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Research report

Sildenafil provides sustained neuroprotection in the absence of learning recovery following the 4-vessel occlusion/internal carotid artery model of chronic cerebral hypoperfusion in middle-aged rats

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ABSTRACT

In this study, we tested whether the phosphodiesterase-5 inhibitor sildenafil protects against neurodegeneration and facilitates recovery from learning deficits examined long after chronic cerebral hypoperfusion (CCH) induced by the 4-vessel occlusion/internal carotid artery (4-VO/ICA) model in middle-aged rats. Male Wistar rats (12–15 months of age) were subjected to permanent 3-stage 4-VO/ICA with an interstage interval of 4 days. Sildenafil (3 mg/kg, p.o.) was administered at one dose per day for 10 days, beginning soon after the first occlusion stage. Three months later, learning in a non-food-rewarded, eight-arm radial maze task was tested. Learning performance is expressed as the latency to find a goal box and the number of reference or working memory errors. Histological examination was performed 1–3 days after behavioral testing. In the vehicle-treated group, permanent 4-VO/ICA markedly disrupted learning performance and caused moderate-to-severe neurodegeneration in the CA1-CA4 subfields of the hippocampus (56.2%), dentate gyrus (DG; 19.2%), retrosplenial cortex (RS cortex; 47.4%), and parietal association cortex (PtA cortex; 38.2%). Sildenafil treatment did not prevent 4-VO/ICA-induced learning deficits, whereas neurodegeneration was significantly reduced in the CA1-CA4 subfields (30.5%), DG (7.2%), RS cortex (11.8%), and PtA cortex (6.5%). Advancing previous findings from our laboratory, this study suggests that while sildenafil can provide important neuroprotection in different brain regions of middle-aged rats subjected to CCH, such histological effect does not translate into cognitive recovery.

1. Introduction

Ample experimental and clinical observations support the hypothesis that a state of chronic cerebral hypoperfusion (CCH) represents an etiologic factor of age-related neurodegenerative diseases and dementia (de la Torre et al., 2002). Prevention against CCH-related risk factors, mainly hypertension and chronic heart disease, constitutes the primary recommendation to reduce the prevalence of neurodegenerative disease associated with CCH (de la Torre, 2009). However, once CCH occurs and evolves, the question arises whether the progression of brain damage and cognitive impairment can be mitigated pharmacologically. Drugs such as sildenafil (Viagra) possess pharmacological properties by which their therapeutic indication transcends the area of erectile dysfunction and pulmonary hypertension to include the treatment of vascular-related CNS disorders among other conditions (Vlachopoulos et al., 2009). Sildenafil acts primarily by selectively inhibiting phosphodiesterase type-5 (PDE5) and increasing intracellular cyclic guanosine monophosphate (cGMP) that in turn leads to vascular smooth muscle relaxation, vasodilation, and perhaps overall cerebral circulation improvement (Ghofrani et al., 2006). Beyond its direct action on vascular smooth muscles, sildenafil exerts important preconditioning and antiapoptotic actions in the myocardium, followed by reduced heart infarct size and improved myocardial function after experimental ischemia (Das et al., 2005; Kukreja et al., 2005). As defined elsewhere, "pharmacological preconditioning refers to the action of compounds that trigger the preconditioning signaling cascades without a physical stimulus" (Dimagl et al., 2009). Whether sildenafil can induce preconditioning in the brain is unknown. However, sildenafil modulates the nitric oxide (NO)/cGMP pathway, the signaling of which promotes angiogenesis, neurogenesis, axonal outgrowth, and synaptic plasticity during development and in the adult animal (Prickaerts et al., 2002; Zhang et al., 2005). In a rat model of stroke, sildenafil induced

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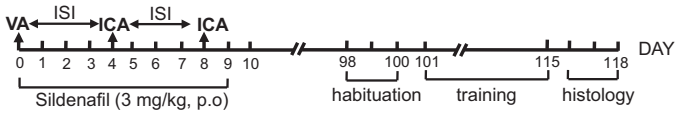

Fig. 1. Schematic representation of the experimental protocol that delineates the phases of the 3-stage 4-VO/ICA model with an ISI of 4 days, periods of sildenafil administration, habituation to the test environment, acquisition training, and histology. VA, vertebral artery; ICA, internal carotid artery; ISI, interstage interval.

angiogenesis, neurogenesis, and axonal remodeling, improved cerebral blood flow in the periinfarct area, and facilitated functional recovery (Zhang et al., 2006; Li et al., 2007; Ding et al., 2011). Acutely, sildenafil improves learning and memory function in several rodent species tested in different behavioral tasks (Reneerkens et al., 2009).

However, whether sildenafil is effective against the neurohistological and behavioral outcomes of CCH has not yet been systematically investigated (Romanini et al., 2010). In that study, young rats were subjected to a 4-vessel occlusion/internal carotid artery (4-VO/ICA) model of CCH, which is based on the permanent, stepwise occlusion of the vertebral arteries (VAs) and internal carotid arteries (ICAs; Neto et al., 2005; Barros et al., 2009). Sildenafil was found to reduce both the rate of mortality and hippocampal neurodegeneration, but its effect on behavior was not evaluated. Unexpectedly, permanent 4-VO/ICA did not impact the ability of young rats to perform the radial maze task. When imposed to middle-aged rats, however, permanent 4-VO/ICA caused not only hippocampal and cortical neurodegeneration, but also learning and memory deficits (Ferreira et al., 2011). Therefore, and given continuity to our previous investigation, in the present study we sought to examine whether the treatment with sildenafil could provide both neurohistological protection and behavioral recovery after permanent 4-VO/ICA in middle-aged rats.

2. Materials and methods

2.1. Subjects

Seventy-one middle-aged (12–15-month-old, 490–600 g body weight) male Wistar rats (inbred strain) were assigned to the following three groups: sham operation ($n = 13$), 4-VO/ICA + vehicle ($n = 34$), and 4-VO/ICA + sildenafil ($n = 24$). The rats were housed at a controlled temperature ($22 \pm 1\,^{\circ}C$) on a 12 h/12 h light/dark cycle (lights on at 7:00 AM). Food and water were provided *ad libitum* throughout the experiment. The materials and methods were approved by our Ethics Committee on Animal Experimentation (protocol no. 044/2008).

2.2. Surgery

Fig. 1 provides a schematic of the entire experimental protocol. The animals were anesthetized with ketamine (15 mg/kg) plus xylazine (1.0 mg/kg) administered intramuscularly. Permanent 4-VO/ICA or sham surgery was performed gradually in three stages according to the sequence VA → ICA → ICA, with an interstage interval (ISI, →) of 4 days. For bilateral occlusion of the VAs, the tip of a unipolar electrode was inserted into the alar foramen of the first cervical vertebra and gently rotated until the presence of hemorrhage ensured vessel rupture. The hemorrhage was then immediately staunched by a 3–4 mA electrical current. This procedure ensures complete and irreversible VA occlusion. The ICAs were carefully dissected from adjacent tissues and permanently ligated using cotton thread. After each occlusion stage, the incision was sutured, and the animal was returned to its home cage until the next surgery. Rectal temperature was monitored with a digital thermometer (Minipa, APPA MT-520, São Paulo, Brazil) using a rectal probe inserted to a depth of approximately 6 cm. Core temperature was controlled only during surgery and maintained at approximately 37.5 °C by a heating blanket. The animals assigned to the sham surgery group were subjected to the same surgical procedures as their counterparts but did not receive vessel occlusions.

2.3. Drug preparation and treatment

Sildenafil citrate (1-[4-etoxido-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pirazolo[4,3-*d*]pyrimidine-5-yl)phenylsulfonyl]-4-methylpiperazine citrate; Gamma Pharmaceuticals, Beijing, China) was dissolved in sterile 0.9% saline (vehicle) and administered by gavage at 3.0 mg/kg in a volume of 1.0 ml/kg. We choose this dose because it effectively affords neuroprotection in young rats (Romanini et al., 2010). Moreover, 3 mg/kg was found to be at the peak of the dose-response curve for the effects of sildenafil on performance in rats tested in the object recognition task (Prickaerts et al., 2005). Drug administration began soon after the first occlusion stage (VA) and continued for 9 days, with one dose per day. Sildenafil solution was freshly prepared before administration.

2.4. Behavioral testing

Learning and memory performance was measured in the aversive, eight-arm radial maze (AvRM) model, which works on the basis of the rat's natural behavior of avoiding open and illuminated areas and its preference for darkened and enclosed places (in this case, a shelter or goal box). A detailed description of the AvRM apparatus has been provided elsewhere (Romanini et al., 2010; Ferreira et al., 2011). After habituation, acquisition training began according to a schedule of three trials per session and one session per day for 15 days (5 days per week). In each trial, the rat was placed into the center of the arena, with all arms closed and the video camera turned on. Thirty seconds later, the arms were opened simultaneously, and the animal was allowed to explore the entire maze. When the rat entered halfway down a non-rewarded arm (*i.e.*, an arm that contained a false goal box), the guillotine doors of the remaining arms were lowered simultaneously. Upon the rat's return to the central area, the newly visited arm was closed immediately, and the animal was again confined in the arena for a further 10 s (delay). When the rat found and entered halfway down the rewarded arm (*i.e.*, the arm that contained the true goal box), the guillotine door of that arm was lowered, forcing the animal to enter the correct goal box where it was left for 1 min. If the rat did not find the correct arm within 4 min, then it was placed into it and gently introduced into the shelter. If the rat inserted only its head into an incorrect opening and remained there for more than 1 min, then it was repositioned to the center of the maze, and the trial was restarted. If such atypical behavior persisted for more than four consecutive sessions (days), then the animal was excluded. Between trials, the rat was moved from the maze (or goal box) to its individual home cage where it was left in a separate room until the maze was cleaned of excrement and randomly rotated on its central axis. The goal box was randomly moved to any of the other seven arms, although its spatial position in relation to the extra-maze cues remained unchanged across trials and sessions and was the same for all of the rats. This procedure took approximately 90 s, after which the subsequent trial began. Learning and memory performance was measured by three parameters: (i) the latency to find the goal box, (ii) the number of reference memory errors, and (iii) the number of working memory errors. Within a given trial, a reference error was counted when the rat visited an arm that contained a false goal box for the first time. However, if the rat returned to an arm that had been visited previously during that trial, then a working memory error was recorded. The animal was considered to have left an arm when it placed all four paws on the central platform.

2.5. Histology

Under anesthesia (Thiopental, 50 mg/kg, i.v.), the animals were transcardially perfused with 0.9% saline followed by Bouin's fixative (20 ml/min for 5 min). Following decapitation, the head was immersed in crushed ice (1–2 °C) for at least 2 h to avoid the appearance of so-called "dark" neurons. The brain was then removed and immersed in the same fixative for 2–3 h. The forebrain was sectioned into two parts and conserved in the same fixative for an additional 3–5 days, after which the parts were embedded in paraffin. For each brain, 12 coronal sections (7 μm thick, 70 μm apart) were cut at the medial level of the hippocampus (−3.60 to −4.30 mm) and processed for Nissl staining. Pyramidal cell loss was quantified bilaterally across the CA1–CA4 subfields of the hippocampus, in the retrosplenial (RS) cortex, and in the parietal association (PtA) cortex. Neurodegeneration (or atrophy) was also examined in the hippocampal dentate gyrus (DG). Because the high density of granular cells in this region precludes the reliable determination of cell number, the thickness of the granular cell layer was measured in the suprapyramidal blade, infrapyramidal blade, and crest of the DG. In both the hippocampus and cerebral cortex, the number of pyramidal cells and thickness of the DG granular cell layer averaged from the various measurements in each individual were transformed into a percentage. The mean of the sham-operated group was considered 100%. Each individual value was then normalized with respect to the mean of the sham. The identity of the groups was not revealed during histological assessment.

2.6. Statistical analysis

When learning and memory performance was analyzed across the five session blocks, a normal distribution (D'Agostino and Pearson omnibus test), sphericity (Mauchly's test), and homocedasticity (Lavene's test) were not consistently achieved among the various groups, within the same group, or among the different parameters (*i.e.*, latency, reference memory errors, and working memory errors). In this case, nonparametric Friedman's analysis of variance (ANOVA) was used to quantify within-group differences, which tested the slope of the learning curve for each individual group. If a global significant value was found, then Dunn's *post hoc* test was applied to localize the session block when the reduction of the latency or number of errors (*i.e.*, learning) was significant. Subsequently, the Kruskal–Wallis ANOVA was used for between-group comparisons in each session block. However, when

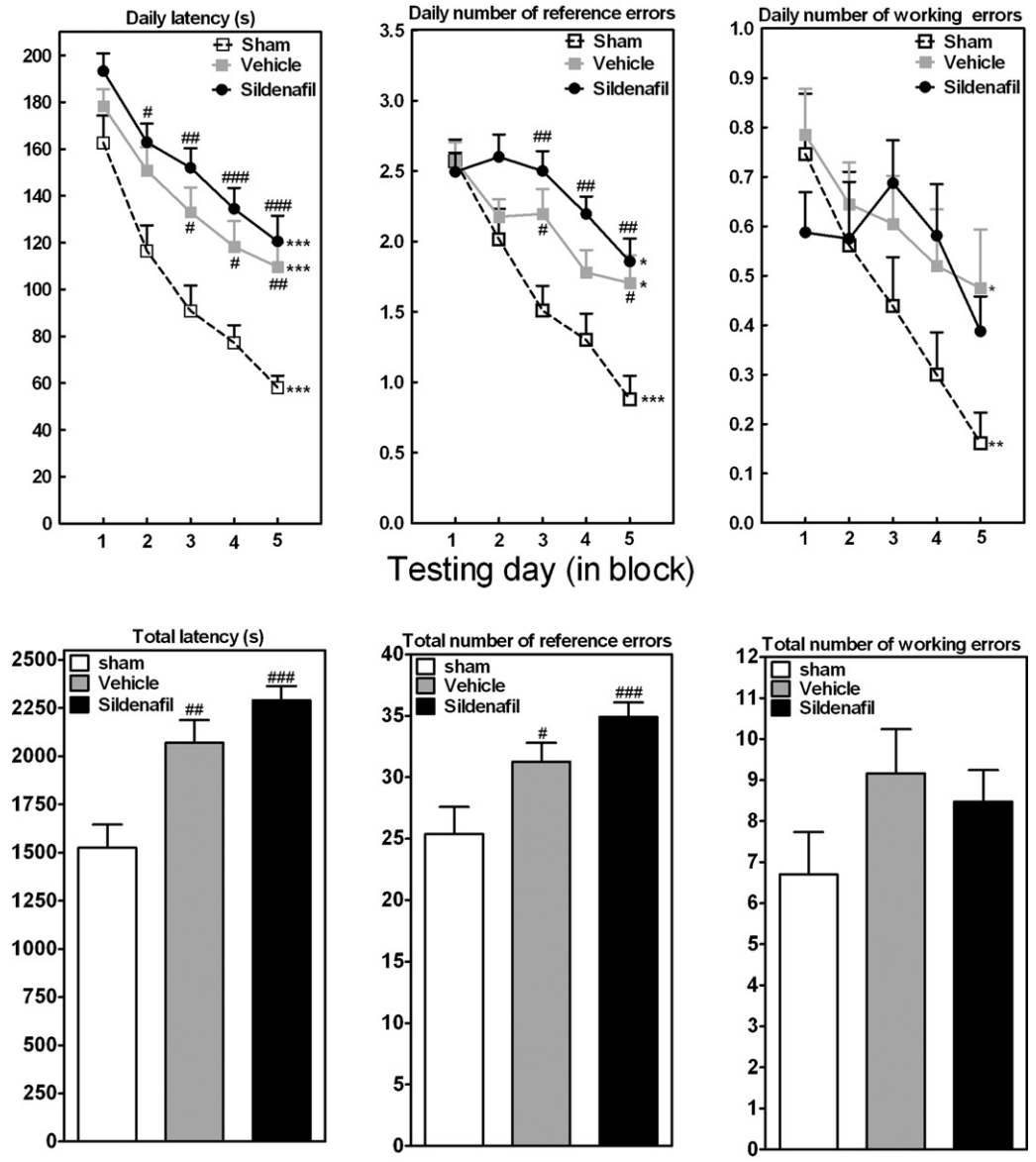

Fig. 2. Chronic cerebral hypoperfusion-induced learning deficits in middle-aged rats and the effects of sildenafil. (*Upper panels*) Temporal distribution of learning performance expressed as latency (left), number of reference memory errors (middle), and number of working memory errors (right). (*Lower panels*) Total latency and total number of errors summed over the 15 training sessions. Permanent 4-VO/ICA disrupted learning performance, an effect that was not changed by sildenafil. The data are expressed as mean ± SEM. Sham, $n = 11$; vehicle, $n = 20$; sildenafil, $n = 16$. Within-group comparisons (curve slope): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* block 1. Between-group comparisons in each testing block: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* sham.

learning performance was expressed as "total latency" and "total number of errors" (summed across 15 consecutive sessions), both a normal distribution and homocedasticity were achieved. In this case, a one-way ANOVA followed by Bonferroni's *post hoc* test was used for between-group comparisons. The Kruskal–Wallis ANOVA was also used to quantify the results of the histological analysis. A proportion-like *t*-test was used to quantify mortality data.

3. Results

3.1. Mortality rate

Overall, 23.4% of the rats died at some time point after the completion of 4-VO/ICA when the animals were already conscious, likely reflecting the fatal effect of severe CCH. In the vehicle-treated group, 26% of the rats died (7/27, $p < 0.001$ *vs.* sham). The mortality rate was lower in the sildenafil-treated group (20%, 4/20, $p < 0.05$ *vs.* sham) but not statistically different from the vehicle group. No death occurred after the sham operation.

3.2. Learning performance

Excluding the habituation period, learning performance assessment began 93 days after 4-VO/ICA and 92 days after the last dose of sildenafil (see Fig. 1). Fig. 2 shows the disruptive effect of 4-VO/ICA on acquisition performance in middle-aged rats and the failure of sildenafil to prevent this. The examination of performance across training Sessions (Fig. 2, upper panels) revealed that the parameters "latency" and "number of reference errors" decreased significantly in all three groups ($S = 10.34–39.70$, $p < 0.0001–0.05$). As training proceeded, the "number of working errors" also decreased in the sham group ($S = 17.42$, $p < 0.01$) and vehicle group ($S = 12.82$, $p < 0.05$) but not in the sildenafil-treated group. Dunn's *post hoc* test showed that the rate of learning was similar between the sham and vehicle groups, with values of "latency" and "number of reference errors" that were significantly lower for blocks 3, 4, and 5 compared with block 1 ($p < 0.001–0.05$). A less robust learning rate was observed in the

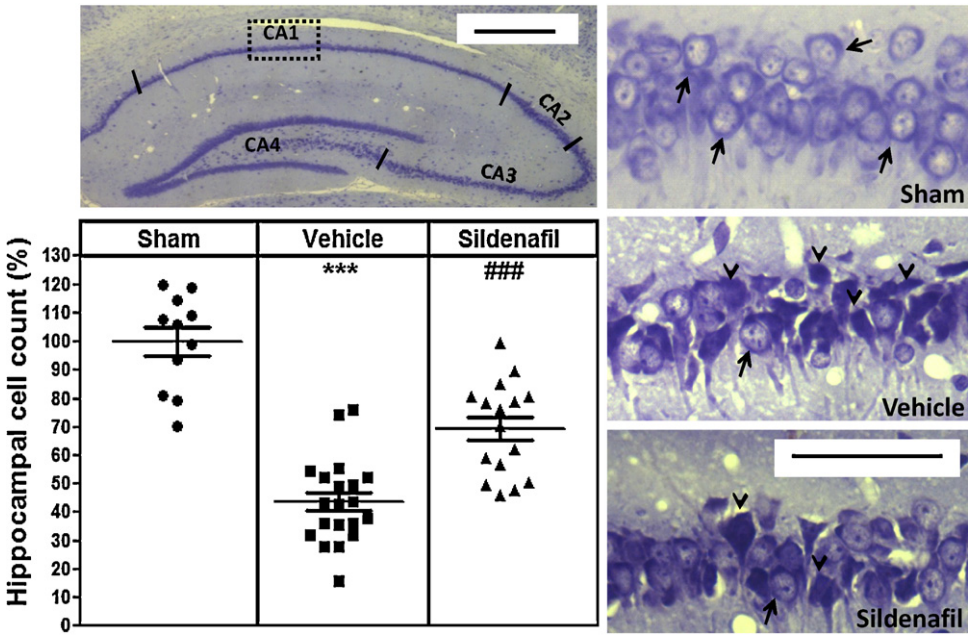

Fig. 3. Chronic cerebral hypoperfusion-induced neurodegeneration over the CA1–CA4 subfields of the hippocampus and the effect of sildenafil. In each rat, a mean value of hippocampal cells was taken from six measurements (3 sections × 2 hemispheres). Each dot represents an individual, averaged cell count, which was normalized to the mean of the sham-operated group (100%). The bars denote the mean ± SEM of the group. (*Left, upper panel*) Low-magnification (50×, scale bar = 500 μm), Nissl-stained coronal brain section at the intermediate level of the hippocampus obtained from a sham-operated rat that shows the CA1–CA4 subfields. (*Right panel*) High-magnification (400×, scale bar = 50 μm), representative photomicrographs of CA1 pyramidal cells (rectangle) of rats assigned to sham operation, 4-VO/ICA + vehicle, or 4-VO/ICA + sildenafil. The arrows and arrowheads indicate normal-looking and degenerating neurons, respectively. $^{***}p < 0.0001$ *vs.* sham, $^{\#\#\#}p < 0.0001$ *vs.* vehicle. The brains of two sham-operated rats were lost during histological preparation. Sham, $n = 11$; vehicle, $n = 20$; sildenafil, $n = 16$.

sildenafil-treated group (latency, block 5 *vs.* block 1, $p < 0.05$). These data indicate that hypoperfused rats also learned the task during training. However, between-group comparisons in each training block revealed that the vehicle-treated group spent more time to find the goal box (K–$W = 12.98$–16.61, $p < 0.01$–0.05) and committed more reference memory errors (K–$W = 12.04$–12.42, $p < 0.05$) than the sham surgery group, reflecting 4-VO/ICA-induced learning impairment. This learning deficiency was not prevented by sildenafil ($p < 0.001$–0.05 *vs.* sham; $p > 0.05$ *vs.* vehicle). The failure of sildenafil to reduce 4-VO/ICA-induced learning deficits became more evident when the cumulative latency and number of reference errors were analyzed ($F_{2,47} = 7.64$–10.83, $p < 0.01$–0.05 vehicle *vs.* sham, $p < 0.001$ sildenafil *vs.* sham; $p > 0.05$ sildenafil *vs.* vehicle; Fig. 2, lower panels).

3.3. Histological analysis

Compared with sham operation, permanent 4-VO/ICA caused moderate-to-severe neurodegeneration over the entire CA1–CA4 pyramidal stratum (56.2% cell loss, K–$W = 30.61$, $p < 0.001$; Fig. 3), DG (19.2% cell loss, K–$W = 19.61$, $p < 0.001$; Fig. 4), and cerebral cortex (47.4% cell loss in the RS cortex, K–$W = 30.37$, $p < 0.001$; 38.2% cell loss in the PtA cortex, K–$W = 33.62$, $p < 0.001$; Fig. 5). Sildenafil treatment significantly reduced neurodegeneration in all of these brain regions (30.5% cell loss in the CA1–CA4, 7.8% cell loss in the DG, 11.8% cell loss in the RS cortex, 6.5% cell loss in the PtA cortex; $p < 0.001$–0.05 sildenafil *vs.* vehicle).

4. Discussion

In this study, sildenafil treatment reduced neurodegeneration in both the CA1–CA4 stratum of the hippocampus and cerebral cortex in middle-aged rats subjected to CCH. Granular cells in the DG were also preserved by sildenafil, reflected by the maintenance of the thickness of the cell layer. In the RS and PtA cortices, the number of normal-looking, Nissl-stained pyramidal cells in the 4-VO/ICA group treated with sildenafil approached the number in the sham-operated animals, indicating robust histological protection. Despite this, however, sildenafil failed to promote learning recovery, clearly indicating a dissociation between neurohistological and functional protection.

Compared to our previous findings (Romanini et al., 2010), the present study demonstrated that the neuroprotective effect of sildenafil goes beyond the pyramidal stratum of the hippocampus, extending to the granular cell layer of the DG and cerebral cortex. This is important, since it suggests that other brain regions that are possibly damaged by CCH, mainly the striatum and thalamus, might also have been protected by sildenafil. Another important aspect of the present histological data refers to the long survival time (*i.e.*, 107 days) elapsed between the end of treatment and the histological analysis. Compared to the 27-day survival time used in Romanini's study, the present findings suggest more convincingly that sildenafil-mediated histological neuroprotection after CCH is a consistent and enduring effect. This is relevant because the degree of neuroprotection afforded by other drugs, such as the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist NBQX, N-channel calcium blocker SNX, and noncompetitive *N*-methyl-D-aspartate antagonist MK801, or non-pharmacologically (*e.g.*, hypothermia and preconditioning) was shown to decline when the postischemic survival time of the analysis was extended from the first 7 days to 30 days overall (Corbett and Crooks, 1997). Moreover, the present study extended to middle-aged rats the analysis on the effects of sildenafil after CCH. This may render the data more relevant from a clinical point of view, since aging-related morbidities such as hypertension, atherosclerosis and chronic heart disease are major risk factors for CCH (de la Torre, 2009).

Our study does not provide mechanistic insights into how sildenafil afforded long-lasting histological protection. In this regard and considering the primary action of sildenafil on vascular smooth muscles, one may speculate whether vascular or nonvascular

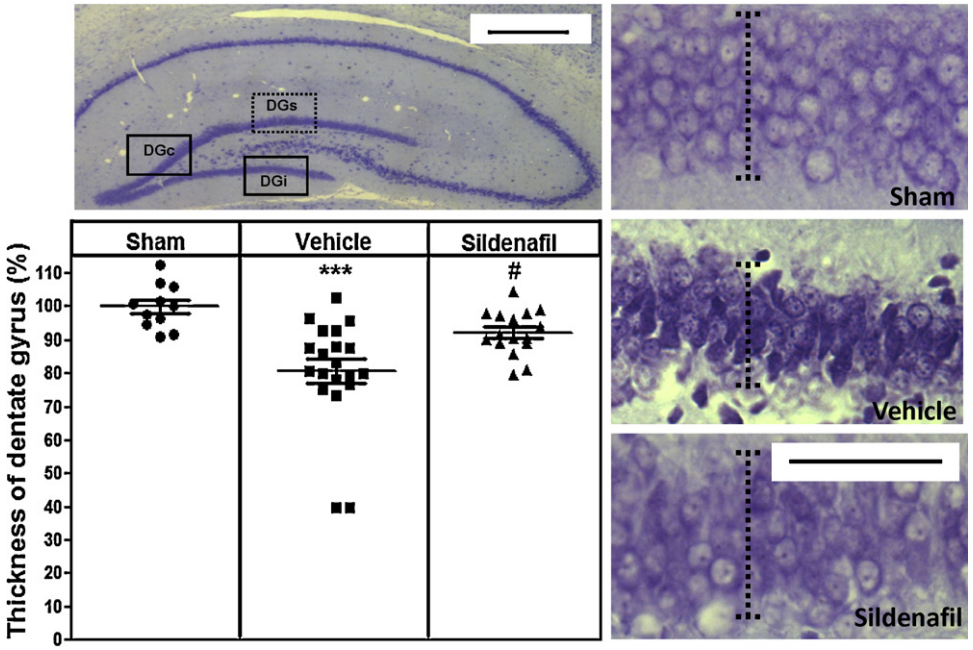

Fig. 4. Chronic cerebral hypoperfusion-induced atrophy in the DG granular cell layer and the effect of sildenafil. In each rat, a mean value of granular cell layer thickness was taken from 18 measurements (3 locations × 3 sections × 2 hemispheres). Each dot represents an individual, averaged thickness measurement, which was normalized to the mean of the respective sham-operated group (100%). The bars denote the mean ± SEM of the group. (*Left, upper panel*) Low-magnification (50×, scale bar = 500 μm) image obtained from a sham-operated rat that shows the locations (rectangles) where the thickness of the DG granular cell layer was measured at the suprapyramidal blade (DGs), infrapyramidal blade (DGi), and crest (DGc). (*Right panels*) High-magnification (400×, scale bar = 50 μm), representative photomicrographs of the DG granular cell layer (dotted rectangle) of rats assigned to sham operation, 4-VO/ICA + vehicle, or 4-VO/CA + sildenafil. Dotted vertical lines outline the qualitative thickness of the DG granular layer. $^{***}p < 0.0001$ *vs.* sham; $^{\#}p < 0.05$ *vs.* vehicle. Sham, $n = 11$; vehicle, $n = 20$; sildenafil, $n = 16$.

mechanisms play a role. Whether the primary action of sildenafil (*i.e.*, vasorelaxation in the vascular bed of the corpus cavernosum and lungs) can translate into increased global cerebral blood flow (CBF) is unclear (Ghofrani et al., 2006). Unfortunately, we were not instrumentally equipped to investigate whether sildenafil improves CBF after permanent 3-stage 4-VO/ICA, which may have helped answer this question. In rats, cerebral PDE5 protein was predominantly found in vascular smooth muscles of meningeal arteries and also in some deeper parenchymal microvessels (Menniti et al., 2009). The PDE5A enzyme is also present and active in the basilar artery in guinea pigs (Kruuse et al., 2003). Moreover, sildenafil was found to increase the level of cGMP in the cerebral cortex (Zhang et al., 2002, 2005) and hippocampus (Prickaerts et al., 2002). These findings suggest that PDE5 may also exert a vascular effect in the brain. However, whether sildenafil increases global CBF is controversial. To our knowledge, only one research group has evaluated the effect of sildenafil on CBF under conditions of cerebral ischemia. Administered 24 h after stroke and daily for 6 or 7 days, sildenafil slightly but significantly increased regional CBF in both young rats (Li et al., 2007) and aged rats (Ding et al., 2011). Such an effect persisted for up to 6 weeks post-stroke and coincided with improved functional recovery in young rats (Li et al., 2007). In these previous studies, however, CBF was estimated in the boundary region of the infarcted tissue where increased angiogenesis and axonal remodeling was also observed. This may have accounted for the sildenafil-induced improvement of regional CBF (Ding et al., 2011). In a similar study, treatment with the potent PDE5A inhibitor PF-5 began 24 h after stroke and continued daily for 7 days, resulting in nearly complete recovery of sensorimotor function measured 3 months later (Menniti et al., 2009). Although CBF was not measured in that study, the authors speculated that the acute effects of sildenafil on cerebral perfusion may have accounted for the rapid onset of sensorimotor function recovery. If so, then we may also suggest that a global and acute effect of sildenafil on brain perfusion contributed to the reversal of the high mortality rate observed after permanent 2-stage 4VO/ICA (Romanini et al., 2010). These findings or interpretations derived from animal experimentation suggest that sildenafil may improve CBF globally. This is not supported, however, by observations in humans, in which sildenafil treatment did not change either blood flow velocity (Arnavaz et al., 2003) or the diameter of the middle cerebral artery, indicating that sildenafil did not dilate cerebral or extracerebral arteries (Kruuse et al., 2003). Remaining uncertain, therefore, is whether sildenafil-mediated neuroprotection could be associated with improved global CBF.

Alternatively, sildenafil may afford histological protection through a preconditioning-like action. Sildenafil-induced preconditioning has been consistently observed in the rabbit and mouse myocardium subjected to ischemia, an effect that appears to be primarily mediated by NO derived from inducible nitric oxide synthase (iNOS; Kukreja et al., 2005). The cardioprotective effect of sildenafil was abolished in cardiomyocytes derived from iNOS gene knockout mice, clearly demonstrating the critical role played by NO-related pathways in mediating the beneficial effect of sildenafil on the myocardium (Das et al., 2005). The activation of mitochondrial adenosine triphosphate-sensitive K^+ channels and mitochondrial $Ca2^+$-activated K^+ channels has also been found to represent majors signaling mechanisms by which sildenafil-induced preconditioning results in cardioprotection (Wang et al., 2008). In association with or apart from preconditioning, sildenafil also reduced apoptosis in the ischemic myocardium (Das et al., 2005). Interestingly, sildenafil given for 8 days also reduced apoptosis in the mouse cerebral cortex after hypoxia (Caretti et al., 2008). Additionally, sildenafil stimulated the expression of vascular endothelial growth factor (Caretti et al., 2008), an effect consistent with the observation that sildenafil administered after stroke promoted angiogenesis in the periinfarct region (Li et al., 2007; Ding et al., 2011). Considering the presence and activity of both PDE5 and NO/cGMP in the brain and

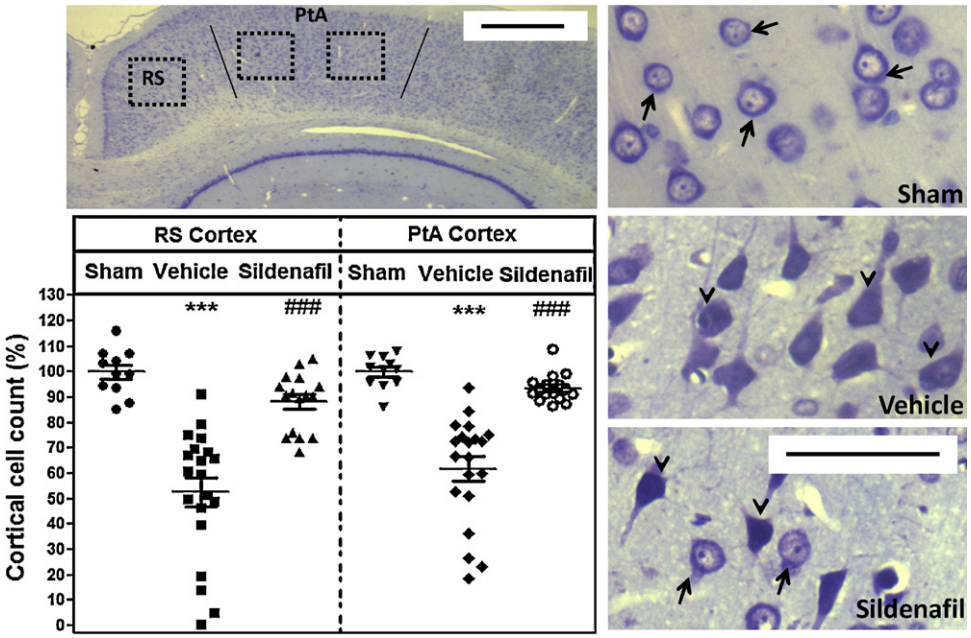

Fig. 5. Chronic cerebral hypoperfusion-induced neurodegeneration in the retrosplenial cortex (RS) or parietal association (PtA) cortex. Digital microscopic areas (rectangles, 361 μm × 270 μm, 40× objective) were captured from the RS or PtA cortex. In each animal, a mean value for the RS cortex was obtained from six measurements (1 area × 3 sections × 2 hemispheres). In the PtA cortex, 12 measurements were made for each rat (2 areas × 3 sections × 2 hemispheres). Each dot represents an individual, the value of which was normalized to the mean of the sham-operated group (100%). The bars denote the mean ± SEM of the groups. (*Left, upper panel*) Low-magnification (50×, scale bar = 500 μm), Nissl-stained coronal brain section that shows the location of the digital areas captured from the RS and PtA cortices. (*Right panel*) High-magnification (400×, scale bar = 50 μm), representative photomicrographs of pyramidal cells in the RS cortex of rats assigned to sham operation, 4-VO/ICA + vehicle, or VO/ICA + sildenafil. The arrows and arrowheads indicate normal-looking and degenerating neurons, respectively. $^{***}p < 0.0001$ *vs.* sham; $^{\#\#\#}p < 0.0001$ *vs.* vehicle. Sham, $n = 11$; vehicle, $n = 20$; sildenafil, $n = 16$.

involvement of NO-related pathways in the mediation of the protective effect of sildenafil in the myocardium (Das et al., 2005), we may speculate that sildenafil-mediated preconditioning *via* activation of the NO/cGMP pathway may have accounted for its neuroprotective effect after CCH. If so, then the question arises how such an action preserved neurons for at least 107 days of brain hypoperfusion, despite the discontinuation of sildenafil treatment soon after the completion of permanent 4-VO/ICA.

Finally, the present study demonstrated that sildenafil-mediated hippocampal and cortical neuroprotection did not translate into recovery from learning deficit after CCH. This information was lacking in the study by Romanini and cols. (2008), as young rats were not impacted cognitively by permanent 4-VO/ICA. This was confirmed later, when 3-stage 4-VO/ICA (*i.e.*, AV → ICA → ICA) with an ISI (→) as shorter as 2 days also failed to cause learning deficit in young rats, but disrupted it in older rats (Ferreira et al., 2011). The impact of permanent, 3-stage 4-VO/ICA on memory performance of middle-aged rats was confirmed subsequently (Pereira et al., 2012). Therefore, the use of middle-aged rats or older ones seems to be a requisite if both neurohistological and cognitive outcome are to be measured after permanent 4-VO/ICA.

Because long-lasting neuroprotection was provided by sildenafil in both hippocampus and cerebral cortex, the lack of behavioral recovery is intriguing. The cerebral cortex and its reciprocal connections with the hippocampus appear to be crucial for processing complex learning and memory tasks, including the radial maze task (Eichenbaum, 2000; Wall and Messier, 2001; Dalley et al., 2004; Lee and Kesner, 2003). Also, damage restricted to the RS cortex has been consistently shown to compromise the ability of rats to solve the radial maze task (Keene and Bucci, 2009). Therefore, disruption of hippocampal/cortical circuitry may at least partially underlie the effect of chronic 4-VO/ICA on learning and memory performance in middle-aged rats (Ferreira et al., 2011; Pereira et al., 2012). Ample evidence also indicates that sildenafil and other selective PDE5 inhibitors administered soon before or after training improve learning and memory performance in different behavioral tasks (Reneerkens et al., 2009). Although a direct influence of PDE5 inhibitors on overall CBF cannot be discarded, the behavioral effects described in response to these agents have been attributed to neuronal alterations (Ghofrani et al., 2006). Based on these assumptions, a key aspect of the present study is that a period greater than 3 months elapsed between the end of sildenafil treatment and the beginning of behavioral testing. Any acute effect of sildenafil exerted during the administration period on neuronal or vascular domains unlikely influenced learning performance measured 3 months later. More likely is that sildenafil-mediated neuroprotection persists as a major, if not unique, effect that theoretically could ensure recovery from learning deficits. This was not the case, however. Neurohistological protection was not accompanied by functional improvement, at least under the present experimental conditions. Similar phenomena have been found by others. For example, robust neuroprotection was observed in the CA1 subfield of the hippocampus after ischemic preconditioning in gerbils. Such a degree of neuroprotection was not only devoid of recovery of locomotor behavior in the open field but also associated with a decrease in locomotor behavior by approximately 30% when the postischemic survival time was extended from 10 to 30 days (Corbett and Crooks, 1997). Robust CA1 neuroprotection without behavioral recovery has also been observed after postischemic treatment with hypothermia (Colbourne and Corbett, 1995). Altogether, these data and the present study indicate that histological preservation does not necessarily translate into long-term functional recovery after brain damage (Green et al., 1992; Colbourne and Corbett, 1995; Corbett and Nurse, 1998). One possible reason for such a dissociation between histological and functional preservation is that the neurons that appeared to be normal in the morphometric analysis no longer functioned normally or were insufficient to ensure behavioral recovery. For example,

phenobarbital provided complete protection against ischemia-induced hippocampal CA1 cell death, but the response of those cells to KCl-stimulated acetylcholine release remained impaired. Additionally, acetylcholinesterase immunoreactivity was almost absent in CA1 neurons that exhibited normal Nissl staining (Ishimaru et al., 1995). Furthermore, ischemic preconditioning also preserved CA1 pyramidal cells from death, demonstrated by Nissl staining, but they were found to be electrophysiologically dysfunctional because their dendritic extracellular field potentials were significantly smaller than those recorded from non-ischemic animals (Corbett and Nurse, 1998). Similar subcellular disturbances may have occurred in the present case, determining the observed learning impairment despite marked neuronal preservation by sildenafil. This interpretation agrees with the general consensus that "dysfunction of complex behaviors and recovery may reflect alterations at the subcellular, synaptic or electrophysiological level, or even of widespread morphological changes that cannot be quantified by a simple cell count in a restricted region of a given structure" (Aronowski et al., 1996). Accordingly and despite the importance of preserving as many neurons as possible after an hypoxic/ischemic event (Corbett and Nurse, 1998), true neuroprotection goes beyond simple cells preservation to include neurochemical and electrophysiological normalization followed by long-term behavioral recovery.

In conclusion, the present study demonstrated that sildenafil afforded sustained histological neuroprotection after chronic cerebral hypoperfusion in middle-aged rats, an effect that was not accompanied by the recovery of learning performance assessed long after sildenafil treatment. Our interpretation is that the neurons rescued by sildenafil in the hippocampus, RS and PtA cortices, and perhaps other brain structures not examined here were no longer functional or insufficient to ensure behavioral recovery. The present findings do not discard, however, the possibility that sildenafil effectively preserves cognitive function after CCH. For example, the PDE3 inhibitor cilostazol administered continuously during the entire duration of behavioral testing in the water maze task preserved learning performance after CCH (Watanabe et al., 2006). Therefore, future studies will need to examine whether other administration or dosing regimens and other behavioral protocols could reveal the efficacy of sildenafil in promoting functional recovery after CCH. Furthermore, a comparative study of sildenafil and cilostazol should be pursued, the results of which may shed light on the role of different PDE isoforms in the mediation of the therapeutic potential of PDE inhibitors in the setting of CCH.

Conflict of interest

The authors state that there is no conflict of interest between this study and any people or organizations, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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