

Beneficial Effects of Delayed P7C3-A20 Treatment After Transient MCAO in Rats

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Abstract Despite ischemic stroke being the fifth leading cause of death in the USA, there are few therapeutic options available. We recently showed that the neuroprotective compound P7C3-A20 reduced brain atrophy, increased neurogenesis, and improved functional recovery when treatment was initiated immediately post-reperfusion after a 90-min middle cerebral artery occlusion (MCAO). In the present study, we investigated a more clinically relevant therapeutic window for P7C3-A20 treatment after ischemic stroke. MCAO rats were administered P7C3-A20 for 1 week, beginning immediately or at a delayed point, 6 h post-reperfusion. Delayed P7C3-A20 treatment significantly improved stroke-induced sensorimotor deficits in motor coordination and

symmetry, as well as cognitive deficits in hippocampal-dependent spatial learning, memory retention, and working memory. In the cerebral cortex, delayed P7C3-A20 treatment significantly increased tissue sparing 7 weeks after stroke and reduced hemispheric infarct volumes 48 h after reperfusion. Despite no reduction in striatal infarct volumes acutely, there was a significant increase in spared tissue volume chronically. In the hippocampus, only immediately treated P7C3-A20 animals had a significant increase in tissue sparing compared to vehicle-treated stroke animals. This structural protection translated into minimal hippocampal-dependent behavioral improvements with delayed P7C3-A20 treatment. However, all rats treated with delayed P7C3-A20 demonstrated a significant improvement in both sensorimotor tasks compared to vehicle controls, suggesting a somatosensory-driven recovery. These results demonstrate that P7C3-A20 improves chronic functional and histopathological outcomes after ischemic stroke with an extended therapeutic window.

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Introduction

Stroke is a devastating cerebrovascular condition with limited therapeutic approaches available. During focal cerebral ischemia, blood flow is drastically reduced in the ischemic core, triggering a complex ischemic cascade and eventual cell death [1]. Cells in the penumbra are also vulnerable but can survive longer due to enhanced tissue perfusion. Currently, the only FDA-approved treatment for acute ischemic stroke is tissue plasminogen activator (tPA), which works by breaking down clots to allow blood reperfusion [2]. Restoring blood flow is critical to treating stroke, because 1.9 million neurons are

estimated to die for every minute that cerebral ischemia persists [3]. In clinical trials, tPA was shown to be effective when administered early, but when it was administered past 4.5 h, there were greater risks for hemorrhagic transformation with only limited benefits [4, 5]. Unfortunately, the majority of ischemic patients require more than 3 h after stroke onset to reach an emergency department (ED) [6], and the average ED door to needle time is over an hour [7]. These factors drastically reduce the number of acute stroke patients eligible for tPA therapy. Endovascular thrombectomy has also demonstrated success as a therapy for ischemic stroke, due in part to their prolonged therapeutic window. Those procedures can be utilized up to 7 h after onset of patient symptoms and when combined with tPA demonstrate increased efficacy [8]. However, even if blood flow is restored by spontaneous or therapeutic interventions, neurons may still undergo delayed cell death [9]. There is therefore a critical need to develop new cytoprotective agents for patients that directly support the survival of injured neurons after ischemic stroke.

A promising approach to treat ischemic stroke is the use of combination therapies, including neuroprotective strategies that aim to extend the time window for thrombolytic intervention [10]. Neuroprotective drugs could delay cell death until blood flow is restored and also counteract the deleterious effects of reperfusion injury [1]. For example, in experimental models, positive synergistic effects have been observed when thrombolysis was combined with some neuroprotective strategies [11, 12]. However, few neuroprotective compound's therapeutic window have been established, which may have contributed to the poor translation to the clinic of some neuroprotective agents [13].

We have recently reported a novel therapeutic treatment for focal cerebral ischemia using the neuroprotective compound P7C3-A20 [14]. P7C3-A20 was selected because it has attractive pharmacological properties and has been shown to be efficacious in multiple preclinical injury models [15–22]. When administered immediately after a 90-min transient middle cerebral artery occlusion (tMCAO) in rats, P7C3-A20 protected both mature and immature neurons from degeneration. This translated into preservation of sensorimotor and cognitive behavioral function as well as maintenance of brain tissue volume at chronic time points. Improved functional recovery directly correlated with increased tissue sparing and increased neurogenesis in both the subventricular zone and hippocampal dentate gyrus subgranular zone [14]. However, the therapeutic utility of P7C3-A20 would be quite limited if it could only be administered during the early periods after stroke, as evidenced from the current limited utility of tPA.

Encouraged by the positive effects of immediate P7C3-A20 treatment, we next sought to determine whether delayed treatment with P7C3-A20 beginning 6 h after reperfusion, a more clinically relevant time point, would also provide significant protection from chronic deficits. We hypothesized that

P7C3-A20 would reduce infarct volume, increase tissue sparing, and improve functional recovery when delayed 6 h after cerebral ischemia. Accordingly, a 1-week course of P7C3-A20 treatment was initiated immediately (iA20) or delayed, 6 h (dA20) after reperfusion in rats. Behavioral and histopathological outcome were then assessed chronically over 7 weeks. Both iA20- and dA20-treated animals displayed reduced sensorimotor deficits on the cylinder and grid-walk tasks, as well as improved cognitive performance in the Morris water maze task. In addition, there was a reduction in cortical infarct volume 48 h after cerebral ischemia with both iA20 and dA20 treatment. Both groups of A20-treated animals also displayed increased cortical tissue volume compared to vehicle-treated stroke controls at 7 weeks after injury, suggesting that this protection was sustained chronically. These results support our previous findings and demonstrate for the first time a persistent therapeutic effect with delayed administration of P7C3-A20 after stroke in mitigating neurodegeneration and promoting chronic functional recovery.

Methods

Transient Focal Cerebral Ischemia

All procedures involving animal care were approved by the Institutional Animal Care and Use Committee at the University of Miami, Miller School of Medicine. Rats were fasted for 12 h prior to tMCAO. To induce ischemia, male Sprague-Dawley rats (300–330 g) were anesthetized with 3% isoflurane in a mixture of 70% N₂O/30% O₂ for 5 min for induction and then maintained via nose cone at 1–2% isoflurane. The animal's brain and body temperatures were monitored and maintained to a normothermic temperature range (36.5 to 37.5 °C). As previously described, the right common carotid artery was dissected with a midline neck incision making sure not to damage the surrounding nerves [14]. The external carotid artery (ECA) and superior thyroid artery were then ligated. A small incision was made in the ECA, and a 20-mm nylon monofilament with a silicone rubber-coated tip (Doccol Corporation, Sharon, MA) was inserted slowly and then advanced to the middle cerebral artery. During the 90-min occlusion period, the ECA was temporarily ligated, and the animal was placed back in the cage. Surgeries for sham rats were identical to tMCAO, except no suture was used. Since certain anesthetics, including isoflurane can be neuroprotective, the total anesthetic time was minimized and animals were awakened during the period of occlusion [23, 24].

Before re-anesthetizing the rats, a neurological score was conducted using a five-point scale to determine the behavioral severity of ischemic insult, as previously described [14, 25]. A score of zero indicated no neurological deficit, a score of two

indicated a moderate neurological deficit where rats circled to the left in their cage, a score of three indicated a severe neurological deficit as animals fell to the left, and a score of four indicated animals that displayed a depressed level of consciousness as indicated by lack of movement. After 90 min, the suture was slowly retracted from the animal and the ECA was sutured shut. Animals were administered buprenorphine 0.03 mg/kg (s.c.) and placed on soft bedding in their cages, which were placed on a heated pad (Peco Services Ltd., England, UK) at 37 °C. Rats were monitored twice daily, and daily weight assessments were conducted to ensure that no animals lost more than 20% total body weight. A total of 148 animals were used in this study.

P7C3 Compound

As previously described, P7C3-A20 and vehicle were prepared by combining DMSO, 5% Dextrose, and Kolliphor. Animals were injected intraperitoneally (10 mg/kg) either immediately or 6 h after reperfusion, and then injections were continued twice daily for 7 days. This dosing regimen was based on previous neurodegenerative studies that demonstrated efficacy of P7C3-A20 [14, 16].

Randomization and blinding procedures were performed by a researcher not associated with the study. Consistent with animal research reporting guidelines [26], a power analysis was performed prior to the experiment to determine the necessary animal number. Using our laboratory working memory, published data, and historical data, we determined that 12 animals were needed per group with a power of 0.8 and an alpha of 0.05.

Behavioral Analysis

Behavioral assessments were conducted as previously described [14]. The experimenter was blinded to treatment groups while performing and evaluating all behavioral tests. Only after the data analysis was completed were the treatment groups decoded.

Sensorimotor To evaluate sensorimotor deficits, we used the cylinder and grid-walk tasks at 1 week post-injury. All tests were 5-min long and were recorded for subsequent post hoc analysis in a slow motion. For the cylinder task, animals were placed in a clear cylinder and independent forelimb paw placements were counted 1 week after injury. The cylinder test measures weight-bearing shifts in forelimbs by encouraging vertical exploratory behavior. Asymmetry was calculated by $[(\text{number of paw placements of the affected forelimb})/(\text{total number of placements of both paws})]$ [27].

For the grid-walk task, rats were placed on an 11 × 11-cm grid that was raised off the surface. Using somatosensory stimuli, animals walk along the outside grid before placing

full weight on their paws in order to not fall through the spaces in the grid. If a paw penetrated through the grid, it was considered a misstep as it did not provide support. The percentage of missteps was calculated by $[(\text{number of missteps})/(\text{total number of paw placements}) + (\text{number of missteps})]$ [27].

Learning and Memory All cognitive assessments were conducted using the Morris water maze and were recorded with the EthoVision software (Noldus Information Technology, Leesburg, VA) in order to analyze time spent in each quadrant, path length, and swim speed [14]. Water maze experiments began 4 weeks after injury based on the temporal maturation of newly generated hippocampal neurons [28]. Rats were placed in a black tub filled with warm water for up to 60 s. Using only visual cues located around the room, rats immediately tried to escape and find a hidden platform that was slightly submerged underneath the water. Animal latencies were recorded during each trial. Four trials were conducted each day (four different starting locations) over 4 days of training, with a 4-min interval between trials. On the fifth day, the platform was removed for the probe trial. Long-term memory was analyzed by measuring the percentage of time animals spent in the target quadrant (quadrant that previously housed the platform).

On the following day, the working memory was evaluated using paired trials: a location trial and match trial. Similar to the hidden platform, animals were placed in a novel starting location and then had 60 s to find the novel hidden platform location (location trial). Next, rats were given a 10-s rest and then placed immediately back in the same starting location to rerun the trial (match trial). Five paired trials were performed (five different starting and ending locations) over 2 days, with the first day considered to be a training day. In each trial, the escape latency was measured and the percent improvement was calculated by $[(\text{location trial} - \text{match trial})/(\text{location trial} \times 100)]$ [14].

Histopathology

Subacute Survival Studies To determine whether delayed P7C3-A20 treatment after tMCAO could reduce acute lesion volume, triphenyl tetrazolium chloride (TTC) histochemistry was utilized at 2 days post-injury. Animals were deeply anesthetized with 3% isoflurane, 70% nitrous oxide, and 30% O₂ and then transcardially perfused with saline (0.9% NaCl; 75 ml). For the infarct volumes, brains were quickly dissected and placed in a brain mold. To improve cutting, the mold was placed in a – 75 °C freezer for 7 min to lightly harden the tissue. Next, nine sections of tissue were cut coronally (every 2 mm) and incubated in 1.5% warmed (37 °C) TTC for 7 min [29]. The sections were then flipped to the other side and incubated for seven additional minutes. The sections were then placed in formaldehyde for 5 min to slightly preserve

the tissue. As previously described, infarct quantification were performed on the cerebral cortex and striatum using the direct method: $[(\text{infarct} / (\text{infarct} + \text{live tissue}) \times 100)]$ [30].

Chronic Survival Studies Seven-week post-injury animals were transcatheterially perfused with saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer pH 7.4 (150 ml). Brains were then dissected, post-fixed for 48 h, and incubated in 20% sucrose for cryoprotection. The tissue was then embedded in an optimal cutting temperature compound (Fisher Scientific, Waltham, MA) and flash frozen. Brains were cut coronally at 30 μm in a stereologic series and mounted. Sections were stained with hematoxylin and eosin (H&E) and then imaged with PathScan Enabler IV (Meyer Instruments, Houston, TX). Using white matter tracts to separate zones, the ipsilateral cortex, striatum, and hippocampus were contoured, and the volumes were quantified with StereoInvestigator (MicroBrightField Bioscience, Williston, VT) between bregma levels 1.2 and -4.3 mm. Healthy intact tissue volume was calculated by $[(\text{total ipsilateral live tissue volume}) / (\text{average of all 12 sham animal's ipsilateral tissue volumes}) \times 100]$.

Statistical Analysis

Tissue volume assessments were expressed mean \pm standard deviation. All other data were expressed as mean \pm standard error of the mean (SEM). After a normal distribution of the data was first confirmed, the following tests were conducted: for comparison of three or more groups with one independent variable, a one-way analysis of variance (ANOVA) with Tukey's honest significance difference (HSD) correction was used; for comparison of three or more groups with two independent variables, a two-way repeated measures ANOVA with Bonferroni correction was performed. Power analysis was conducted with a power of 0.8 and an alpha of 0.05.

Results

Inclusion, Exclusion, and Mortality

A power analysis determined that 12 animals were required for each of the five experimental groups: sham, tMCAO + immediate vehicle, tMCAO + immediate P7C3-A20 treatment (iA20), tMCAO + delayed vehicle, and tMCAO + delayed P7C3-A20 treatment (dA20). Since there were no significant differences between vehicle groups, animals were pooled. There were two sets of animals used in the experimental paradigm (Fig. 1): 60 animals for chronic behavioral and tissue volume assessments and 48 animals for acute infarct volume (TTC) assessments.

A neurological score was performed prior to MCA reperfusion to assess ischemic-induced functional deficits, and a total of 14 animals were excluded, because they did not demonstrate an acute behavioral deficit. In addition, eight statistical outliers were determined using a box plot, which eliminated four rats based on performance in the cylinder task and four rats based on performance in the water maze task. Also, within 3 days of cerebral ischemia, 18 animals died or were excluded from future testing because they lost more than 20% of their body weight. Body weight and brain and body temperatures were the only physiological parameters assessed during the induction of cerebral ischemia, and there were no significant changes between experimental groups. An extensive physiological assessment battery was not conducted in this study because of the specific surgical approach used. Moreover, there have been no reported side effects of P7C3-A20 using this treatment protocol in rats, such as alterations in blood pressure or glucose (16).

Immediate and Delayed P7C3-A20 Treatment Reduce Acute Cortical Infarct Volume

We previously reported that after tMCAO, immediate treatment with P7C3-A20 reduced tissue loss chronically and restored nicotinamide adenine dinucleotide (NAD) levels at 48 h. Here, we compared the efficacy of this week-long treatment with P7C3-A20 as a function of whether it was initiated immediately (iA20) or delayed 6 h (dA20) after reperfusion. Infarct volumes were first assessed 48 h after reperfusion using TTC staining, and representative images are presented in Fig. 2a. In the cortex, there was a significant reduction in cortical infarct volume with iA20 treatment as compared with tMCAO + vehicle ($p < 0.001$) (Fig. 2b). This protection with P7C3-A20 was sustained when treatment was delayed 6 h, as dA20-treated animals also had significantly reduced infarct volumes compared to tMCAO + vehicle animals ($p < 0.01$). Furthermore, there was no significant difference in cortical infarct volume between iA20 and dA20 treatment groups. Since the infarct core develops throughout the striatum with the filament MCAO model, striatal TTC volumetric assessments were also performed [31]. At 48 h, there was no significant difference between any experimental groups in striatal infarct volumes (Fig. 2c).

Sensorimotor Deficits Are Reduced by Both iA20 and dA20 Treatment

Since dA20 treatment reduced infarct volumes acutely, we next investigated whether this tissue protection translated into functional improvements. Sensorimotor ability was assessed using the cylinder and grid-walk tasks 7 days after tMCAO. The 7-day post-ischemic period was chosen, because in our

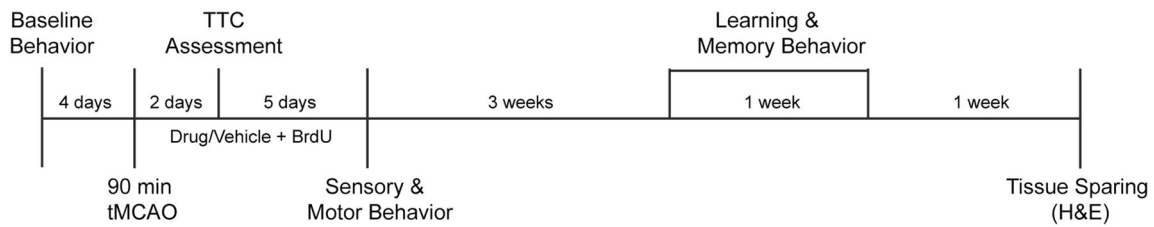


Fig. 1 Experimental paradigm. Rats were acclimated to the new environment for 1 week before surgery. Stroke was induced using a 90-min tMCAO, and rats were then administered vehicle or P7C3-A20 either immediately or 6 h post-reperfusion. Forty-eight hours after reperfusion, infarct volume was assessed in the cortex and striatum. At 7 days,

sensorimotor ability was determined with the cylinder and grid-walk task. Hippocampal-dependent spatial memory tasks began 4 weeks post-injury and lasted for 1 week. Six weeks after ischemia, rats were sacrificed for cortical, striatal, and hippocampal histological assessments

previous study, iA20 improved sensorimotor ability at this time point [14]. Therefore, we wanted to determine whether delaying P7C3-A20 treatment would also improve sensorimotor function.

Since tMCAO is a unilateral injury, it causes deficits in symmetry that can be critically analyzed with the cylinder task, which examines spontaneous weight-bearing shifts of forelimbs during vertical exploratory behavior. As expected, sham animals had no deficit in symmetry (Fig. 3a). After cerebral ischemia, however, there was a significant reduction in symmetry in the tMCAO + vehicle treatment group as compared to sham animals ($p < 0.001$). dA20 treatment significantly increased symmetry, as animals used their contralateral forelimb more than tMCAO + vehicle animals ($p < 0.01$). There was no significant difference between iA20- and dA20-treated animals (Fig. 3a).

On the same day, impairments in limb function were assessed using the grid-walk task. Placed on a wire grid,

animals actively explore the novel environment while positioning their paws along the wire frame. Motor coordination was determined by measuring the percentage of missteps that occurred in the rat's contralateral forelimb. Due to the unfamiliarity of the task, all animals will make some degree of misstep (~10% in sham animals; Fig. 3b). Cerebral ischemia significantly impaired motor coordination, as all treatment groups had an elevated number of missteps as compared to sham rats. There was a significant reduction in missteps, however, with both iA20 and dA20 treatment as compared with tMCAO + vehicle ($p < 0.05$). Sham, iA20 treatment, and dA20 treatment were not significantly different (Fig. 3b).

Immediate and Delayed P7C3-A20 Treatment Improve Hippocampal-Dependent Spatial Memory

Based on our previous findings that iA20 treatment protects rats from learning and memory deficits after tMCAO, we

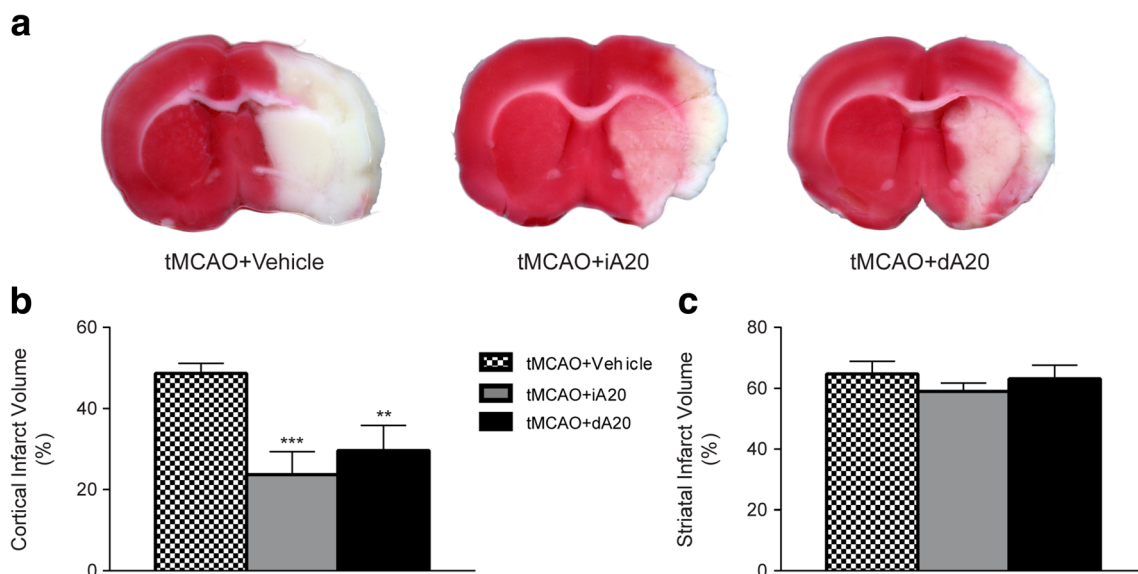


Fig. 2 iA20 and dA20 treatment reduce cortical infarct volume. Forty-eight hours after reperfusion, rats were sacrificed and cortical and striatal infarct volumes were determined using TTC staining (a). Immediate (iA20) and 6 h delayed (dA20) A20 treatment significantly reduced cortical infarct compared to tMCAO + vehicle (b). There was no significant

change in striatal infarct volumes (c). For all groups, $n = 12$; mean \pm SEM. Double asterisks indicate $p < 0.01$; triple asterisks indicate $p < 0.001$ versus tMCAO + vehicle. One-way ANOVA with Tukey's HSD correction for multiple comparisons

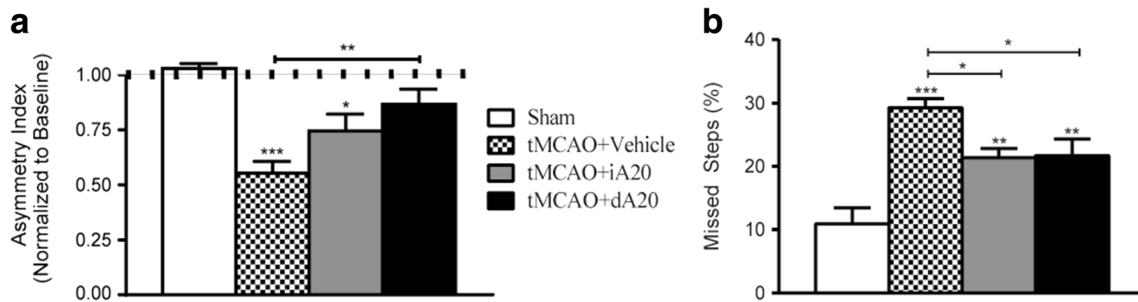


Fig. 3 iA20 and dA20 treatment both improve sensorimotor ability 1 week after stroke. One week after injury, sensorimotor ability was assessed using the cylinder and grid-walk test. **a** In the cylinder task, sham animals demonstrated normal symmetry. However, cerebral ischemia significantly impaired forelimb use in tMCAO + vehicle rats. There was significant improvement in symmetry with dA20 treatment as compared with vehicle treatment after tMCAO ($p < 0.01$). Between iA20 and dA20 treatment, there was no significant difference in symmetry. **b** On the same day, motor coordination was assessed with the grid-walk task. Due to the

novel environment, sham animals had missteps as they explored. Cerebral ischemia significantly impaired motor coordination in all groups, and treatment with both iA20 and dA20 significantly reduced the percentage of missteps ($p < 0.05$) compared to tMCAO + vehicle. For all groups, $n = 12$; mean \pm SEM. Single asterisk indicates $p < 0.05$, double asterisks indicate $p < 0.01$, and triple asterisks indicate $p < 0.001$ versus sham. One-way ANOVA with Tukey's HSD correction for multiple comparisons

next tested whether this same therapeutic benefit would be documented with delayed treatment. Four weeks after cerebral ischemia, cognitive ability was examined using a hippocampal-dependent spatial test, the Morris water maze (MWM). Acquisition was assessed over four training days, as animals learned the location of a hidden platform using spatial cues located around the room. All group's escape latencies were reduced daily, demonstrating normal acquisition learning in all animals. There was no significant interaction between escape latency and days (Fig. 4a). However, there was a significant main effect on treatment ($F_{(4,44)} = 13.06$, $p < 0.001$) as tMCAO + vehicle was significantly different from sham, iA20, and dA20 treatment. On the final day of acquisition, both sham and iA20-treated animals found the hidden platform more quickly than the other groups. Cerebral ischemia significantly impaired spatial memory, as the tMCAO + vehicle rat's escape latency was twice that of the iA20 treatment group ($p < 0.01$) (Fig. 4b). Despite observing no significant difference between dA20 and tMCAO + vehicle, there was also no significant difference between iA20, dA20, and sham animals.

Using a probe trial, memory retention was evaluated next by removing the hidden platform and measuring the time spent in the quadrant that previously housed the platform (Fig. 4c). Focal ischemia impaired memory retention as tMCAO + vehicle rats performed significantly worse than sham animals ($p < 0.01$). However, treatment with iA20 improved memory retention, as rats spent significantly more time in the target quadrant ($p < 0.01$). There was no significant improvement with dA20 treatment, although the results were trending ($p = 0.058$). In addition, there was no significant difference between the sham, iA20, and dA20 experimental groups. Total path length and swim velocity were also examined during the probe trial, and there was no difference between treatment groups (Fig. 4d, e).

After the probe trial, working memory was evaluated with a MWM paradigm that used five paired trials, consisting of a location and match trial (Fig. 4f). The starting location of the rat and hidden platform location were changed at the start of each location trial. Working memory was determined as the difference between the match and the location trial. There was no significant interaction between treatment groups and trials, but it was trending ($p = 0.068$) (Fig. 4g). All treatment groups performed better on the match trial (Fig. 4h), with sham animals demonstrating the greatest improvement (62%). There was a significant reduction in working memory ability of tMCAO + vehicle animals as compared to sham ($p < 0.001$), but this was recovered with iA20 treatment ($p < 0.001$). There was also significant improvement with dA20 treatment as compared with vehicle treatment ($p < 0.05$). Sham, iA20, and dA20 groups were not significantly different in the MWM working memory paradigm.

Cortex and Striatum Are Protected Chronically with Both iA20 and dA20 Treatment

We next speculated that P7C3-A20's ability to increase cell survival could be responsible for the sensorimotor and cognitive improvements observed in treated animals. Seven weeks after injury, rat brains were stained with H&E in order to quantify cortical, striatal, and hippocampal tissue volumes (Fig. 5a, d). In the cortex, both iA20- and dA20-treated animals had significantly more cortical volume as compared to tMCAO + vehicle (Fig. 5b). There was a similar trend in the striatum, as iA20- ($p < 0.01$) and dA20 ($p < 0.01$)-treated animals had significantly more tissue volume than tMCAO + vehicle (Fig. 5c). In the hippocampus, there was minimal tissue loss in the tMCAO + vehicle group, and there was only significant tissue preservation with iA20 treatment ($p < 0.05$), but not dA20 treatment (Fig. 5d). In all histopathological assessments

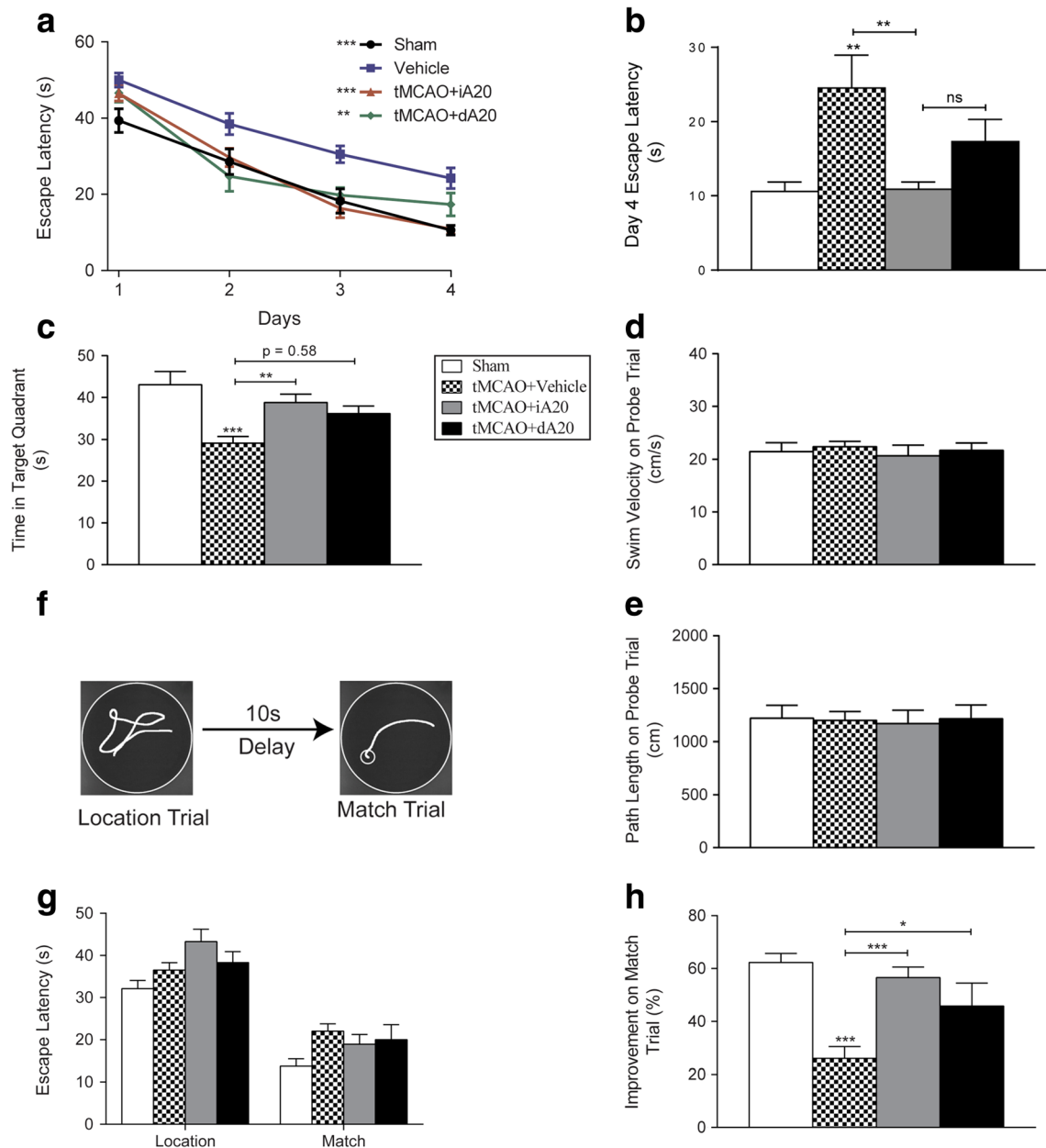


Fig. 4 iA20 and dA20 treatment reduces stroke-induced cognitive deficits. Four weeks after stroke, learning, memory retention, and working memory were assessed using the Morris water maze. **a** Over the course of four training days, all experimental groups improved in their escape latencies. There was no significant interaction between treatment and days, but there was a significant main effect of treatment between sham, iA20 treatment, and dA20 treatment compared to tMCAO + vehicle. **b** On the final day of acquisition, sham animals reached the target quadrant in 10 s, whereas tMCAO + vehicle spent twice as long. Administering iA20 significantly reduced escape latency compared to tMCAO + vehicle ($p < 0.01$). There was no improvement with dA20 treatment. **c** After training days, memory retention was assessed with a probe trial. tMCAO + vehicle animals spent significantly less time in the target quadrant as compared to sham ($p < 0.001$) and iA20-treated ($p < 0.01$) animals. dA20 did not significantly improve memory retention, but the results were

trending ($p = 0.058$). **d, e** There was no change in swim velocity or path length on the probe trial between all groups. **f** Working memory was determined using paired trials, consisting of a location trial and then a match trial. **g** There was no significant interaction between treatment group and trial. **h** Between the two trials, there was a significant reduction in working memory after cerebral ischemia ($p < 0.001$). Both iA20 ($p < 0.001$) and dA20 ($p < 0.05$) treatment significantly improved working memory on the match trial as compared to tMCAO + vehicle. For all groups, $n = 12$; mean \pm SEM. **a, g** Two-way ANOVA with Bonferroni correction for multiple comparisons. **b–e, h** One-way ANOVA with Tukey's HSD correction for multiple comparisons

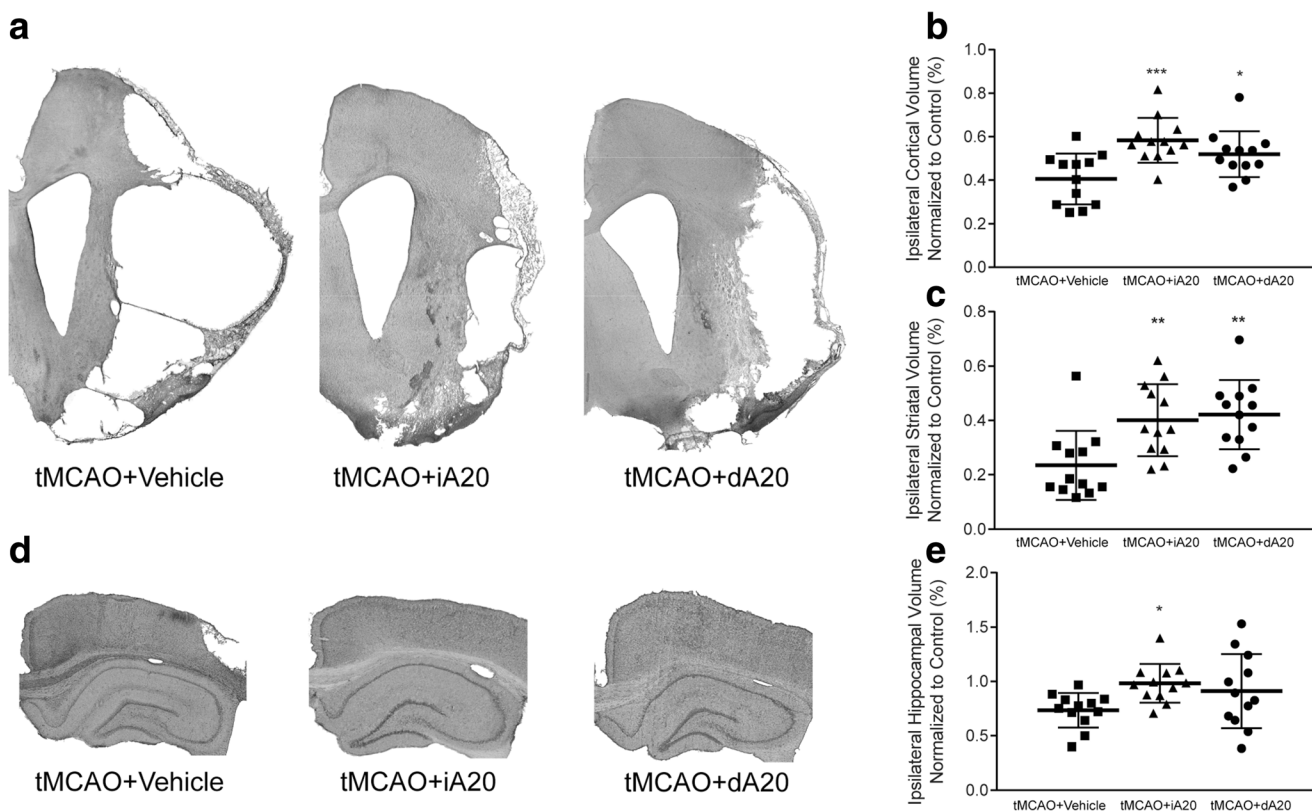


Fig. 5 iA20 and dA20 treatment increases cortical and striatal tissue volume. Seven weeks after tMCAO, H&E staining was performed in order to calculate cortical, striatal, and hippocampal tissue volume (**a, d**). There was a significant increase in cortical tissue volume with iA20 ($p < 0.001$) and dA20 treatment ($p < 0.05$) when compared with tMCAO + vehicle (**b**). In the striatum, there was a significant increase in tissue volume with both A20 treatment groups ($p < 0.01$) compared to tMCAO + vehicle (**c**). Only iA20 treatment had significantly ($p < 0.01$)

increased tissue volume compared to tMCAO + vehicle in the hippocampus. Percent tissue volume was normalized to the average of all sham animals for each region. Each point represents the calculated volume for an individual animal; wider horizontal bars represent the group average; smaller horizontal bars represent one standard error from the mean. For all groups, $n = 12$. Single asterisk indicates $p < 0.05$ versus tMCAO + vehicle. One-way ANOVA with Tukey's HSD correction for multiple comparisons (**b–d**)

at 7 weeks, there was no significant difference between iA20 and dA20 treatment groups.

Discussion

The major goal of this study was to determine whether the therapeutic benefits of P7C3-A20 would be sustained if the treatment window was extended beyond immediate initiation of treatment after tMCAO. We show for the first time that delaying treatment of P7C3-A20 to 6 h after a 90-min tMCAO significantly reduces cortical infarct volume 48 h after reperfusion, as well as increases cortical and striatal tissue sparing 7 weeks after injury. These reductions in tissue damage translated into significant improvements in sensorimotor and hippocampal-dependent learning and memory ability. In each outcome measured, there was no significant difference between either iA20 or dA20 treatment, suggesting that the beneficial effects of P7C3-A20 did not significantly diminish when treatment was delayed 6 h post-reperfusion. Taken

together, these studies support and extend our previous work demonstrating that P7C3-A20 is efficacious as a treatment for focal cerebral ischemia.

Despite iA20's ability to ameliorate many stroke-induced deficits, initiation of treatment immediately after reperfusion is not a practical expectation for treating the majority of acute stroke patients. This limitation has been made especially evident in regard to the use of tPA, which the majority of ischemic patients do not receive because it can only be administered up to 4.5 h after the onset of stroke-related neurological symptoms [5]. Previous evidence suggested that an extended therapeutic window for P7C3-A20 might exist in stroke. In blast-mediated traumatic brain injury, for example, delaying P7C3 treatment 24 h blocked axonal degeneration and preserved learning and memory [22]. P7C3-A20's mechanism of action also provided additional evidence for extending P7C3-A20's therapeutic window after focal stroke. By enhancing flux of the NAD salvage pathway via stimulation of nicotinamide phosphoribosyltransferase (NAMPT), P7C3-A20 stabilizes levels of NAD after injury [14]. The coenzyme

NAD is rapidly depleted after 30 min of ischemia, which is predominantly due to DNA damage and activation of poly(ADP-ribose) polymerases. A second and third NAD depletion occurs at 6 and 25 h when necrosis and apoptosis are most prominent, respectively [32]. Therefore, salvaging NAD at 6 h after focal stroke could have a significant beneficial effect on recovery.

Since NAD levels are reduced after stroke, there have been many therapies aimed at targeting NAMPT in order to restore intracellular NAD. For example, administering eNAMPT after focal cerebral ischemia protects against white matter injury by increasing the amount of myelinated fibers in the striatum and corpus callosum [33–35]. Conversely, inhibiting NAMPT increases glutamate excitotoxicity via Sirt1 [36], which expands the infarct volume and the number of degenerating neurons after photothrombosis [34, 37]. Recently, Wang et al. (2016) demonstrated that P7C3-A20 elevated NAD levels 24 h after permanent MCAO [38], and in our previous manuscript, we also observed P7C3-A20-mediated NAD recovery 48 h after a tMCAO [14]. We therefore suspected that treating with P7C3-A20 at that same time point would translate into reduced infarct volumes. Indeed, in this study, we detected a significant reduction in cortical infarct with both iA20 and dA20 treatment, which suggests that P7C3-A20's ability to protect the cortex was preserved when treatment was delayed 6 h after reperfusion.

By contrast, we observed no significant change between iA20 or dA20 treatment in striatal infarct volumes 2 days post-injury. Chronically, however, there was a significant increase in tissue sparing in the cortex and striatum with both P7C3-A20 treatment groups compared to tMCAO + vehicle animals. In this regard, we were somewhat surprised to observe chronic protection in the striatum, considering that there was no difference in the striatal infarct acutely. One possible explanation is that animals that underwent infarct assessments had a curtailed treatment regimen. Since infarct volume was assessed only 48 h after injury, the same time point that NAD was quantified in our previous P7C3-A20 stroke publication, rats only received one third the total cumulative dose of P7C3-A20 that chronic rats received [14]. Therefore, P7C3-A20's striatal protection may require an extended treatment paradigm. Moreover, since the striatum represents the ischemic core of the insult, it is particularly vulnerable to a lack of collateral circulation after focal stroke [31]. Cells in the striatum might simply require longer treatment regimens to attenuate the mechanisms underlying progressive atrophy in order to augment cell survival [31, 39]. In addition, dA20 treatment did not significantly protect the hippocampal volume chronically after stroke, which correlated with minimal improvements observed in hippocampal-dependent spatial memory tests. Only in a working memory paradigm did dA20 treatment reduce stroke-induced cognitive deficits. However, the results in

memory retention with dA20 treatment were trending towards a protective effect.

Since we previously reported that iA20 treatment enhanced motor functional recovery [14], we also wanted to determine if this was seen in the dA20 group. In the grid-walk task, both iA20- and dA20-treated rats demonstrated significant improvements over tMCAO + vehicle animals, indicating that delayed treatment of P7C3-A20 had a sustained therapeutic effect. In the cylinder task, tMCAO significantly impaired symmetry and use of the animal's contralateral forelimb, which was subsequently recovered with both iA20 and dA20 treatment. Despite observing no improvement with iA20 animals compared with tMCAO + vehicle animals, there was also no significant difference between iA20 and dA20 rats.

Delayed treatment of P7C3-A20 also reduced learning and memory behavioral deficits, which are commonly reported after focal cerebral ischemia. In the MWM, dA20-treated rats showed improvement in spatial learning, memory retention, and working memory. To confirm that the cognitive improvements observed with P7C3-A20 treatment were not due to impairments in motor ability, swimming performance was also evaluated using total path length and swim velocity during the probe trial [40–42]. There was no significant difference in path length or swim velocity between experimental groups, suggesting that tMCAO did not contribute to learning and memory deficits in the MWM. Interestingly, the improvements in hippocampal-dependent learning were not as great as the gains observed in sensorimotor function, possibly indicating a somatosensory-driven recovery with dA20 treatment.

Our studies support and extend the previous findings that the therapeutic effects of iA20, including improved functional performance and increased tissue sparing, are sustained when treatment is delayed 6 h [14, 38]. Since we previously observed that iA20 treatment increased neurogenesis at 7 weeks post-injury, future studies are now needed to determine the role of neurogenesis with dA20 treatment. Specifically, does dA20 treatment also increase neurogenesis in the SGZ and SVZ, and if so, does this phenomenon participate in the functional recovery that is observed? P7C3-A20 was thought to enhance neurogenesis by protecting vulnerable immature neurons without affecting the amount of astrocytes or oligodendrocytes in the hippocampus [43]. However, little is known about the other cell types in the neurovascular unit, and future studies are now needed to determine their role. Studies are also required to determine whether P7C3-A20 is efficacious in female animals since preclinical and clinical studies have demonstrated sex differences in pathomechanisms and outcomes after ischemic stroke [44]. Although physiological differences in sex may change the pharmacokinetics of a compound [45], we felt it was outside the scope of the current project. However, based on the current findings demonstrating that P7C3-A20 is efficacious when administered at a clinically

relevant time point, we plan in the future to compare P7C3-A20 treatment in female and male MCAO animals.

Finally, it would be useful to determine whether P7C3-A20 has an additive or synergistic effect when combined with other treatment methods, such as tPA or thrombectomy. For example, tPA in combination with a stent retrieval protocol performed better than tPA alone in the SWIFT PRIME trial for large vessel occlusions [46]. Neuroprotective agents such as P7C3-A20 might have synergistic effects with direct intravascular treatments by protecting vulnerable neurons from acute and more progressive injury mechanisms. In the past, neuroprotective compounds have been combined with thrombolysis and evaluated as a treatment strategy for ischemic stroke, such as AMPA and NMDA receptor antagonists, MMP inhibitors, immunosuppressants, scavengers of ROS, and hypothermia [11]. However, since the therapeutic window was not established beforehand for some drugs, mixed results were reported [10]. Because P7C3-A20 demonstrated a persistent therapeutic effect when treatment was delayed 7.5 h after the primary ischemic insult, it would be important to evaluate a combination therapy including tPA with P7C3-A20 in a thrombotic stroke model. Ultimately, if P7C3 compounds are also shown to be effective in hemorrhagic stroke, they would be an ideal therapy for first responders to protect and delay vulnerable cells from dying until recanalization can occur.

In summary, our studies demonstrate for the first time that delaying the initiation of P7C3-A20 treatment to 6 h after cerebral reperfusion has a significant beneficial effect on long-term stroke outcome. P7C3-A20 treatment reduced acute and chronic tissue damage, which was associated with improvements in sensorimotor ability and cognitive performance. In addition, this study replicated our original findings of the beneficial role of iA20 treatment after transient focal cerebral ischemia [14, 43]. Ultimately, since the majority of ischemic patients take more than 4 h after stroke onset to receive treatment [6, 7], being able to initiate treatment at 7.5 h after the initial ischemic insult makes P7C3-A20 an appealing strategy for acute ischemic stroke.

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Compliance with Ethical Standards

Conflict of Interest Andrew A. Pieper owns patents related to the P7C3 series of neuroprotective compounds.

Research Involving Animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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