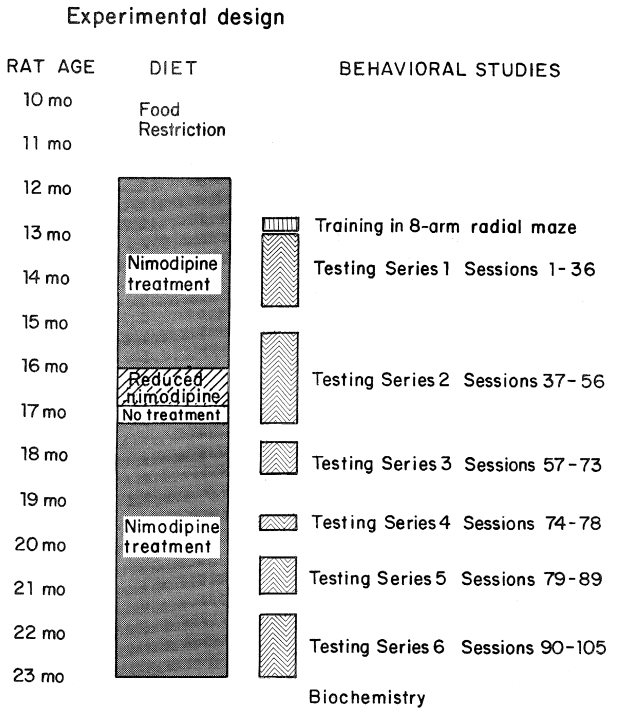

Fig. 1. Experimental design. The rats used (19) were 10 months old when the experiment began. They were all food restricted until the body weight was 80% of the original one. From then onwards, 10 rats received the nimodipine diet as indicated in Section 2 and 9 rats received the same diet lacking nimodipine. The periods of training and testing on the radial 8-arm maze (series 1 to 6) are indicated. Behavioral testing was carried out daily with interruptions (weekends, etc.) within each test series.

fed on standard food pellets (Letica, Barcelona, Spain) to which nimodipine (1 $g\,kg^{-1}$, Bayer, Leverkusen, Germany) was added. The individual dosage corresponded to a daily dose of approximately 30 mg/kg body weight over the total period of 12 months. Treatment was only interrupted once, after 4 months and two weeks, for 38 days during which the dosage was gradually reduced to 15 mg/kg body weight (2 weeks) and 7.5 mg/kg (2 weeks) and totally suspended (9 days). From then onwards, rats again received 30 mg/kg nimodipine daily.

2.3. Behavioral testing

Behavioral testing was started when rats were 13 months old and was performed during the daylight period at the same time of the day (see Fig. 1 for details). Spatial working memory was tested in an 8-arm radial maze (Huidobro et al., 1993) similar to that described by Olton and Samuelson (1976). During the initial training sessions (5 days, one session per day), rats were given approximately 15 min to explore the apparatus, and food pellets were present in the central platform and along the arms. In the last training session and during the test sessions that were conducted from then onwards, a 45-mg pellet of Letica dry rat food, which served as reinforcement, was placed in each of the food receptacles at the end of each arm. Daily feeding took place within 1 to 3 h after the training or testing sessions. At the start of each test session each rat was placed on the center platform of the apparatus in the same orientation and allowed to make up to 16 choices or given a total of 15 min if 16 choices were not made. A choice was defined as the rat proceeding to the end of an arm. Re-entries into an arm previously visited during that session (up to 1/2 of its length) were counted as errors.

Choice accuracy was scored in all animals by the following methods: number of correct choices in the first 8–12 visits (CC8, CC10, CC12) and number of errors made in the first 8–12 visits (ER8, ER10, ER12). These scores were used to compare the learning ability of nimodipine- and vehicle-treated animals in sessions where none of the rats had learnt the task yet. The mobility of each rat during the first test series was scored as NS7 or NS8, the *n*umber of sessions (out of a block of 10) in which 7 or 8 arms were visited. Rats were tested until a performance criterion of visiting 8 arms in 8 or 9 choices during 3 consecutive days was reached. In order to evaluate the working memory of the rats in the execution of post-delay tests (Nakamura-Palacios et al., 1996) a delay time was then introduced between the fourth and the fifth choice, and rats were tested until the performance criterion of visiting 8 arms in 8 or 9 choices during 3 consecutive days was again reached (Huidobro et al., 1993). Data from these experiments with delays are not used in this study because of the small number of animals from the control group that were used in the delay experiments. Regular testing was continued with the remaining rats.

All animals were tested for working memory performance in the radial 8-arm maze in a total of 105 test sessions divided into 6 series (Fig. 1). Behavioral testing between these series was interrupted, and at the start of a new series all rats were tested again (with no delay times). Two rats (one control and one nimodipine-treated) were killed after 36 test sessions had been completed in order to perform a pathological test. After 73 sessions, one of the nimodipine-treated rats was no longer used for behavioral testing and another control rat died after 79 sessions. At the end of the 12-month experiment, all rats were killed for biochemical analysis.

2.4. Biochemical analysis

The biochemical analysis was carried out with the 23-month old animals used in the behavioral studies and with 3-month old rats processed in parallel. Every experiment included one 3-month old animal and two old ones (one nimodipine treated and one control). The experimenters were unaware of each subjects group assignment. Brain regions (hippocampus, cerebral cortex) were removed (Villalba et al., 1995), and synaptosomes were prepared as described earlier (Gómez-Puertas et al., 1994). The final pellets were resuspended in 0.12 ml (hippocam-

pus) or 0.24 ml (cerebral cortex) of 0.32 M sucrose adjusted to pH 7.4, and kept in ice until used.

2.5. Ca^{2+} uptake

$^{45}Ca^{2+}$ accumulation was determined as described in Martínez-Serrano et al. (1992) with modifications. Synaptosomes (10 μl, 40–80 μg protein) were preincubated in low K^+ medium (5 mM KCl, 145 mM NaCl, 1 mM $MgCl_2$, 0.4 mM KPO_4H_2, 10 mM glucose, 10 mM Tris pH 7.4) for 5 min at 37°C with gentle shaking, and then $^{45}Ca^{2+}$ uptake was started by addition of high K^+ medium (83.5 mM KCl, 66.4 mM NaCl, 1.3 mM $CaCl_2$, 38 nM ruthenium red, $^{45}Ca^{2+}$ (1.94 μCi ml^{-1}), 1 mM $MgCl_2$, 0.4 mM KPO_4H_2, 10 mM glucose, 10 mM Tris pH 7.4) so that the final concentrations of $^{45}Ca^{2+}$, K^+, Na^+ and Ca^{2+} were, 0.75 μCi ml^{-1}, 68.8 mM, 84.8 mM and 1 mM, respectively. 29 nM ruthenium red (final concentration) was present in the assay to avoid any contribution from contaminating free mitochondria (Villalba et al., 1995). The incubation lasted 10 min and then the reaction was stopped by addition of a Ca^{2+}-chelating solution: 3 mM EGTA (ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid), 5 mM NaOH, 7 μM ruthenium red, final concentrations. Samples were vortexed and 8 s later were filtered (glass fiber filters) and washed twice with 5 ml 0.32 M sucrose, 10 mM Tris pH 7.4. Filters were dried and counted for radioactivity. $^{45}Ca^{2+}$ uptake was corrected for the Ca^{2+} bound to the filter and that bound to synaptosomes at time zero. Experiments were performed in triplicate or quadruplicate.

2.6. Western blots

Quantitation of the cytosolic, high-affinity, Ca^{2+}-binding protein, calbindin-D28K and the endoplasmic reticulum, high-capacity, Ca^{2+}-binding protein, calreticulin, was carried out by Western blotting. Antibodies (polyclonal) against calreticulin were obtained from Affinity Bioreagents. Antibodies against calbindin-D28K were raised in rabbits. Calbindin-D28K was purified from intestine of rachitic chicks treated for 2 days with cholecalciferol, following published methods (Wasserman et al., 1968; Maruyama et al., 1985). Synaptosomal proteins (100 μg sample^{-1}) were resolved by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). Proteins were transferred from gels to nitrocellulose by using a Bio-Rad transblot apparatus at 32 V overnight. For immunoblot analysis, membranes were blocked with 5% skimmed milk (Molico, Nestle®) in phosphate-buffered saline (PBS) for 1 h at room temperature, washed three times with PBS (15 min each), and incubated for 2 h with the antibodies against calreticulin (1:1000) or calbindin-D28K (1:5000). A secondary antibody linked to peroxidase (Boehringer) was then used (90 min incubation) and chemiluminescence (ECL Western blotting analysis system, Amersham) was used for detection of secondary antibody binding. The immunoblots were scanned with a densitometer (Shimadzu). Control experiments showed a linear relationship between the protein content per sample (within the range 40 to 140 μg) and the optical density obtained in the calbindin or calreticulin bands, under the conditions used. In every experiment, samples from control and nimodipine-treated rats were processed in parallel. The different nitrocellulose blots obtained were processed simultaneously with each primary and secondary antibody, to minimize the variability among experiments.

Statistical analysis was carried out using the BMDP package (BMDP Statistical Software, Los Angeles, CA, USA).

3. Results

3.1. Behavior

Performance in the 8-arm maze of the rats from the control group at 13–14 months of age (during the first test series) may be judged from their ability to learn the task in the first test sessions (Fig. 3) and from the number of animals that reached criterion in the course of the experiment (Fig. 2). According to both criteria, the learning ability of these animals was substantially impaired with respect to that of younger animals in a similar test (Huidobro et al., 1993). The ability to learn the task in the first testing sessions was clearly lower than that of younger (6–8 month-old) animals: CC8 in sessions 1–10 was 5.30 $\pm$ 0.18 in this study, 6.57 $\pm$ 0.21 for 6–8 month-old rats (Huidobro et al., 1993), and 7.2 $\pm$ 0.07 for 3-month-old rats (Luine et al., 1990). Only 22% of the 13- to 14-month-old animals from the present study reached criterion in the first test series (in session 29 and 34) whereas 71% of the 6- to 8-month-old rats did (in sessions 14–32, Huidobro et al., 1993). However the 13- to 14-month-old animals were not so severely impaired as those of 24 months of age that never reached criterion on a similar task (Huidobro et al., 1993; Blanco et al., 1994). Taken together, these results confirm that behavioral performance in the 8-arm radial maze declines before 8 months of age (Stewart et al., 1989).

The performance of the nimodipine-treated rats on the 8-arm maze was significantly improved compared to that of the controls from the first to the last series of the test sessions. All of the nimodipine-treated rats reached criterion in either the first or the second series of tests, whereas 45% of the controls never learned the task even after 105 test sessions (Fig. 2). Seven out of the ten nimodipine-treated rats that were tested in the maze learned the task during the first 36 sessions whereas only two (out of nine) of the controls did. In fact, the performance of the 13- to 14-month-old nimodipine-treated animals was similar to that of the 6- to 8-month-old rats reported previously

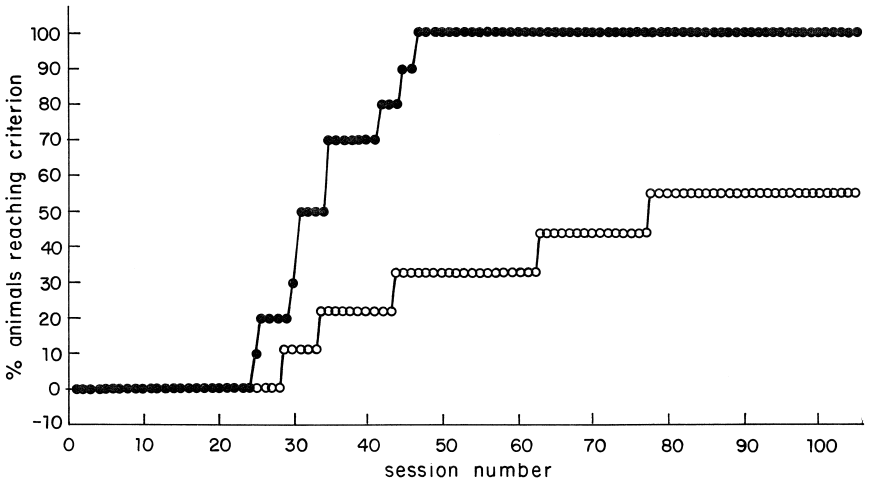

Fig. 2. Performance of nimodipine-treated and control rats in the 8-arm radial maze over the 105 test sessions, cumulative data. Nimodipine-treated rats (closed circles) and control animals (open circles) were tested in 6 different series. The results show the percentage of the animals in each group that reached criterion at least once during testing.

(Huidobro et al., 1993), in that 70% or 71%, respectively, of the animals reached criterion in 36 test sessions.

Differences between nimodipine-treated and age-matched controls were also obtained in the later series: in the case of nimodipine-treated animals, 8 out of 9 (2nd series), 7 out of 9 (3rd series), and 6 out of 8 (4th, 5th and 6th series) learned the task, whereas only 2 out of 8 (2nd series), 2 out of 8 (3rd series), 1 out of 7 (4th and 5th series), or none out of 7 (6th series) of the controls did.

Fig. 3 shows the progress of the rats during the first test series and before any of the animals had reached criterion. The number of correct choices, CC8, was significantly higher in nimodipine-treated animals than controls from the start of the experiment, and a clear trend towards improvement was observed in the nimodipine-treated animals (sessions 1–5 and 6–10 were different from 21–25, analysis of variance (ANOVA), post-hoc Bonferroni test, $P < 0.05$) which was not seen during the sessions with control animals (ANOVA, $P = 0.157$). An increase in test performance occurred with further testing and differences were even more pronounced during the later test series, when the animals were 19 to 22 months old. Fig. 4 shows the number of correct choices, CC8, and the number of errors, ER8, made by the nimodipine-treated and control rats in the first sessions of test series 4, 5 and 6 (sessions 74–76, 79–81, 90–92). Nimodipine-treated rats performed better than controls every time that a new series was started after a period when training in the maze was interrupted. This better performance was also manifested by the striking easiness with which the nimodipine-treated rats reached criterion every time a new test series was initiated as compared with the control aged rats. As ob-

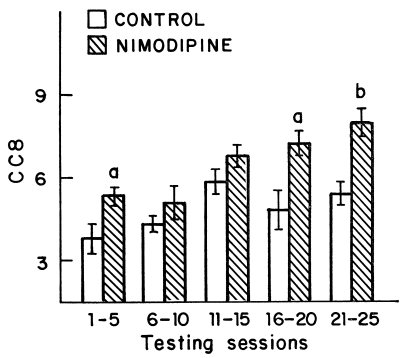

Fig. 3. Evolution of the performance of the rats in the 8-arm radial maze over the first 25 test sessions. 10 nimodipine-treated animals and 9 control rats were tested daily. CC8 indicates the number of correct choices in the first 8 visits. In these initial sessions some of the rats failed to move in the maze; when calculating mean CC8, these sessions were not used. The results are means ± S.E.M. $^{a}P < 0.05$; $^{b}P < 0.01$. (Mann–Whitney test).

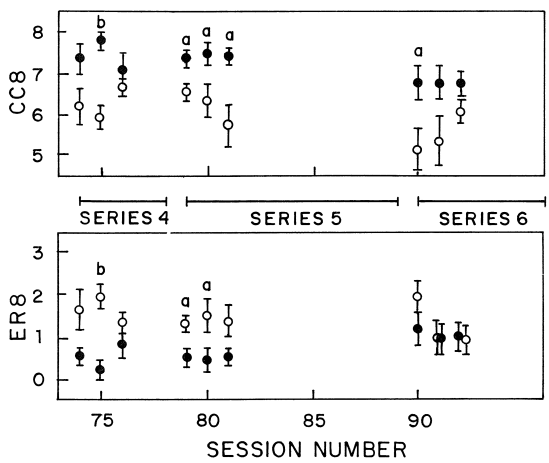

Fig. 4. Behavioral performance at the start of test series 4, 5 and 6. CC8 and ER8 indicate the number of correct choices and the number of errors in the first 8 visits for nimodipine-treated rats (closed circles) or controls (open circles). Results are means ± S.E.M. $^{a}P < 0.05$; $^{b}P < 0.005$. (Mann–Whitney test).

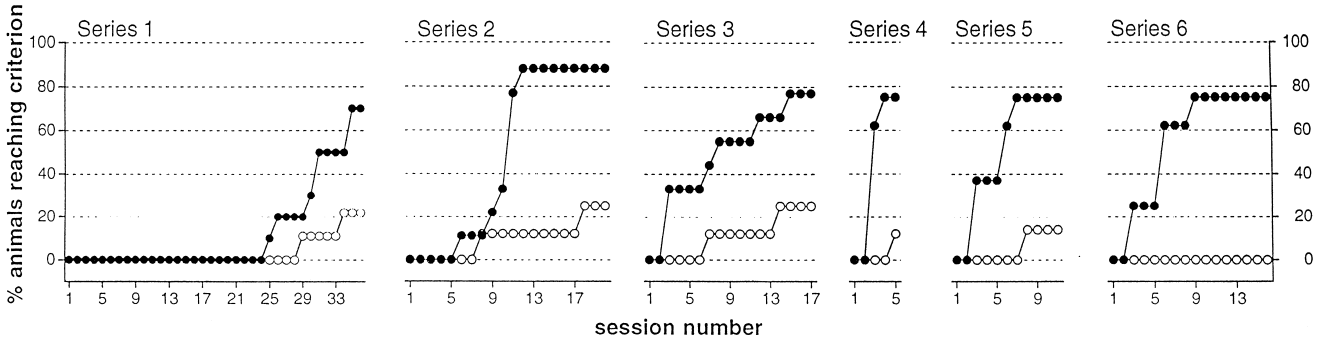

Fig. 5. Effect of nimodipine administration on the ability to learn the 8-arm maze task over the 6 different test series. The percentage of nimodipine-treated (closed circles) and control rats (open circles) reaching criterion in each of the test series is shown.

served in Fig. 5 (series 4 to 6), a substantial proportion of these rats (62%, 37% and 25% in series 4, 5 and 6 respectively) reached criterion at the start of each series (note that according to our definition, the criterion cannot be reached before 3 sessions) whereas 12%, 14% or none of the control rats learned the task. In the same series, 50% of the nimodipine rats reached criterion in 3 or 5.5 sessions (Fig. 5). Table 1 shows that nimodipine treatment was also followed by a significant increase in ambulation, as reflected in the number of sessions in which the rat visited at least 8 arms, NS8, during the first test series.

Interestingly, a reduction in the dose of nimodipine did not eliminate the beneficial effects of the treatment. Thus, during the second test series, when the nimodipine dose was reduced to 15 mg/kg body weight and to 7.5 mg/kg body weight (see Fig. 1), the ability to learn the task was still strikingly increased (Fig. 5).

3.2. Biochemistry

Synaptosomes obtained from cerebral cortex and hippocampus were used to study $^{45}Ca^{2+}$ accumulation and calbindin-D28K and calreticulin levels. For comparative purposes, adult, 3-month-old rats were also included in the biochemistry studies. The experimental design was a paired one. Every day a young adult rat was studied in parallel with two old rats, control and nimodipine-treated. Experiments where data from one of these groups of rats was missing were eliminated in the statistical analysis.

Table 1
Choice accuracy and rat ambulation during the first test series

	Control rats	Nimodipine-treated rats
NS8 Sessions 1–10	4.67 ± 0.99	6.9 ± 0.75
NS8 Sessions 10–20	6.33 ± 0.85	8.5 ± 0.64[a]
NS8 Sessions 1–20	11.0 ± 1.42	15.4 ± 1.13[b]
CC8 1–10	5.30 ± 0.18	5.78 ± 0.13[a]
CC8 1–20	5.58 ± 0.13	6.11 ± 0.08[c]

CC8 and NS8 scores during the first 10 sessions. Results are means ± S.E.M. [a]$P < 0.05$ [b]$P < 0.025$; [c]$P < 0.005$ (Mann–Whitney test).

We did not find any significant effect of chronic nimodipine administration either on calbindin-D28K or calreticulin levels or on $^{45}Ca^{2+}$ uptake in synaptosomes derived from hippocampus (results not shown). However, Table 2 shows that the levels of cytosolic calbindin-D28K and the endoplasmic reticulum-resident calreticulin were higher in cerebral cortex synaptosomes from old rats treated with nimodipine than in those from vehicle-treated rats, with increases of about 40% and 24%, respectively. It should be noted that the mean calbindin-28 K and calreticulin levels in cerebrocortical synaptosomes were still higher than those of controls when data obtained from all the nimodipine-treated rats were used (including those eliminated in the statistical analysis) (calbindin-D28K levels: 1.39 ± 0.25, $n = 8$; calreticulin levels 1.10 ± 0.07, $n = 9$, see values in Table 2 for comparison). Thus, even though the effects of nimodipine treatment were relatively small, they suggest a possible effect of this chronic treatment on Ca^{2+}-binding proteins in cerebral cortex synaptosomes.

$^{45}Ca^{2+}$ uptake in whole rat brain synaptosomes decreases with age and this has been shown to be accounted

Table 2
Calbindin-28 K and calreticulin levels and $^{45}Ca^{2+}$ uptake in synaptosomes derived from cerebral cortex of nimodipine-treated and control 23-month-old rats

	Cerebral cortex synaptosomes	
	Old, untreated	Old, nimodipine-treated
calbindin-28 K	1.02 ± 0.18 (6)	1.43 ± 0.25 (6)[a]
calreticulin	0.87 ± 0.03 (7)	1.11 ± 0.08 (7)[a]
$^{45}Ca^{2+}$ uptake	18.31 ± 1.92 (7)	22.7 ± 2.21 (7)

Western blots for calbindin-28 K and calreticulin were carried out as described in methods. The appropriate protein bands were quantitated by scanning densitometry. Intensities are expressed as ratio values using as control the sample from the 3-month-old animal in each experiment. The results from Ca^{2+} uptake experiments are given as nmol $^{45}Ca^{2+}$/mg protein. Results represent means ± S.E.M. The number of animals in each group is shown in brackets. Ca^{2+} uptake in cerebral cortex synaptosomes from the 3-month-old rats was 22.37 ± 1.96 nmol $^{45}Ca^{2+}$/mg protein. The differences between groups were analyzed by Wilcoxon signed rank test (one-tailed). [a]$P < 0.05$.

for by a decrease in the capacity for Ca^{2+} accumulation in synaptosomal mitochondria (Vitórica and Satrústegui, 1986; Martínez-Serrano et al., 1992). Table 2 also shows that $^{45}Ca^{2+}$ uptake in cerebral cortex synaptosomes was also greater in nimodipine-treated rats than in controls. However, in this case, the difference was not statistically significant. There was no correlation between $^{45}Ca^{2+}$ uptake and the levels of the Ca^{2+}-binding proteins.

4. Discussion

In the present study we used chronic treatment with nimodipine to investigate the neuroprotective and behavioural effects of L-type Ca^{2+} channel blockade. The results show that chronic nimodipine administration (daily oral intake, 30 mg/kg) from 12 to 23 months of age resulted in a very pronounced increase in learning ability in Wistar rats, which manifested itself over the whole of the 12-month period of administration. This treatment did not have adverse effects on rat survival (none of the nimodipine-treated died during the experiment).

Nimodipine treatment was also associated with a significant increase in ambulation (NS8, Table 1) during the first test series. This increase in NS8 may be associated with an improved working memory performance but may be also due to an effect of nimodipine on exploratory behaviour, arousal or attention, as described earlier in old rats (De Jong et al., 1993), since there was a positive correlation between NS8 and CC8 during the first 10 test sessions in nimodipine-treated animals ($r = 0.73$, $P = 0.009$) but not in controls ($r = 0.497$, $P = 0.157$). However, this does not appear to be the case. There was no correlation between NS8 and CC8 for sessions 1–20 ($r = 0.333$, $P = 0.142$ for all 19 rats, and $r = -0.311$, $P = 0.331$ for the 10 nimodipine-treated rats), suggesting that nimodipine's effects on learning (CC8) are not related to effects of the treatment on arousal or ambulation.

The fact that the nimodipine-treated animals maintained an improved performance during the whole of the experiment indicates that the effects of the drug do not wear off with treatment (no habituation). The improvement of working memory was observed also in sessions where the daily dose was reduced to 15 mg/kg or 7.5 mg/kg body weight, suggesting that lower doses may be also effective. In fact, Riekkinen et al. (1997) have also observed a facilitation in spatial learning tasks (water maze) in old rats with lower nimodipine doses (3 and 10 mg/kg).

It has been reported that the beneficial effects of nimodipine on learning are lost when its administration is suspended (Levere and Walker, 1991). The present study was not designed to test this specifically, but we noticed that at the start of the third series, i.e., 2 weeks after a 9-day interruption of nimodipine administration, the behavior of the nimodipine-treated and control animals was very similar (CC8 values in sessions 57, 58 and 59, were 6.33 ± 0.44, 6.67 ± 0.24 and 7 ± 0.29 for nimodipine-treated animals and 6.25 ± 0.89, 6.38 ± 0.38 and 6.50 ± 0.27 for aged-matched control rats, respectively), suggesting that nimodipine's effects on learning might indeed fade away when its administration is stopped. However, even though the initial performance scores were similar, 77% of the nimodipine-treated rats reached criterion in the same series whereas only 22% of the controls did (Fig. 5), indicating that nimodipine's effects reappear some time after the treatment is restarted.

It has been shown that chronic nimodipine administration has direct effects at the brain level, on enzyme (nitric oxide synthase) activity in cerebral cortex and on neurotransmitter levels (5 months treatment; Kabuto et al., 1995; Inada et al., 1997). Moreover, in the senescence-accelerated prone mouse (SAMP8), nimodipine administration for three weeks caused increases in the B_{max} values for Ca^{2+} channel ligands in cerebral cortex and hippocampus and a marked decrease in brain Ca^{2+} content (Yamada et al., 1996). Chronic s.c. infusion of nimodipine ($1\ \mu g\,h^{-1}$) for seven days in rats also induced an increase in dihydropyridine binding sites in cerebral cortex (Diaz et al., 1995). The increased binding of Ca^{2+} channel ligands to receptors believed to be localized to synaptic sites is thought to reflect an up-regulation of these receptors as a result of their prolonged blockade by nimodipine (Yamada et al., 1996). These results support the notion that low doses of nimodipine cross the blood–brain barrier and may have effects on age-related brain dysfunction.

The results from this study support the notion that long, chronic nimodipine treatment may result in long-term changes at the level of synaptosomal Ca^{2+}-binding proteins. We did not find any changes in the hippocampus, but a small increase in calbindin-D28K and calreticulin levels was observed in the cerebral cortex. Amenta et al. (1995) have also reported that treatment of aged rats with the dihydropyridine-type Ca^{2+} antagonist darodipine for six months increased calbindin-D28K immunoreactivity in the cerebellar cortex. Interestingly, postnatal induction of calbindin-D28K in rat forebrain was also enhanced by nimodipine administration (Luiten et al., 1994).

Changes in intracellular Ca^{2+} dynamics, as achieved with Ca^{2+} entry blockers, may be accompanied by changes in Ca^{2+}-dependent gene expression. Ca^{2+} modulates the expression of calbindin in peripheral tissues (Huang and Christakos, 1988) and calreticulin levels are up-regulated in different cell lines upon depletion of intracellular Ca^{2+} stores (Krause and Michalak, 1997; Waser et al., 1997). Therefore, the increased levels of these two proteins upon persistent Ca^{2+} channel blockade could be initiated by a common Ca^{2+}-dependent mechanism. Calbindin-D28K, a major Ca^{2+}-binding protein in the brain, is localized in neurons which are relatively resistant to seizure-induced insults (Schwob et al., 1980) and ischemic injury (Goodman et al., 1993). Calbindin-positive hippocampal neurons are protected against damage induced by glutamate and

Ca^{2+} ionophore and are better equipped to reduce $[Ca^{2+}]_i$ after a Ca^{2+} load than calbindin-negative neurons (Mattson et al., 1991). Chard et al. (1993) and Reddy et al. (1997) have directly demonstrated the intracellular impact of the Ca^{2+}-buffering capacity of calbindin by showing that the introduction of exogenous calbindin into dorsal root ganglion neurons or insulin-secreting cells resulted in a reduction of the depolarization- or ionophore-induced rate of increase in intracellular Ca^{2+}. In addition, calbindin overexpression in hippocampal neurons was found to strongly affect synaptic plasticity, by suppressing posttetanic potentiation (Chard et al., 1995). Interestingly, in Aplysia sensory neurons, calreticulin levels increase in response to long-term sensitization (Kennedy et al., 1992).

The mechanism by which nimodipine works to facilitate learning in the old rat is still an unresolved issue. Hippocampal high threshold L-type Ca^{2+} currents are increased in neurons from aged rats (Campbell et al., 1996) and this is inversely correlated with the learning ability of the old animals (Thibault and Landfield, 1996). In old, memory impaired rats, $^{45}Ca^{2+}$ entry through synaptosomal voltage-gated Ca^{2+} channels is also inversely correlated with working memory performance (Blanco et al., 1994). Thus, the beneficial effects of nimodipine administration on learning observed in this and other studies may involve, at least in part, its acute effects on neurons as a Ca^{2+} channel blocker (Disterhoft et al., 1996). In addition, the chronic presence of nimodipine appears to give rise to long-term changes at a protein level, including the Ca^{2+}-binding proteins calbindin-D28K and calreticulin from cerebral cortex synaptosomes. Ca^{2+} homeostatic mechanisms are altered in the aged brain both at pre- and post-synaptic levels (Satrústegui et al., 1996; Verkhratsky and Toescu, 1998). Increased levels of these Ca^{2+}-binding proteins in synaptosomes may be important in the regulation of synaptosomal Ca^{2+} homeostasis and neurotransmitter release in the cerebral cortex. Moreover, since synaptosomes and cell bodies of individual brain regions show similar changes with aging (Villalba et al., 1995), it is possible that nimodipine also induces long-term increases in the levels of these proteins in cell bodies in the cerebral cortex. Ca^{2+}-dependent signaling pathways are involved in transcriptional responses associated with learning and memory (Chen and Tonegawa, 1997), and both the hippocampus and the cerebral cortex participate in memory consolidation and retrieval (Izquierdo and Medina, 1997).

Thus, it is possible that the acute effects of nimodipine as a Ca^{2+} channel blocker in addition to its long-term effects on protein levels may help to overcome the alterations in Ca^{2+} homeostatic mechanisms that occur in old age. It is also tempting to speculate that chronic treatment with nimodipine may alter the naturally occurring process of neuron aging and atrophy, halting or slowing down neurodegeneration and facilitating in this way cognitive function at advanced age.

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