Published in final edited form as:

Pharmacol Res. 2018 October ; 136: 121–132. doi:10.1016/j.phrs.2018.09.001.

Treatment duration affects cytoprotective efficacy of positive allosteric modulation of $\alpha 7$ nAChRs after focal ischemia in rats.

Nikhil Gaidhani and Victor V. Uteshev*

Abstract

To minimize irreversible brain injury after acute ischemic stroke (AIS), the time to treatment (i.e., *treatment delay*) should be minimized. However, thus far, all cytoprotective clinical trials have failed. Analysis of literature identified short treatment durations (< 72 h) as a common motif among completed cytoprotective clinical trials. Here, we argue that short cytoprotective regimens even if given early after AIS may only slow down the evolution of ischemic brain injury and fail to deliver sustained long-term solutions leading to relapses that may be misinterpreted for conceptual failure of cytoprotection. In this randomized blinded study, we used young adult male rats subjected to transient 90 min suture middle cerebral artery occlusion (MCAO) and treated with acute vs. subchronic regimens of PNU120596, a prototypical positive allosteric modulator of $\alpha 7$ nicotinic acetylcholine receptors with anti-inflammatory cytoprotective properties to test the hypothesis that insufficient treatment durations may reduce therapeutic benefits of otherwise efficacious cytoprotectants after AIS. A single acute treatment 90 min after MCAO significantly reduced brain injury and neurological deficits 24 h later, but these effects vanished 72 h after MCAO. These relapses were avoided by utilizing sub-chronic treatments. Thus, extending treatment duration augments therapeutic efficacy of PNU120596 after MCAO. Furthermore, sub-chronic treatments could offset the negative effects of prolonged treatment delays in cases where the acute treatment window after MCAO was left unexploited. We conclude that a combination of short treatment delays and prolonged treatment durations may be required to maximize therapeutic effects of PNU120596, reduce relapses and ensure sustained therapeutic efficacy after AIS. Similar concepts may hold for other cytoprotectants including those that failed in clinical trials.

Graphical Abstract

*Corresponding author: Department of Pharmacology and Neuroscience, University of North Texas Health Science Center, 3500 Camp Bowie Blvd., Fort Worth, TX 76107, Victor.Uteshev@unthsc.edu.

Conflict of interest: None

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Extending Treatment Extends Therapeutic Benefits

Brain Injury 24 h After Focal Ischemia
Without Treatment:

Brain Injury 24 h After Focal Ischemia
+ Single Acute Treatment:

Brain Injury 72 h After Focal Ischemia
+ Single Acute Treatment:

Brain Injury 72 h After Focal Ischemia
+ Daily Sub-Chronic Treatments:

Keywords

ischemic stroke; MCAO; acetylcholine; PNU120596; PNU-120596; cholinergic anti-inflammatory pathway

INTRODUCTION

Despite multiple failures in pre-clinical and clinical trials, cytoprotection remains a promising approach to treatment of acute ischemic stroke (AIS) [1–4]. The impact of clinically effective cytoprotectants cannot be overestimated as cytoprotectants may be effective in both ischemic and hemorrhagic strokes and thus, functional imaging is not required prior to cytoprotective therapies considerably reducing time to treatment initiation. Furthermore, intranasal route of administration of safe cytoprotectants does not require medical training and can be performed by stroke patients or friends and family members immediately after the onset of AIS symptoms [5]. Thus, cytoprotectants may serve as both a first-aid therapeutic bridge to intra-arterial interventions (e.g., thrombolysis, thrombectomy) and a monotherapy. A potent and safe cytoprotectant could considerably elevate the success rate of thrombolysis and/or thrombectomy providing a tremendous benefit to stroke patients. There is also not a rational basis to expect that cytoprotection is incompatible with rehabilitation treatments (e.g., targeted plasticity [6] and neurogenesis [7,8]).

The $\alpha 7$ nicotinic acetylcholine receptors (nAChR) is uniquely positioned as a therapeutic target in stroke due to its anti-inflammatory cytoprotective action resulting from its activation and ubiquitous expression in neuronal and glial/immune cells [9–13]. To date, selective positive allosteric modulators (referred to hereafter as PAMs) of $\alpha 7$ nAChRs have not been tested as cytoprotective stroke therapies in clinical trials. However, the results of pre-clinical studies hold significant promise [5,10,13–19]. Activation of $\alpha 7$ nAChRs is required for activation of certain endogenous central and peripheral anti-inflammatory cytoprotective mechanisms including the cholinergic anti-inflammatory pathway [20–22],

The therapeutic efficacy of these endogenous mechanisms after stroke can be significantly augmented by PAMs [9,10,13–19,23,24] creating a rational basis for pharmacological vagus nerve stimulation [23,25] and a strong case for further testing according to the mission of the US National Institutes of Health and the Stroke Treatment Academic Industry Roundtable (STAIR) guidelines [26–28], Ongoing pre-clinical trials in young adult and aged male/female rodents indicate that PAMs generate significant therapeutic benefits by reducing neurological deficits and brain injury after focal ischemia specifically, in a transient 90 min suture middle cerebral artery occlusion (MCAO) model of AIS [5,14–17], By inhibiting receptor desensitization, PAMs prolong activation of $\alpha 7$ nAChRs by endogenous agonists (i.e., ACh, released by the vagus nerve in response to inflammation; and choline, released by injured brain tissue near the time and site of injury as a result of breakdown of phosphatidylcholine) creating significant therapeutic benefits after ischemic [5,14,15] and traumatic brain injuries [9,13], Activation of both central and peripheral anti-inflammatory cytoprotective $\alpha 7$ -dependent pathways have been proposed [12,22,23,29] as contributing mechanisms responsible for therapeutic efficacy of PAMs and the involvement of JAK2/CaMKII-dependent intracellular pathway has been evident [5,12,23,30–39],

PAMs can be Type I or II [24,40], Both types potentiate $\alpha 7$ nAChRs, but only Type II PAMs (referred to here as PAMs) can reactivate pre-desensitized $\alpha 7$ nAChRs allowing persistent $\alpha 7$ activation to extend therapeutic efficacy. PAMs are inactive without $\alpha 7$ agonists [41] and thus, the challenge of therapeutic targeting is naturally resolved as a systemically applied PAM is expected to be relatively homogeneously distributed throughout the body by circulation, but its therapeutic effect would take place only in target sites with elevated ACh [24,25] and/or choline [42,43] after AIS: i.e., in the ischemic penumbra and central/peripheral inflammatory sites where and when it is most needed [10,23], to elicit a high spatiotemporal precision of PAMs, contrasting PAMs from $\alpha 7$ agonists that activate $\alpha 7$ nAChRs indiscriminately.

In our previous studies, we used PNU120596 (i.e., PNU), a prototypical PAM developed by Pfizer [44] with anti-inflammatory cytoprotective properties linked to pharmacological vagus nerve stimulation [13,23,25,29] to demonstrate its therapeutic utility after MCAO [5,14,15], In these studies, a single acute PNU treatment significantly reduced neurological deficits and brain injury measured 24 h after MCAO even if the time to treatment was delayed by 6 h after MCAO [14], However, only acute PNU therapy was tested in the past. The possibility of post-treatment relapses, the impact of treatment duration and the early-phase evolution of neurological deficits, ischemic injury and therapeutic benefits of acute vs. sub-chronic PNU treatments have not been previously evaluated.

In AIS, the time to treatment (i.e., *treatment delay*) should be kept to a minimum to minimize irreversible brain injury. The FAST-Mag study demonstrated that intravenous (i.v.) medications could be successfully delivered by ambulance personnel in ~73% of AIS within 1 h and ~99% within 2 h of onset [45], Thus, cytoprotectants may serve as both a therapeutic bridge to intra-arterial interventions and a monotherapy. However, all previous cytoprotective clinical trials for stroke have failed [46–48], Analysis of literature indicates that short (<72 h) treatment regimens were a common motif in most completed cytoprotective clinical trials for ischemic stroke [46–50], Short treatment delays (6–48 h

after the stroke onset) were utilized in both acute (24 h) and sub-chronic (72 h) regimens (Table 1). Here we reason that insufficient treatment durations may leave AIS patients undertreated because an early therapy may alleviate only the primary ischemic injury which occurs in the first 24 h after stroke. By contrast, the secondary injury linked to activation of the immune system and elevated inflammation develops slowly over several days after stroke and may not be effectively counteracted by acute cytoprotective therapies. In addition, severe adverse effects and toxicity of long-term exposures to NMDAR antagonists may have been possible reasons for skepticism in the past and why chronic cytoprotectants may have been generally avoided [51,52]. However, the therapeutic machineries of NMDARs and $\alpha 7$ nAChRs are not only different, but in many ways antipodal [40]: cytotoxicity is often linked to excessive activation of NMDARs, while cytoprotection is linked to augmented activation of $\alpha 7$ nAChRs. Importantly, activation of $\alpha 7$ nAChRs inhibits immune responses and serves as a potent central and peripheral anti-inflammatory mechanism [11,12,23,24,29],

This fully randomized blinded pilot study was driven by an idea that insufficient treatment durations may reduce therapeutic benefits of an otherwise efficacious cytoprotective therapy after AIS. To test for this possibility, we used a transient MCAO model of ischemic stroke and PNU to compare acute vs. sub-chronic treatment paradigms. We hypothesized that extending treatment duration extends therapeutic efficacy of PNU after MCAO. The results of this study supported this hypothesis. Furthermore, sub-chronic PNU treatments also effectively offset the negative effects of prolonged treatment delays. We conclude that a combination of short treatment delays and prolonged treatment durations may be required to maximize therapeutic effects of PNU, reduce relapses and ensure sustained therapeutic efficacy after AIS. Similar concepts may hold for other anti-inflammatory cytoprotectants including those that failed in clinical trials.

MATERIALS AND METHODS

Animals:

Young adult male Sprague Dawley (SD) rats (~280 g) were purchased from Envigo (Somerset, NJ) and used in accordance with the Guide for the Care and Use of Laboratory Animals (NIH 865–23, Bethesda, MD). All experimental protocols complied with the ARRIVE guidelines and were approved by the University of North Texas Health Science Center (UNTHSC) Institutional Animal Care and Use Committee. Animals were allowed to accommodate for at least 5 days after arrival to UNTHSC before any procedures were done. A total of 78 animals were used in experiments to test the effects of PNU in acute and subchronic treatment paradigms after tMCAO. A retrospective analysis (Figure 6) was done using existing data already collected from 40 animals in this and our previous studies. Additional animals were not used for Figure 6.

Anesthesia:

Isoflurane (4% induction; 1.8% maintenance) was purchased from Henry Schein Animal Health (Dublin, OH) and used to induce and maintain deep anesthesia during all surgical manipulations and drug/vehicle injections. Isoflurane was delivered by a mask as a gaseous mixture with 70% N₂O +30% O₂. Animals were maintained under spontaneous ventilation

and subjected to identical procedures. Blood electrolytes, glucose, gases, pH, cardiorespiratory and other physiological parameters were not monitored. The animal body temperature during anesthesia was measured by rectal thermometer and maintained at 37°C using a heating pad. Anesthesia duration was kept at 20 min for all animals to minimize neuroprotective effects of isoflurane as we reported [53], Animals requiring longer anesthesia durations were discarded.

Transient 90 min suture middle cerebral artery occlusion (MCAO):

Our approach was to induce reliable reproducible focal ischemia and ischemic injury by conducting MCAO surgeries within a narrow window of experimental parameters including animal weight (280±10 g) and source (only Envigo), accommodation window (5–7 days after arrival and prior to MCAO surgery), anesthesia duration (20 min), sedation duration for reperfusion (5 min), isoflurane dose (4% induction +1.8% maintenance), room temperature (24°C) [53,54], Experiments were not continued if experimental parameters fell outside of the specified range. The MCAO procedures and effective surgical parameters were established and confirmed by Laser Doppler measurements of regional cerebral blood flow (rCBF) as we reported [53], These parameters were kept constant in all experiments to ensure reliable reproducible focal MCAO and infarct volumes as confirmed by intermittent rCBF measurements. To initiate MCAO, a 4–0 monofilament nylon suture (Doccol Corporation, Sharon, MA) was used to advance from bifurcation of carotid artery toward MCA (i.e., 19-mm).

After induction of anesthesia, the depth of anesthesia was confirmed by a lack of the paw pinch reflex and a midline incision in the neck was made to expose and permanently ligate the left common carotid artery (CCA), the external carotid artery and the occipital artery. A 4–0 monofilament nylon suture (19-mm) was then inserted from the CCA into the left internal carotid artery to occlude the origin of the left MCA. Rats were allowed to recover from anesthesia in a warm cage for the rest of MCAO. After 90 min of MCAO, the animals were briefly sedated with isoflurane (4% induction + 1.8% maintenance for 5 min), the suture was removed for reperfusion and the wound was closed.

Measurements of infarct volume:

Immediately prior to euthanasia by decapitation 24–72 h after MCAO, animals were anesthetized for <1 min with the same anesthetic mixture used for surgical anesthesia and the brains were quickly removed and dissected into coronal sections (2-mm thickness) using a brain matrix. The brain sections were then immersed in 2% 2,3,5- triphenyltetrazolium chloride (TTC) in saline for 20 min at room temperature and images of ischemic infarcts were taken using a low resolution Olympus microscope. Infarct volumes were calculated as a percentage of the contralateral slice volume to account for ipsilateral edema as we previously published [5,53], The infarct and contralateral brain section areas were measured using Image J. Infarct volumes were calculated as products of infarct areas by the section thickness. To reduce the effect of post-ischemic edema, infarct area (IA) was measured as a % of the total brain section area calculated as the total contralateral brain section area (TCA) minus the non-infarcted ipsilateral brain section area (NI IA) divided by the TCA: $IA = 100\% \times (TCA - NI IA) / TCA$.

Neurobehavioral testing:

Neurobehavioral tests were done immediately prior to every s.c. injection and 15 min prior to euthanasia as shown in Figure 1.

Bederson test:

Neurological deficits were evaluated using a four-level scale: 0, normal; 1, forelimb flexion; 2, decreased resistance to lateral push; and 3, circling.

Cylinder test:

The use of forelimb was analyzed by observing the animal's movements over 3- min intervals in a transparent, 18-cm-wide, 30-cm-high poly-methyl-methacrylate cylinder. Forelimb movements of animals were observed from top. After an episode of rearing and wall exploration, forelimb placing on the cylinder wall was scored. The number of independent forelimb placements observed for the right forelimb, left forelimb and both forelimbs simultaneously were recorded. A total of 20 forelimb placements were recorded per animal. Strokes animals displayed an asymmetrical use of forelimbs during the cylinder wall exploration. The % use of impaired forelimb (i.e., right, which is contralateral) was calculated as: $100\% \times (RL + \frac{1}{2} BL) / TL$, where RL is the number of placements of the right/contralateral forelimb; BL is the number of simultaneous placements of the right (contralateral) and the left (ipsilateral) forelimbs; and TL is the total number of forelimb placement.

Drugs:

PNU120596 was purchased from Selleckchem (Houston, TX) and dissolved in dimethyl sulfoxide (DMSO; vehicle) to create a stock solution (10–80 mM) that was kept frozen at –30°C until the day of experiments. PNU 120596 was administered at doses found effective in our previous studies where only single acute treatments were used [5,9,14,15], The main goal of this study is to determine therapeutic utility of sub-chronic PNU 120596 treatments. The amount of DMSO injected in each animal did not exceed 0.5 ml/kg. I.V. injections were given via a tail vein while s.c. injections were given in the animal back or thighs.

Acute and sub-chronic combined vs. delayed treatments:

In the study, each animal was subjected to either combined (i.v.+s.c.1–4 injections) or delayed (only s.c.1–4 injections) PNU treatments (Figure 1): 1) i.v. (2.3 mg/kg) immediately prior to reperfusion; i.e. 90 min after MCAO onset; 2) s.c.1 (11.1 mg/kg) 5 h after i.v. injection; 3) s.c.2 (5.5 mg/kg) 24 h after MCAO onset; 4) s.c.3 (5.5 mg/kg) 48 h after MCAO onset. In this paradigm, the loading i.v. dose was increased ~2-fold from that used in our previous studies where behavioral and histological measurements were done 24 h after MCAO [14,15], The loading i.v. dose (2.3 mg/kg) was ~50% of the maintenance s.c.2–3 doses (i.e., 5.5 mg/kg) to arrest the ischemic insult. The maintenance doses were lower to reduce circulating exposures to PNU as the safety-exposure-efficacy relationship is not known. *Research rigor:* In this study, all experiments and analysis were conducted by an experimenter blinded to experimental conditions and treatments. Animals were randomly assigned to groups and treatments. Anesthesia duration was kept constant at 20 min across

animals to minimize cytoprotective effects of isoflurane as we reported [53], Animals that required longer anesthesia durations were discarded.

Statistical Analysis:

Differences between groups were tested for statistical significance using a non-parametric unpaired two-tailed Mann-Whitney U-test. The sample size $n=6$ was predicted from analysis of the statistical power (G*Power 1.3.9.2, Duesseldorf, Germany) using the effect size and data from our recent study of the effects of PNU after MCAO[5]: the sample size $n=6$ yields $1-\beta>0.8$ ($p=0.05$). In experiments where pilot data were available ($n=3$ per group), these data were included in the analysis increasing the sample size to $n=9$. A total of three animals did not survive the study: one control animal and one treated animal did not survive the combined therapy and one control animal did not survive the delayed therapy. One animal treated by the combined therapy refused to make contacts with the cylinder wall after day 1 and was excluded from behavioral analysis on days 2–3. This animal was still used in histological TTC analysis. One outlier was identified by the ROUT method ($Q=1\%$) in the data pool collected on day 1 after the combined therapy. However, the statistical significance was achieved both in the presence or absence of this outlier. The results of experiments were presented as mean+S.E.M. The threshold for statistical significance (the p-value) was set at 0.05.

RESULTS

In this fully randomized blinded pilot study, we use combined and delayed (Figure 1; see *Materials and Methods*) sub-chronic PNU treatments after MCAO in young adult male rats. The *combined therapy* was defined as a single i.v. injection followed by multiple sub-chronic s.c. injections as illustrated in Figure 1A. In the *delayed therapy*, i.v. injection was omitted, but subchronic s.c. injections remained as illustrated in Figure 1B. I.V. injections modeled first-aid therapy at home or in the ambulance, while multiple sub-chronic s.c. injections modeled a drip infusion in the hospital. Throughout the study, the term “after MCAO” was referred to the time after MCAO onset.

Evolution of ischemic brain injury in the first 12 h after MCAO.

To evaluate the evolution of ischemic infarcts in the first 72 h after focal ischemia, young adult male rats were subjected to a 90 min MCAO and euthanized for histological TTC staining at various time points after MCAO: i.e., 2.5, 12, 24 and 72 h. The results of these tests are illustrated in Figure 2. The infarct volume at 2.5 h after MCAO (i.e., 1 h after reperfusion) was minimal ($0.6\pm0.6\%$, $n=3$) despite the deficient Bederson's score (3 ± 0 , $n=3$). The infarct volume increased to $25.9\pm1.7\%$ ($n=3$) 12 h after MCAO (Bederson's score $=3\pm0$, $n=3$) and further increased to $53.2\pm0.6\%$ ($n=4$) 24 h after MCAO (Bederson's score $=3\pm0$, $n=4$) before saturating at $57.5\pm2.9\%$ ($n=8$) 72 h after MCAO (Bederson's score $=3\pm0$, $n=8$). These data are consistent with previous analyses where infarct volume in mice was found to mature between 24 h and 72 h after MCAO [55], Note that some animals used for histological analysis at 72 h after MCAO were also used as controls in various treatment experiments and thus, were injected with vehicle according to the time line of combined therapy illustrated in Figure 1A. Animals used for histological analysis at 2.5, 12 and 24 h

after MCAO were not injected with any solutions. All animals exhibited deficient performance in Bederson's tests (score = 3) regardless of the time window between MCAO and TTC staining suggesting that TTC assays are insufficiently sensitive for reliable detection of the early stages of neuronal injury and death (e.g., <12 h after MCAO) as compared to the Bederson's test. By contrast, the cylinder test could not be used for evaluation of neurological deficits in the first 24 h after MCAO due to the limited kinesis of stroked animals during the first 24 h of post-surgical recovery. These data indicate that brain injury matures around 24 h after MCAO and thus, a 24 h delay was accepted as the earliest time point for behavioral tests and analysis. As a result, the cylinder tests were conducted daily at 24, 48 and 72 h after MCAO.

Time course of cytoprotective effects of PNU in the first 12 h after MCAO.

In this fully randomized blinded experiment, young adult male SD rats were subjected to a 90 min MCAO and randomly assigned to two groups (n=9 each): PNU-treated (a single i.v. injection of 2.3 mg/kg PNU was delivered immediately after reperfusion; i.e., 90 min after MCAO onset) and vehicle-treated (i.e., control; the matching amount of vehicle was injected i.v. at the matching time point). The experimenter was blinded to treatment and used encoded vials with transparent solutions. The results of this experiment are shown in Figure 3. In this experiment, infarct volumes were not determined 24 h after MCAO because daily behavioral tests were conducted until 72 h after MCAO. PNU injected on day 0 significantly reduced neurological deficits 24 h after MCAO/treatment (i.e., day 1) in animals treated with PNU as compared to control animals treated with vehicle as evidenced by animal performance in the cylinder test ($p=0.0211$, $n=9$; Mann-Whitney, unpaired, two-tailed; Figure 3A) and expected from our previous published reports [5,14,15]. However, therapeutic benefits of a single acute i.v. PNU treatment declined 24 h later (i.e., 48 h after MCAO/treatment) and vanished completely 48 h later (i.e., 72 h after MCAO/treatment) (i.e., day 3). Thus, on day 3, both PNU-treated and control animals demonstrated an equally poor performance in the cylinder test ($p=0.8878$, $n=7-8$; Mann-Whitney, unpaired, two-tailed; Figure 3B). The performance of PNU-treated animals in the cylinder test declined as a function of time after treatment (Figure 3C), while control animals exhibited a relatively stable poor performance throughout the entire 72 h time window of experiment (Figure 1D). The analysis of infarct volumes on day 3 (Figure 3E-F) supported behavioral data and showed a lack of statistical significance between PNU-treated and control groups: $p=0.7768$, $n=8$; Mann-Whitney, unpaired, two-tailed; Figure 1E-F. One outlier was identified by the ROUT method ($Q=1\%$) in the data pool collected on day 1 (arrowhead; Figure 3A). Removing this outlier further strengthened statistical significance ($p=0.0091$, $n=8$; Mann-Whitney, unpaired, two-tailed; not shown) and our conclusions. One control animal did not survive past day 1. One treated animal did not survive past day 2. One treated animal refused to make contacts with the cylinder wall after day 1 and was excluded from behavioral analysis on days 2–3. This animal was still used in histological TTC analysis. All available data are presented in Figures 3A-B. However, because multiple control and treated animals generated identical data points, these data cannot be uniquely identified from Figure 3C-D. Taken together, these results demonstrate the intrinsic limitation of a single PNU treatment even if it is administered in the acute phase of stroke (e.g., <90 min after MCAO). These results suggest that multiple sub-chronic treatments may be necessary to generate sustained

therapeutic efficacy of PNU after AIS. This hypothesis was tested in the next set of experiments where multiple s.c. PNU injections were done daily for 72 h in the presence (i.e., *combined therapy*) or absence (i.e., *delayed therapy*) of acute i.v. PNU injection.

Therapeutic efficacy of the combined sub-chronic PNU therapy 72 h after MCAO.

In this fully randomized blinded experiment, we determined the therapeutic efficacy of PNU using the combined sub-chronic paradigm as illustrated in Figure 1A. Young adult male SD rats were subjected to a 90 min MCAO and randomly assigned to two groups (n=9 each): PNU-treated (a single i.v. 2.3 mg/kg PNU injection was given immediately after reperfusion, i.e., 90 min after the onset of MCAO; followed by daily s.c. PNU injections as illustrated in Figure 1A; s.c.1: 11 mg/kg; s.c.2–3: 5.5 mg/kg) and the matching amounts of vehicle injected similarly at the matching time points as controls. Behavioral tests were conducted 72 h after MCAO immediately prior to euthanasia. The results of this experiment are shown in Figure 4. The combined (i.v.+s.c.) subchronic PNU therapy significantly (Mann-Whitney, unpaired, two-tailed) reduced neurological deficits (cylinder test: $p=0.0003$, $n=8$; Figure 4A; and Bederson: $p=0.0014$, $n=8$; Figure 4B) and infarct volume ($p=0.0011$, $n=8$; Figure 4C-D) 72 h after MCAO. Thus, supplementing acute i.v. PNU injection with multiple daily s.c. PNU injections extended therapeutic efficacy of PNU from 24 h (Figure 3) to 72 h (Figure 4) indicating that a continuous exposure to PNU may be required for sustained therapeutic efficacy. This result is consistent with our previous data demonstrating that pre-conditioning with s.c. PNU 24 h prior to MCAO was not effective after MCAO [15] likely due to a relatively short half-life of PNU (~8 h; i.v. PNU) [56].

Therapeutic efficacy of the delayed sub-chronic PNU therapy 72 h after MCAO.

In this fully randomized blinded experiment, we determined the therapeutic efficacy of PNU using the delayed sub-chronic paradigm as illustrated in Figure 1B. Young adult male SD rats were subjected to a 90 min MCAO and randomly assigned to two groups (n=6 each): PNU-treated (daily s.c. PNU injections as illustrated in Figure 1B; s.c.1: 11 mg/kg; s.c.2–3: 5.5 mg/kg) and the matching amounts of vehicle injected similarly at the matching time points as controls. Accordingly, the first s.c. PNU injection (s.c.1; Figure 1B) was done 6.5 h after MCAO onset (i.e., 5 h after reperfusion) followed by s.c.2–3 injections 24 h and 48 h later, respectively. Behavioral tests were conducted 72 h after MCAO immediately prior to euthanasia. The results of this experiment are shown in Figure 5. The delayed (s.c. only) sub-chronic PNU therapy significantly (Mann-Whitney, unpaired, two-tailed) reduced neurological deficits (cylinder test: $p=0.0043$, $n=5/6$; Figure 5A; and Bederson: $p=0.0122$, $n=5/6$; Figure 5B) and infarct volume ($p=0.0043$, $n=5/6$; Figure 5C-D) 72 h after MCAO. One control animal did not survive past day 1. These results demonstrate that the acute i.v. PNU treatment is not required for therapeutic efficacy of PNU after MCAO as long as sub-chronic PNU regimens are implemented. Thus, the combined and delayed PNU regimens appear to deliver similar therapeutic efficacies and the sub-chronic treatment paradigm may be used in cases where the acute treatment window (<6 h) after MCAO has been unexploited.

Significant correlation between behavioral and histological data.

Our results suggest a significant correlation between behavioral (cylinder test) and histological (TTC staining) data measured 72 h after MCAO (Figures 4–5). To determine whether the cylinder test can be used to predict evolution of brain injury and PAM treatment, we conducted a retrospective analysis of experimental data obtained from 40 young adult male rats treated with PNU or vehicle for 72 h after MCAO. This analysis indeed determined significant correlation ($r=-0.8373$, $p<0.0001$, 95% CI: -0.9128 to -0.7066 , $n=40$) between animal performance in the cylinder test and infarct volume 72 h after MCAO (Figure 6). In this analysis, data from both combined (i.v.+s.c.; Figures 1A and 4) and delayed (s.c only; Figures 1B and 5) treatment paradigms were pooled together. Significant correlations were also detected if data from PNU and vehicle treatments were analyzed separately (PNU: $r=-0.8093$, $p=0.0006$, 95% CI: -0.9395 to -0.4750 , $n=14$; and vehicle: $r=-0.4982$, $p=0.0096$, 95% CI: 0.7478 to -0.1254 , $n=26$). These results present the cylinder test as a tool for predicting infarct volume 72 h after MCAO and may be used to evaluate the evolution of ischemic injury and PNU therapy in the absence of functional imaging.

Long-lasting therapeutic effects of short sub-chronic PNU treatments.

To test the hypothesis that short sub-chronic PNU treatments can produce long-lasting therapeutic effects and reduce relapses, young adult male rats were subjected to tMCAO, randomly assigned to two groups (treatment vs. control; $n=6$ each) and subjected to a combined sub-chronic treatment paradigm for 72 h (Figure 1A) utilizing either PNU (treatment group) or vehicle (control group). All experiments and analyses were conducted by a researcher blinded to group assignment. The effect of PNU treatment was evaluated by a cylinder test 3–4 times weekly for up to 30 days after MCAO. The results of this experiment are shown in Figure 7. Based on the animal performance, 4 distinct groups of effects were identified (Figure 7A–B): Group 1 (tMCAO+vehicle) was characterized by slow spontaneous recovery after tMCAO without treatment ($n=5$; asterisks, plus signs, grey rhombs, black dots, open squares; Figure 7A); Group 2 (tMCAO+vehicle) did not show any recovery after tMCAO without treatment ($n=1$; open circles; Figure 7A); Group 3 (tMCAO +PNU) was characterized by sustained performance in the cylinder test for up to 30 days after tMCAO with 4 sub-chronic PNU treatments over the first 72 h after tMCAO ($n=4$; grey circles, open triangles, open squares, plus signs; Figure 7B); and Group 4 (tMCAO+PNU) exhibited delayed relapses at around day 11 after MCAO despite 4 sub-chronic PNU treatments over the first 72 h after tMCAO ($n=3$; rhombs, asterisks, black dots; Figure 7B). Data points within groups 1 and 3–4 were averaged for statistical analysis to evaluate the effects of treatment and time factors after MCAO and interactions across treatment and time factors using a repeated measures two-way ANOVA (Figure 7C–D). Several animals were also subjected to a cylinder test immediately prior to MCAO. These data points were illustrated as black and bold symbols on the Y-axis corresponding to day 0; Figure 7). Since only a small subset of animals was subjected to a cylinder test before tMCAO, these data points were presented for illustration purpose only and were not averaged or analyzed for statistical significance.

The statistical analysis revealed significant main effects for the factors of PNU treatment [$F(2,9)=36.27$; $p<0.0001$] and time after MCAO [$F(13,117)=5.421$; $p<0.0001$] and indicated

significant interaction of PNU treatment vs. time after tMCAO [$F(26,117)=13.77$; $p<0.0001$]. The corresponding post-test multiple comparisons tests (Tukey) detected significant differences across PNU- and vehicle-treated data points spanning over 24 days of experiments despite the slow spontaneous recovery observed in five out of six control animals (Figure 7C). This spontaneous recovery was nearly complete by days 29–30 rendering differences between PNU- and vehicle-treated groups insignificant ($p>0.05$). Data between days 25 and 28 were not collected. These results indicate that a short 4-injection PNU treatment spanning over the first 72 h after tMCAO produces significant therapeutic effects that last for days after the end of treatment. Relapses in the treatment group suggest insufficient efficacy which may be remedied by increased treatment duration, administration frequency and/or PNU dose. Relapses may also indicate adverse effects of a rapid PNU withdrawal.

DISCUSSION

Legitimate concerns regarding the conceptual validity of cytoprotection as an approach to stroke therapy have been raised in the past reflecting numerous failures in cytoprotective clinical trials [3,46–48]. However, analysis of stroke literature indicated that short treatment durations (<72 h) were a common motif among most cytoprotective clinical trials (Table 1) [46–50]. Insufficient treatment duration may reduce therapeutic efficacy of an otherwise efficacious cytoprotective therapy leaving patients undertreated leading to relapses that may be misinterpreted for conceptual failures of cytoprotection. Extending treatment duration may extend therapeutic efficacy of treatments after AIS. While acute cytoprotective therapies may alleviate the primary brain injury after AIS, the secondary injury linked to activation of the immune system and elevated central/peripheral inflammation may not be effectively counteracted by only acute treatments as it develops slowly over several days after AIS. In that event, sub-chronic anti-inflammatory cytoprotective treatments may be required to ensure sustained long-term therapeutic efficacy.

In this study, we focus on endogenous $\alpha 7$ nAChR-dependent anti-inflammatory cytoprotective mechanisms [9–13,23,24,57,58] that can be augmented by PNU, a prototypical PAM developed by Pfizer [44]. In our previous studies, we have identified PNU as a potent therapeutic agent with efficacy after focal ischemia [5,14,15,29] and traumatic brain injury [9,13]. We argued that PAMs may generate therapeutic benefits after ischemic and traumatic brain injury via multiple cytoprotective anti-inflammatory mechanisms of action including pharmacological vagus nerve stimulation [10,23–25]. The rationale for the use of PAMs after stroke arises from: 1) ubiquitous expression of $\alpha 7$ nAChRs in neuronal [59–63] and glial/immune cells [18–21,64–69], where $\alpha 7$ activation supports neuronal resistance to ischemic injury [15,70–73] and inhibits central/peripheral immune responses [11,12]; and 2) anti-inflammatory $\alpha 7$ -dependent cholinergic tone [23] elevated by ischemia and inflammation which can be augmented by PAMs [9,13–15,19,24,54]. Thus, significant therapeutic benefits after stroke may be achieved by recruiting a single effector pathway (i.e., the $\alpha 7$ nAChR) that simultaneously activates multiple central/peripheral anti-inflammatory cytoprotective pathways [23].

This randomized blinded pilot study used a transient 90 min suture MCAO model of AIS in young adult male rats to determine the impact of treatment duration on therapeutic benefits of PNU [13,23,25,29]. PNU was administered either as a single acute i.v. injection immediately after reperfusion (i.e., 90 min after the onset of MCAO) and/or as daily s.c. injections for 3 days to determine whether extending PNU treatment duration indeed extends its therapeutic efficacy after MCAO. Our results demonstrate the intrinsic limitation of a single PNU treatment (Figure 1) even if it was administered during the early acute phase of stroke (i.e., immediately after reperfusion): therapeutic efficacy detected 24 h after acute PNU treatment completely vanished 72 h after MCAO suggesting that sub-chronic treatments may be necessary to generate sustained therapeutic efficacy of PNU after AIS. Indeed, this hypothesis was supported by experimental data: supplementing acute i.v. PNU treatment with daily s.c. PNU injections for 3 days prevented relapses at 72 h after MCAO (*combined therapy*; Figures 7A and 2). Thus, extending exposure to PNU may indeed extend its therapeutic efficacy and in fact, may be required for sustained longterm therapeutic benefits after AIS. Taken together these data suggest that to truly maximize therapeutic efficacy of PNU, a short treatment delay may need to be combined with a long treatment duration.

Interestingly, the efficacies of daily s.c. PNU injections in the absence (*delayed therapy*; Figures 7B and 3) and presence (*combined therapy*; Figures 7A and 2) of acute i.v. PNU treatment were very similar supporting our sub-hypothesis that sub-chronic PNU treatments may offset neurological deficits and brain injury resulted from a missed early treatment. These data also demonstrate that acute i.v. PNU treatment may not be required for sustained therapeutic efficacy of PNU as long as sub-chronic PNU regimens are implemented. Thus, the *combined* and *delayed* PNU regimens appear to deliver similar therapeutic efficacies and the sub-chronic treatment paradigm may be useful in cases where the acute treatment window (<6 h) after MCAO was left unexploited.

At least three factors may contribute to relapses after acute PNU treatments: 1) The secondary injury slowly progressing for days after MCAO [55,74,75]; 2) The relatively short (~10 h) half-life time of PNU [56]; and 3) The need for a continuous PNU exposure to achieve therapeutic effects [15]. There is a rational basis to expect that to achieve a sustained long-term therapeutic efficacy, treatment durations may need to be comparable to or greater than the duration of secondary injury after AIS. If the secondary injury which is linked to activation of the immune system and elevated central/peripheral inflammation extends well past the duration of acute treatment (e.g., i.v. PNU), these treatments may not be viewed as sufficient even if they are given at an early phase after AIS, as our data indicate (Figure 1). Similar concepts may hold for other anti-inflammatory cytoprotectants including those that failed in clinical trials [46–48].

In the absence of functional imaging such as MRI, it is challenging to monitor the evolution of brain injury and treatment. One way to resolve this challenge is through monitoring of proven behavioral parameters significantly correlated with the level of brain injury. Figure 4 justifies the use of a cylinder test as a measure of neurological function significantly correlated with infarct volume after tMCAO in young adult male rats. The fact that the same correlation function approximates both PNU- and vehicle-treated data indicates that the

cylinder test score most likely reflects injury, not treatment. Thus, the same linear correlation function obtained for higher PNU doses may be used to predict infarct volumes and/or scores of the cylinder tests for lower or even ineffective PNU doses and possibly other PAMs and neuroprotective agents.

Thus, we conclude that short PNU regimens may only slow down the evolution of ischemic brain injury without delivering sustained longer-term solutions leading to relapses that may be misinterpreted for conceptual failures of cytoprotection. By contrast, extending PNU exposure by daily sub-chronic s.c. PNU treatments extends therapeutic benefits as we have detected in our rat model of AIS. An illustration to this concept is shown in Figure 8, where data from this and our previously study [15] were used: evolution of brain injury in untreated rats ([this study]; *red line*), rats treated only once (i.v. PNU 0.5 h after MCAO with ischemic infarcts analyzed 24 h after MCAO [15] and i.v. PNU 1.5 h after MCAO with ischemic infarcts analyzed 72 h after MCAO [this study]; *blue line*) and rats subjected to a combined therapy (Figure 7A) for 72 h (i.v.+s.c. [this study]; *green line*) are illustrated for comparison. Daily PNU treatments prevented relapses (*green vs. red line*) observed 72 h after acute treatments (*blue vs. red line*). A 2-way ANOVA detected significant dependence of PNU effects on both treatment regimen (no treatment vs. acute i.v. PNU injection vs. sub-chronic i.v.+s.c. PNU injections: $F(2,40)=14.31$, $p<0.0001$) and treatment duration (24 h vs. 72 h: $F(2,40)=8.517$, $p<0.01$) with significant interactions between treatment regimens and durations: $F(2,40)=4.333$, $p<0.0198$. The Tukey's test with multiple comparisons gave significant differences in infarct volumes at both 24 h ($p<0.05$, *blue square vs. red circle*; and $p<0.05$, *green triangle vs. red circle*) and 72 h ($p<0.05$, *blue square vs. green rhomb*; and $p<0.01$, *red circle vs. green rhomb*) after MCAO. By contrast, significant relapses were detected 72 h after acute MCAO even though significant effects were observed 24 h after MCAO: $p<0.01$, *blue squares at 24 vs. 72 h*; and $p>0.05$, *blue square at 72 h vs. red circle at 72 h*. Sub-chronic i.v.+s.c. PNU treatments did not result in relapses ($p>0.05$, *green triangle vs. green rhomb*). These data present duration of PNU treatment as a key therapeutic factor required for sustained therapeutic efficacy.

Note, the discussed cholinergic PNU-based treatments are unrelated to citicoline-based therapy (i.e., cytidine-5'-dihosphocholine) that recently failed the International Citicoline Trial on Acute Stroke (ICTUS) [76], Citicoline repairs cellular membranes and reduces free radicals by stimulating synthesis of phosphatidylcholine [76]. By contrast, PAMs augment therapeutic efficacy of endogenous $\alpha 7$ agonists after MCAO: injury-elevated choline [10,14,15] and inflammation-elevated ACh [11,23],

The mammalian neuro-immune axis[11] and innervation of immune-related sub-diaphragmatic organs (e.g., spleen, liver, gastrointestinal tract) by the efferent vagus nerve make electrical and pharmacological stimulation of the vagus nerve a promising treatment of septic [22,77] and hemorrhagic [78,79] shocks, ischemic[80], hemorrhagic[81] and traumatic brain injuries [82]. VNS decreases production of pro-inflammatory cytokines and reduces inflammation.[11,77] While positive modulation of $\alpha 7$ nAChRs by PNU are expected to be anti-inflammatory, both anti- apoptotic and anti-inflammatory mechanisms are likely to contribute to the observed effects of PNU after MCAO because of the ubiquitous expression of functional $\alpha 7$ nAChRs in neuronal and immune tissues.

Accordingly, direct (central anti-apoptotic) and indirect (central and peripheral anti-inflammatory) neuroprotective effects of PAMs after ischemic and traumatic brain injury have been previously proposed[11,12,23,25,77],

One limitation of this study is the use of relatively short (<72 h) sub-chronic treatments. While four PNU treatments over the first 72 h completely eliminate early relapses 72 h after MCAO as compared to a single acute treatment (Figures 1–3), delayed relapses remained (see days 11– 12; Figure 5B) indicating a lack of long-term efficacy in just under 50% of animals and the need for exploring increased treatment duration, administration frequency and/or PNU dose. These challenges will be addressed in our future studies as we are primarily interested in developing short neuroprotective strategies with long-lasting effects. Interestingly, in our pilot experiments (Figure 5), four PNU treatments (i.e., a combined therapy) given over the first 72 h after tMCAO (as illustrated in Figure 7A) significantly improved neurological function evaluated by a cylinder test between day 1 and day 24 after tMCAO as compared to animals treated with vehicle (Figures 5C-D). However, due to slow spontaneous recovery in the control group (Figures 5A and 5C), differences between the cylinder score in treatment (open circles) vs. vehicle (open squares) groups became insignificant on day 30 (Figure 5C). These pilot results warrant a thorough dedicated study to determine the minimal efficacious PNU treatment duration, administration frequency and/or dose that ensure permanent elimination of relapses. Once these parameters are determined, the next key step would be testing the same strategies in larger species such as rabbits and exploring the impact of other biological variables such as sex and age. These factors have not been explored in this study because of budgetary limitations.

In this study, we focused on the cylinder test because it cannot be learned by animals as it does not involve task performance. Thus, animal performance in the cylinder test reflects accumulation or vanishing of ipsilateral vs. contralateral (to the injury site) sensorimotor functions. Thus, it is highly unlikely that the observed slow spontaneous recovery (Figures 5A and 5C) reflects animal adaptation or learning. In fact, spontaneous recovery after ischemic and traumatic brain injury is not uncommon in rat models [83–86] and presents a serious confounding factor which is more than capable of compromising the apparent efficacy of therapeutic compounds. The mechanisms of spontaneous recovery are unknown but may include synaptic plasticity within the sensorimotor cortex and/or injury-induced neuroprotection including activation of the cholinergic anti-inflammatory pathway [11].

The apparent lack of recovery after relapses in the treatment group (i.e., Group 4) suggests critical differences between untreated injury and relapses. In the absence of treatment, to be effective, the spontaneous recovery mechanisms may require an early start after tMCAO. By contrast, if injury is treated in the first few hours after ischemic insult and as a result, its development in the first few days is inhibited or even eliminated (Figure 5D), the accelerated development of injury due to delayed relapses days after stroke may not induce spontaneous recovery perhaps, because the recovery mechanisms require activation in the first few hours after the initial ischemic insult. To test these hypotheses, a long-term study utilizing measurements of multiple outcomes including functional imaging would be required to compare the time courses of injury and neurological functions in untreated vs. treated but relapsed animals after tMCAO. One possible explanation to relapses in PNU treatment

group (Group 4; Figure 5D) may be adverse reactions to a rapid PNU withdrawal. Nevertheless, the presented data support the central hypothesis of this study that extending duration of PNU treatment is therapeutically beneficial. However, to maximize therapeutic efficacy of sub-chronic PNU treatments and determine the minimal PNU treatment duration that ensures permanent elimination of relapses, a dedicated optimization study is required.

Another limitation of this study is that we did not determine the utility of PNU in combination with tissue plasminogen activator (tPA). Even if prolonged PNU treatments do not permanently eliminate relapses, PAMs may be effective as a therapeutic bridge to intra-arterial interventions (i.e., thrombolysis and thrombectomy): PAMs are expected to considerably reduce the time to treatment as treatments with PAMs may start immediately after the onset of AIS symptoms (e.g., at home or in the ambulance) because functional imaging is not required prior to PAM treatment. This possibility will be explored in our future studies.

Conclusion.

This study determined that acute PAM regimens even if given early after AIS may only slow down the evolution of brain injury after AIS and fail as longer-term therapeutic solutions leading to relapses that may be misinterpreted for conceptual failures of cytoprotection. Extending PAM exposure by daily sub-chronic treatments extends therapeutic benefits of PAM. We conclude that both treatment delay and duration are critical factors that define therapeutic efficacy of PAM. These concepts may hold for other anti-inflammatory cytoprotectants found effective after AIS.

Acknowledgements:

This study was supported in part by the William and Ella Owens Medical Research Foundation and the NIH grant DK082625 to V.U.

Abbreviations:

AIS	Acute ischemic stroke
MCAO	middle cerebral artery occlusion
PAM	positive allosteric modulator
TTC	2,3,5-triphenyltetrazolium chloride

References

1. Neuhaus AA, Couch Y, Hadley G, Buchan AM. Neuroprotection in stroke: The importance of collaboration and reproducibility. *Brain* 2017;140:2079–2092. [PubMed: 28641383]
2. Lapchak PA, Uteshev VV. Stroke cytoprotection: Can repeating history with new expectations really be the path to success in stroke research? *Translational stroke research* 2017;8:104–106. [PubMed: 28251583]
3. Tymianski M Can molecular and cellular neuroprotection be translated into therapies for patients?: Yes, but not the way we tried it before. *Stroke* 2010;41:S87–90. [PubMed: 20876514]

4. Lapchak PA, Zhang JH. The high cost of stroke and stroke cytoprotection research. *Translational stroke research* 2017;8:307–317. [PubMed: 28039575]
5. Sun F, Johnson SR, Jin K, Uteshev VV. Boosting endogenous resistance of brain to ischemia. *Mol Neurobiol* 2017;54:2045–2059. [PubMed: 26910820]
6. Hays SA, Rennaker RL, Kilgard MP. Targeting plasticity with vagus nerve stimulation to treat neurological disease. *Prog Brain Res* 2013;207:275–299. [PubMed: 24309259]
7. Lindvall O, Kokaia Z. Stem cells in human neurodegenerative disorders--time for clinical translation? *J Clin Invest* 2010;120:29–40. [PubMed: 20051634]
8. Hill JJ, Jin K, Mao XO, Xie L, Greenberg DA. Intracerebral chondroitinase abc and heparan sulfate proteoglycan glypican improve outcome from chronic stroke in rats. *Proceedings of the National Academy of Sciences of the United States of America* 2012;109:9155–9160. [PubMed: 22615373]
9. Gatson JW, Simpkins JW, Uteshev VV. High therapeutic potential of positive allosteric modulation of $\alpha 7$ nAChRs in a rat model of traumatic brain injury: Proof-of-concept. *Brain Res Bull* 2015;112C:35–41.
10. Uteshev VV. The therapeutic promise of positive allosteric modulation of nicotinic receptors. *Eur J Pharmacol* 2014;727:181–185. [PubMed: 24530419]
11. Pavlov VA, Tracey KJ. Neural circuitry and immunity. *Immunol Res* 2015
12. Egea J, Buendia I, Parada E, Navarro E, Leon R, Lopez MG. Anti-inflammatory role of microglial $\alpha 7$ nAChRs and its role in neuroprotection. *Biochem Pharmacol* 2015;97:463–472. [PubMed: 26232730]
13. Dash PK, Zhao J, Kobori N, Redell JB, Hylin MJ, Hood KN, Moore AN. Activation of $\alpha 7$ cholinergic nicotinic receptors reduce blood-brain barrier permeability following experimental traumatic brain injury. *J Neurosci* 2016;36:2809–2818. [PubMed: 26937017]
14. Sun F, Jin K, Uteshev VV. A type-II positive allosteric modulator of $\alpha 7$ nAChRs reduces brain injury and improves neurological function after focal cerebral ischemia in rats. *PloS one* 2013;8:e73581. [PubMed: 23951360]
15. Kalappa BI, Sun F, Johnson SR, Jin K, Uteshev VV. A positive allosteric modulator of $\alpha 7$ nAChRs augments neuroprotective effects of endogenous nicotinic agonists in cerebral ischaemia. *Br J Pharmacol* 2013;169:1862–1878. [PubMed: 23713819]
16. Han Z, Shen F, He Y, Degos V, Camus M, Maze M, Young WL, Su H. Activation of $\alpha 7$ nicotinic acetylcholine receptor reduces ischemic stroke injury through reduction of pro-inflammatory macrophages and oxidative stress. *PloS one* 2014;9:e105711. [PubMed: 25157794]
17. Han Z, Li L, Wang L, Degos V, Maze M, Su H. $\alpha 7$ nicotinic acetylcholine receptor agonist treatment reduces neuroinflammation, oxidative stress, and brain injury in mice with ischemic stroke and bone fracture. *J Neurochem* 2014;131:498–508. [PubMed: 25040630]
18. Parada E, Egea J, Buendia I, Negredo P, Cunha AC, Cardoso S, Soares MP, Lopez MG. The microglial $\alpha 7$ -acetylcholine nicotinic receptor is a key element in promoting neuroprotection by inducing heme oxygenase-1 via nuclear factor erythroid-2-related factor 2. *Antioxid Redox Signal* 2013
19. Munro G, Hansen R, Erichsen H, Timmermann D, Christensen J, Hansen H. The $\alpha 7$ nicotinic ACh receptor agonist compound b and positive allosteric modulator pnu-120596 both alleviate inflammatory hyperalgesia and cytokine release in the rat. *Br J Pharmacol* 2012;167:421–435. [PubMed: 22536953]
20. Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ. Nicotinic acetylcholine receptor $\alpha 7$ subunit is an essential regulator of inflammation. *Nature* 2003;421:384–388. [PubMed: 12508119]
21. Vida G, Pena G, Deitch EA, Ulloa L. $\alpha 7$ -cholinergic receptor mediates vagal induction of splenic norepinephrine. *J Immunol* 2011;186:4340–4346. [PubMed: 21339364]
22. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 2000;405:458–462. [PubMed: 10839541]
23. Uteshev VV. Cholinergic protection in ischemic brain injury In: Lapchak P, Zhang JH, ed. *Neuroprotective therapy for stroke and ischemic disease*. Switzerland: Springer International Publishing, 2017.

24. Uteshev VV. Allosteric modulation of nicotinic acetylcholine receptors: The concept and therapeutic trends. *Curr Pharm Des* 2016;22:1986–1997. [PubMed: 26831463]
25. Uteshev VV, Tenovuo O, Gaidhani N. The cholinergic potential, the vagus nerve and challenges in treatment of traumatic brain injury. *Curr Pharm Des* 2016;22:2083–2092. [PubMed: 26818869]
26. Albers GW, Goldstein LB, Hess DC, Wechsler LR, Furie KL, Gorelick PB, Hurn P, Liebeskind DS, Nogueira RG, Saver JL. Stroke treatment academic industry roundtable (stair) recommendations for maximizing the use of intravenous thrombolytics and expanding treatment options with intra-arterial and neuroprotective therapies. *Stroke* 2011;42:2645–2650. [PubMed: 21852620]
27. Lapchak PA, Zhang JH, Noble-Haeusslein LJ. Rigor guidelines: Escalating stair and steps for effective translational research. *Translational stroke research*;4:279–285. [PubMed: 23658596]
28. Fisher M, Albers GW, Donnan GA, Furlan AJ, Grotta JC, Kidwell CS, Sacco RL, Wechsler LR. Enhancing the development and approval of acute stroke therapies: Stroke therapy academic industry roundtable. *Stroke* 2005;36:1808–1813. [PubMed: 16020764]
29. Hoover DB. Cholinergic modulation of the immune system presents new approaches for treating inflammation. *Pharmacol Ther* 2017
30. Pavlov VA, Ochani M, Yang LH, Gallowitsch-Puerta M, Ochani K, Lin X, Levi J, Parrish WR, Rosas-Ballina M, Czura CJ, Larosa GJ, Miller EJ, Tracey KJ, Al-Abed Y. Selective $\alpha 7$ -nicotinic acetylcholine receptor agonist gts-21 improves survival in murine endotoxemia and severe sepsis. *Critical care medicine* 2007;35:1139–1144. [PubMed: 17334244]
31. Baez-Pagan CA, Delgado-Velez M, Lasalde-Dominicci JA. Activation of the macrophage $\alpha 7$ nicotinic acetylcholine receptor and control of inflammation. *J Neuroimmune Pharmacol* 2015;10:468–476. [PubMed: 25870122]
32. Wu H, Li L, Su X. Vagus nerve through $\alpha 7$ nachr modulates lung infection and inflammation: Models, cells, and signals. *BioMed research international* 2014;2014:283525. [PubMed: 25136575]
33. Shin SS, Dixon CE. Alterations in cholinergic pathways and therapeutic strategies targeting cholinergic system after traumatic brain injury. *Journal of neurotrauma* 2015;32:1429–1440. [PubMed: 25646580]
34. Bencherif M, Lippiello PM, Lucas R, Marrero MB. $\alpha 7$ nicotinic receptors as novel therapeutic targets for inflammation-based diseases. *Cell Mol Life Sci* 2011;68:931–949. [PubMed: 20953658]
35. Quik M, Zhang D, McGregor M, Bordia T. $\alpha 7$ nicotinic receptors as therapeutic targets for parkinson's disease. *Biochem Pharmacol* 2015;97:399–407. [PubMed: 26093062]
36. Shaw S, Bencherif M, Marrero MB. Janus kinase 2, an early target of $\alpha 7$ nicotinic acetylcholine receptor-mediated neuroprotection against $\text{A}\beta$ -(1–42) amyloid. *J Biol Chem* 2002;277:44920–44924. [PubMed: 12244045]
37. Akaike A, Takada-Takatori Y, Kume T, Izumi Y. Mechanisms of neuroprotective effects of nicotine and acetylcholinesterase inhibitors: Role of $\alpha 4$ and $\alpha 7$ receptors in neuroprotection. *J Mol Neurosci* 2010;40:211–216.
38. Thomsen MS, Hansen HH, Timmerman DB, Mikkelsen JD. Cognitive improvement by activation of $\alpha 7$ nicotinic acetylcholine receptors: From animal models to human pathophysiology. *Curr Pharm Des* 2010;16:323–343.
39. Bok J, Wang Q, Huang J, Green SH. Camkii and camkiv mediate distinct prosurvival signaling pathways in response to depolarization in neurons. *Mol Cell Neurosci* 2007;36:13–26. [PubMed: 17651987]
40. Uteshev VV. $\alpha 7$ nicotinic ach receptors as a ligand-gated source of Ca^{2+} ions: The search for a Ca^{2+} optimum. *Adv Exp Med Biol* 2012;740:603–638. [PubMed: 22453962]
41. Gusev AG, Uteshev VV. Physiological concentrations of choline activate native $\alpha 7$ -containing nicotinic acetylcholine receptors in the presence of pnu-120596 [1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea]. *J Pharmacol Exp Ther* 2010;332:588–598. [PubMed: 19923442]

42. Friedman SD, Brooks WM, Jung RE, Hart BL, Yeo RA. Proton mr spectroscopic findings correspond to neuropsychological function in traumatic brain injury. *AJNR American journal of neuroradiology* 1998;19:1879–1885. [PubMed: 9874540]
43. Kiewert C, Mdzinarishvili A, Hartmann J, Bickel U, Klein J. Metabolic and transmitter changes in core and penumbra after middle cerebral artery occlusion in mice. *Brain Res* 2010;1312:101–107. [PubMed: 19961839]
44. Hurst RS, Hajos M, Raggenbass M, Wall TM, Higdon NR, Lawson JA, Rutherford-Root KL, Berkenpas MB, Hoffmann WE, Piotrowski DW, Groppi VE, Allaman G, Ogier R, Bertrand S, Bertrand D, Arneric SP. A novel positive allosteric modulator of the alpha7 neuronal nicotinic acetylcholine receptor: In vitro and in vivo characterization. *JNeurosci* 2005;25:4396–4405. [PubMed: 15858066]
45. Saver JL, Starkman S, Eckstein M, Stratton S, Pratt F, Hamilton S, Conwit R, Liebeskind DS, Sung G, Sanossian N, Investigators F-M, Coordinators. Methodology of the field administration of stroke therapy - magnesium (fast-mag) phase 3 trial: Part 2 - prehospital study methods. *Int J Stroke* 2014;9:220–225. [PubMed: 24444117]
46. Xu SY, Pan SY. The failure of animal models of neuroprotection in acute ischemic stroke to translate to clinical efficacy. *Med Sci Monit Basic Res* 2013;19:37–45. [PubMed: 23353570]
47. Grupke S, Hall J, Dobbs M, Bix GJ, Fraser JF. Understanding history, and not repeating it. Neuroprotection for acute ischemic stroke: From review to preview. *Clin Neurol Neurosurg* 2015;129:1–9. [PubMed: 25497127]
48. Labiche LA, Grotta JC. Clinical trials for cytoprotection in stroke. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics* 2004;1:46–70. [PubMed: 15717007]
49. Saver JL, Starkman S, Eckstein M, Stratton SJ, Pratt FD, Hamilton S, Conwit R, Liebeskind DS, Sung G, Kramer I, Moreau G, Goldweber R, Sanossian N. Prehospital use of magnesium sulfate as neuroprotection in acute stroke. *The New England journal of medicine* 2015;372:528–536. [PubMed: 25651247]
50. Lees KR, Zivin JA, Ashwood T, Davalos A, Davis SM, Diener HC, Grotta J, Lyden P, Shuaib A, Hardemark HG, Wasiewski WW, Stroke-Acute Ischemic NXYTTI. Nxy-059 for acute ischemic stroke. *The New England journal of medicine* 2006;354:588–600. [PubMed: 16467546]
51. Davis SM, Lees KR, Albers GW, Diener HC, Markabi S, Karlsson G, Norris J. Selfotel in acute ischemic stroke : Possible neurotoxic effects of an nmda antagonist. *Stroke* 2000;31:347–354. [PubMed: 10657404]
52. Lees KR. Cerestat and other nmda antagonists in ischemic stroke. *Neurology* 1997;49:S66–69. [PubMed: 9371155]
53. Gaidhani N, Sun F, Schreihof D, Uteshev VV. Duration of isoflurane-based surgical anesthesia determines severity of brain injury and neurological deficits after a transient focal ischemia in young adult rats. *Brain Res Bull* 2017;134:168–176. [PubMed: 28755978]
54. Sun F, Johnson SR, Jin K, Uteshev VV. Boosting endogenous resistance of brain to ischemia. *Mol Neurobiol* 2016
55. Harada S, Fujita WH, Shichi K, Tokuyama S. The development of glucose intolerance after focal cerebral ischemia participates in subsequent neuronal damage. *Brain Res* 2009;1279:174–181. [PubMed: 19445903]
56. McLean SL, Idris NF, Grayson B, Gendle DF, Mackie C, Lesage AS, Pemberton DJ, Neill JC. Pnu- 120596, a positive allosteric modulator of alpha7 nicotinic acetylcholine receptors, reverses a sub-chronic phencyclidine-induced cognitive deficit in the attentional set-shifting task in female rats. *J Psychopharmacol* 2012;26:1265–1270. [PubMed: 22182741]
57. Borovikova LV, Ivanova S, Nardi D, Zhang M, Yang H, Ombrellino M, Tracey KJ. Role of vagus nerve signaling in cni-1493-mediated suppression of acute inflammation. *Autonomic neuroscience : basic & clinical* 2000;85:141–147. [PubMed: 11189021]
58. Pavlov VA, Parrish WR, Rosas-Ballina M, Ochani M, Puerta M, Ochani K, Chavan S, Al-Abed Y, Tracey KJ. Brain acetylcholinesterase activity controls systemic cytokine levels through the cholinergic anti-inflammatory pathway. *Brain Behav Immun* 2009;23:41–45. [PubMed: 18639629]
59. Dani JA, Bertrand D. Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *AnnuRevPharmacolToxicol* 2007;47:699–729.

60. McGehee DS, Role LW. Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. *Annu Rev Physiol* 1995;57:521–546. [PubMed: 7778876]
61. Breese CR, Adams C, Logel J, Drebing C, Rollins Y, Barnhart M, Sullivan B, Demasters BK, Freedman R, Leonard S. Comparison of the regional expression of nicotinic acetylcholine receptor alpha7 mrna and [125i]-alpha-bungarotoxin binding in human postmortem brain. *J Comp Neurol* 1997;387:385–398. [PubMed: 9335422]
62. Whiteaker P, Davies AR, Marks MJ, Blagbrough IS, Potter BV, Wolstenholme AJ, Collins AC, Wonnacott S. An autoradiographic study of the distribution of binding sites for the novel alpha7-selective nicotinic radioligand [3h]-methyllycaconitine in the mouse brain. *Eur J Neurosci* 1999;11:2689–2696. [PubMed: 10457165]
63. Clarke PB. Nicotinic receptors in mammalian brain: Localization and relation to cholinergic innervation. *Prog Brain Res* 1993;98:77–83. [PubMed: 8248540]
64. Hao J, Simard AR, Turner GH, Wu J, Whiteaker P, Lukas RJ, Shi FD. Attenuation of cns inflammatory responses by nicotine involves alpha7 and non-alpha7 nicotinic receptors. *Exp Neurol* 2010;227:110–119. [PubMed: 20932827]
65. Koval L, Lykhmus O, Zhmak M, Khrushov A, Tsetlin V, Magrini E, Viola A, Chernyavsky A, Qian J, Grando S, Komisarenko S, Skok M. Differential involvement of alpha4beta2, alpha7 and alpha9alpha10 nicotinic acetylcholine receptors in b lymphocyte activation in vitro. *The international journal of biochemistry & cell biology* 2011;43:516–524. [PubMed: 21146628]
66. Kawashima K, Fujii T. Expression of non-neuronal acetylcholine in lymphocytes and its contribution to the regulation of immune function. *Frontiers in bioscience : a journal and virtual library* 2004;9:2063–2085. [PubMed: 15353271]
67. De Rosa MJ, Dionisio L, Agriello E, Bouzat C, Esandi Mdel C. Alpha 7 nicotinic acetylcholine receptor modulates lymphocyte activation. *Life Sci* 2009;85:444–449. [PubMed: 19632243]
68. Shytle RD, Mori T, Townsend K, Vendrame M, Sun N, Zeng J, Ehrhart J, Silver AA, Sanberg PR, Tan J. Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors. *J Neurochem* 2004;89:337–343. [PubMed: 15056277]
69. Sharma G, Vijayaraghavan S. Nicotinic cholinergic signaling in hippocampal astrocytes involves calcium-induced calcium release from intracellular stores. *Proceedings of the National Academy of Sciences of the United States of America* 2001;98:4148–4153. [PubMed: 11259680]
70. Meyer EM, Tay ET, Zoltewicz JA, Papke RL, Meyers C, King M, Fiebre CMd Neuroprotective and memory-related actions of novel a7 nicotinic agents with different mixed agonist/antagonist properties. *JPharmacolExpTher* 1998;284:1026–1032.
71. Shimohama S, Greenwald DL, Shafron DH, Akaike A, Maeda T, Kaneko S, Kimura J, Simpkins CE, Day AL, Meyer EM. Nicotinic a7 receptors protect against glutamate neurotoxicity and neuronal ischemic damage. *Brain Res* 1998;779:359–363. [PubMed: 9473725]
72. Li Y, Papke RL, He YJ, Millard WJ, Meyer EM. Characterization of the neuroprotective and toxic effects of alpha7 nicotinic receptor activation in pc12 cells. *Brain Res* 1999;830:218–225. [PubMed: 10366678]
73. Rosa AO, Egea J, Gandia L, Lopez MG, Garcia AG. Neuroprotection by nicotine in hippocampal slices subjected to oxygen-glucose deprivation: Involvement of the alpha7 nachr subtype. *JMolNeurosci* 2006;30:61–62.
74. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: An integrated view. *Trends Neurosci* 1999;22:391–397. [PubMed: 10441299]
75. Janowski M, Wagner DC, Boltze J. Stem cell-based tissue replacement after stroke: Factual necessity or notorious fiction? *Stroke* 2015;46:2354–2363. [PubMed: 26106118]
76. Davalos A, Alvarez-Sabin J, Castillo J, Diez-Tejedor E, Ferro J, Martinez-Vila E, Serena J, Segura T, Cruz VT, Masjuan J, Cobo E, Secades JJ, International Citicoline Trial on acUte Stroke trial i. Citicoline in the treatment of acute ischaemic stroke: An international, randomised, multicentre, placebo-controlled study (ictus trial). *Lancet* 2012;380:349–357. [PubMed: 22691567]
77. Bernik TR, Friedman SG, Ochani M, DiRaimo R, Ulloa L, Yang H, Sudan S, Czura CJ, Ivanova SM, Tracey KJ. Pharmacological stimulation of the cholinergic antiinflammatory pathway. *J Exp Med* 2002;195:781–788. [PubMed: 11901203]

78. Guarini S, Altavilla D, Cainazzo MM, Giuliani D, Bigiani A, Marini H, Squadrito G, Minutoli L, Bertolini A, Marini R, Adamo EB, Venuti FS, Squadrito F. Efferent vagal fibre stimulation blunts nuclear factor-kappaB activation and protects against hypovolemic hemorrhagic shock. *Circulation* 2003;107:1189–1194. [PubMed: 12615800]
79. Guarini S, Cainazzo MM, Giuliani D, Mioni C, Altavilla D, Marini H, Bigiani A, Ghiaroni V, Passaniti M, Leone S, Bazzani C, Caputi AP, Squadrito F, Bertolini A. Adrenocorticotropin reverses hemorrhagic shock in anesthetized rats through the rapid activation of a vagal anti-inflammatory pathway. *Cardiovascular research* 2004;63:357–365. [PubMed: 15249194]
80. Khodaparast N, Hays SA, Sloan AM, Fayyaz T, Hulsey DR, Rennaker RL, 2nd, Kilgard MP. Vagus nerve stimulation delivered during motor rehabilitation improves recovery in a rat model of stroke. *Neurorehabil Neural Repair* 2014;28:698–706. [PubMed: 24553102]
81. Hays SA, Khodaparast N, Hulsey DR, Ruiz A, Sloan AM, Rennaker RL, 2nd, Kilgard MP. Vagus nerve stimulation during rehabilitative training improves functional recovery after intracerebral hemorrhage. *Stroke* 2014;45:3097–3100. [PubMed: 25147331]
82. Pruitt DT, Schmid AN, Kim LJ, Abe CM, Trieu JL, Choua C, Hays SA, Kilgard MP, Rennaker RL. Vagus nerve stimulation delivered with motor training enhances recovery of function after traumatic brain injury. *Journal of neurotrauma* 2016;33:871–879. [PubMed: 26058501]
83. Grin'kina NM, Abdel-Baki SG, Bergold PJ. Reversible behavioral deficits in rats during a cycle of demyelination-remyelination of the fimbria. *PloS one* 2013;8:e53775. [PubMed: 23349742]
84. Liu FJ, Lim KY, Kaur P, Sepramaniam S, Armugam A, Wong PT, Jeyaseelan K. Micromas involved in regulating spontaneous recovery in embolic stroke model. *PloS one* 2013;8:e66393. [PubMed: 23823624]
85. Sommer CJ. Ischemic stroke: Experimental models and reality. *Acta neuropathologica* 2017;133:245–261. [PubMed: 28064357]
86. Wahl AS, Schwab ME. Finding an optimal rehabilitation paradigm after stroke: Enhancing fiber growth and training of the brain at the right moment. *Frontiers in human neuroscience* 2014;8:381. [PubMed: 25018717]
87. Davis SM, Albers GW, Diener HC, Lees KR, Norris J. Termination of acute stroke studies involving selfotel treatment. *Assist steering committed. Lancet* 1997;349:32. [PubMed: 8999265]
88. Albers GW, Atkinson RP, Kelley RE, Rosenbaum DM. Safety, tolerability, and pharmacokinetics of the n-methyl-d-aspartate antagonist dextrorphan in patients with acute stroke. *Dextrorphan study group. Stroke* 1995;26:254–258. [PubMed: 7831698]
89. Albers GW, Goldstein LB, Hall D, Lesko LM, Aptiganel Acute Stroke I. Aptiganel hydrochloride in acute ischemic stroke: A randomized controlled trial. *JAMA : the journal of the American Medical Association* 2001;286:2673–2682. [PubMed: 11730442]
90. Wahlgren NG, Ranasinha KW, Rosolacci T, Franke CL, van Erven PM, Ashwood T, Claesson L. Clomethiazole acute stroke study (class): Results of a randomized, controlled trial of clomethiazole versus placebo in 1360 acute stroke patients. *Stroke* 1999;30:21–28. [PubMed: 9880383]
91. Krams M, Lees KR, Hacke W, Grieve AP, Orgogozo JM, Ford GA, Investigators AS. Acute stroke therapy by inhibition of neutrophils (astin): An adaptive dose-response study of uk-279,276 in acute ischemic stroke. *Stroke* 2003;34:2543–2548. [PubMed: 14563972]
92. Clark WM, Raps EC, Tong DC, Kelly RE. Cervene (nalmefene) in acute ischemic stroke : Final results of a phase iii efficacy study. The cervene stroke study investigators. *Stroke* 2000;31:1234–1239. [PubMed: 10835438]
93. Saver JL, Starkman S, Eckstein M, Stratton SJ, Pratt FD, Hamilton S, Conwit R, Liebeskind DS, Sung G, Kramer I, Moreau G, Goldweber R, Sanossian N, Investigators F-M, Coordinators. Prehospital use of magnesium sulfate as neuroprotection in acute stroke. *N Engl J Med* 2015;372:528–536. [PubMed: 25651247]
94. Lyden P, Weymer S, Coffey C, Cudkowicz M, Berg S, O'Brien S, Fisher M, Haley EC, Khatri P, Saver J, Levine S, Levy H, Rymer M, Wechsler L, Jadhav A, McNeil E, Waddy S, Pryor K. Selecting patients for intra-arterial therapy in the context of a clinical trial for neuroprotection. *Stroke* 2016;47:2979–2985. [PubMed: 27803392]

95. Diener HC, AlKhedr A, Busse O, Hacke W, Zingmark PH, Jonsson N, Basun H, Study g. Treatment of acute ischaemic stroke with the low-affinity, use-dependent nmda antagonist ar-15896ar. A safety and tolerability study. *Journal of neurology* 2002;249:561–568. [PubMed: 12021946]
96. Shuaib A, Lees KR, Lyden P, Grotta J, Davalos A, Davis SM, Diener HC, Ashwood T, Wasiewski WW, Emeribe U, Investigators SIT. Nxy-059 for the treatment of acute ischemic stroke. *The New England journal of medicine* 2007;357:562–571. [PubMed: 17687131]
97. Sacco RL, DeRosa JT, Haley EC, Jr., Levin B, Ordronneau P, Phillips SJ, Rundek T, Snipes RG, Thompson JL, Glycine Antagonist in Neuroprotection Americas I. Glycine antagonist in neuroprotection for patients with acute stroke: Gain americas: A randomized controlled trial. *JAMA : the journal of the American Medical Association* 2001;285:1719–1728.
98. Lees KR, Lavelle JF, Cunha L, Diener HC, Sanders EA, Tack P, Wester P, Group GPIES. Glycine antagonist (gv150526) in acute stroke: A multicentre, double-blind placebo-controlled phase ii trial. *Cerebrovasc Dis* 2001;11:20–29.
99. Elting JW, Sulter GA, Kaste M, Lees KR, Diener HC, Hommel M, Versavel M, Teelken AW, De Keyser J. Ampa antagonist zk200775 in patients with acute ischemic stroke: Possible glial cell toxicity detected by monitoring of s-100b serum levels. *Stroke* 2002;33:2813–2818. [PubMed: 12468775]
100. RANTTAS ST. A randomized trial of tirilazad mesylate in patients with acute stroke (ranttas). The ranttas investigators. *Stroke* 1996;27:1453–1458. [PubMed: 8784112]
101. Emsley HC, Smith CJ, Georgiou RF, Vail A, Hopkins SJ, Rothwell NJ, Tyrrell PJ, Acute Stroke I. A randomised phase ii study of interleukin-1 receptor antagonist in acute stroke patients. *J Neurol Neurosurg Psychiatry* 2005;76:1366–1372. [PubMed: 16170078]
102. Muir KW, Holzapfel L, Lees KR. Phase ii clinical trial of sipatrigine (619c89) by continuous infusion in acute stroke. *Cerebrovasc Dis* 2000;10:431–436. [PubMed: 11070372]
103. Teal P, Davis S, Hacke W, Kaste M, Lyden PD, Modified Randomized Exposure Controlled Trial Study I, Fierus M, Bayer HealthCare AG. A randomized, double-blind, placebo-controlled trial to evaluate the efficacy, safety, tolerability, and pharmacokinetic/pharmacodynamic effects of a targeted exposure of intravenous repinotan in patients with acute ischemic stroke: Modified randomized exposure controlled trial (mrect). *Stroke* 2009;40:3518–3525. [PubMed: 19745176]
104. Lodder J, van Raak L, Hilton A, Hardy E, Kessels A, Group ES. Diazepam to improve acute stroke outcome: Results of the early gaba-ergic activation study in stroke trial. A randomized double-blind placebo-controlled trial. *Cerebrovasc Dis* 2006;21:120–127. [PubMed: 16340187]
105. Saxena R, Wijnhoud AD, Carton H, Hacke W, Kaste M, Przybelski RJ, Stern KN, Koudstaal PJ. Controlled safety study of a hemoglobin-based oxygen carrier, dclhb, in acute ischemic stroke. *Stroke* 1999;30:993–996. [PubMed: 10229733]
106. Horn J, de Haan RJ, Vermeulen M, Limburg M. Very early nimodipine use in stroke (venus): A randomized, double-blind, placebo-controlled trial. *Stroke* 2001;32:461–465. [PubMed: 11157183]
107. Enlimomab Acute Stroke Trial I. Use of anti-icam-1 therapy in ischemic stroke: Results of the enlimomab acute stroke trial. *Neurology* 2001;57:1428–1434. [PubMed: 11673584]
108. Schneider D, Berrouschot J, Brandt T, Hacke W, Ferbert A, Norris SH, Polmar SH, Schafer E. Safety, pharmacokinetics and biological activity of enlimomab (anti-icam-1 antibody): An open-label, dose escalation study in patients hospitalized for acute stroke. *European neurology* 1998;40:78–83. [PubMed: 9693236]
109. Diener HC, Cortens M, Ford G, Grotta J, Hacke W, Kaste M, Koudstaal PJ, Wessel T. Lubeluzole in acute ischemic stroke treatment: A double-blind study with an 8-hour inclusion window comparing a 10mg daily dose of lubeluzole with placebo. *Stroke* 2000;31:2543–2551. [PubMed: 11062273]
110. Ogawa A, Yoshimoto T, Kikuchi H, Sano K, Saito I, Yamaguchi T, Yasuhara H. Ebselen in acute middle cerebral artery occlusion: A placebo-controlled, double-blind clinical trial. *Cerebrovasc Dis* 1999;9:112–118. [PubMed: 9973655]

111. Lapchak PA. A critical assessment of edaravone acute ischemic stroke efficacy trials: Is edaravone an effective neuroprotective therapy? Expert opinion on pharmacotherapy 2010;11:1753–1763. [PubMed: 20491547]
112. Gelmers HJ, Gorter K, de Weerd CJ, Wiezer HJ. A controlled trial of nimodipine in acute ischemic stroke. The New England journal of medicine 1988;318:203–207. [PubMed: 3275894]
113. Nimodipine ASGf. Clinical trial of nimodipine in acute ischemic stroke. The american nimodipine study group. Stroke 1992;23:3–8. [PubMed: 1731418]
114. Muresanu DF, Heiss WD, Hoemberg V, Bajenaru O, Popescu CD, Vester JC, Rahlfs VW, Doppler E, Meier D, Moessler H, Guekht A. Cerebrolysin and recovery after stroke (cars): A randomized, placebo- controlled, double-blind, multicenter trial. Stroke 2016;47:151–159. [PubMed: 26564102]
115. Gharagozli K, Harandi AA, Houshmand S, Akbari N, Muresanu DF, Vester J, Winter S, Moessler H. Efficacy and safety of cerebrolysin treatment in early recovery after acute ischemic stroke: A randomized, placebo-controlled, double-blinded, multicenter clinical trial. Journal of medicine and life 2017; 10:153–160. [PubMed: 29075343]
116. Clark WM, Wechsler LR, Sabounjian LA, Schwiderski UE, Citicoline Stroke Study G. A phase iii randomized efficacy trial of 2000 mg citicoline in acute ischemic stroke patients. Neurology 2001;57:1595–1602. [PubMed: 11706098]
117. De Deyn PP, Reuck JD, Deberdt W, Vlietinck R, Orgogozo JM. Treatment of acute ischemic stroke with piracetam. Members of the piracetam in acute stroke study (pass) group. Stroke 1997;28:2347–2352. [PubMed: 9412612]
118. Fassoulaki A, Kostopanagiotou G, Kaniaris P, Varonos DD. Piracetam attenuates the changes in the surface potential of the phosphatidylcholine monolayer produced by alcohols. Acta anaesthesiologica Belgica 1985;36:47–51. [PubMed: 4013633]
119. Winblad B Piracetam: A review of pharmacological properties and clinical uses. CNS drug reviews 2005;11:169–182. [PubMed: 16007238]
120. Peuvot J, Schanck A, Deleers M, Brasseur R. Piracetam-induced changes to membrane physical properties. A combined approach by ³¹p nuclear magnetic resonance and conformational analysis. Biochem Pharmacol 1995;50:1129–1134. [PubMed: 7488225]
121. Muller WE, Koch S, Scheuer K, Rostock A, Bartsch R. Effects of piracetam on membrane fluidity in the aged mouse, rat, and human brain. Biochem Pharmacol 1997;53:135–140. [PubMed: 9037245]

Figure 1. Time lines of PNU regimens used in this study.

A) The *combined* therapy is defined as single i.v. + multiple s.c. injections for 72 h after MCAO. **B)** In the *delayed* therapy, i.v. injection is omitted. Single i.v. injections model a first-aid therapy given at home or in ambulance. Subchronic s.c. injections model a drip infusion in the hospital. Note, the term “after MCAO” used in the text is referred to time after MCAO onset: i.e., immediately after reperfusion. Behavioral tests were conducted prior to each s.c. injection and 15 min prior to euthanasia.

Evolution of ischemic injury without treatment

Figure 2. Evolution of ischemic injury without treatment.

Young adult male rats were used to build the time course of ischemic brain injury after MCAO in the absence of treatment: red circles. Brains were collected for TTC analysis at different time points after the onset of MCAO (in h): 2.5, 12, 24 and 72. Representative brain sections corresponding to specific time points after MCAO are illustrated in frame inserts. The following data points are illustrated (time, mean infarct volume $\pm$ SEM; #of animals): (2.5, 0.6 $\pm$ 0.6; n=3), (12, 25.9 $\pm$ 1.7; n=3), (24, 53.2 $\pm$ 0.6; n=4), (72, 57.5 $\pm$ 2.9; n=8). Some animals used for histological analysis at 72 h after MCAO were also used as controls in various treatment experiments and thus, were injected with vehicle according to the time line of combined therapy illustrated in Figure 7 A. Animals used for histological analysis at 2.5, 12 and 24 h after MCAO were not injected with any solutions.

Figure 3. Time course of cytoprotective effects of a single acute i.v. PNU treatment.

In this fully randomized blinded experiment, a single i.v. injection of 2.3 mg/kg PNU or vehicle was delivered immediately prior to reperfusion (i.e., 90 min after MCAO onset; day 0) to determine the time course of therapeutic effects of PNU. As expected from our previous reports [5,14,15], PNU significantly reduced neurological deficits vs. vehicle as evidenced by the cylinder test conducted 24 h after treatment (day 1; **A**). These protective effects declined in the following 48 h and eventually, vanished completely 72 h after treatment (day 3; **B**). The cylinder test was given to each animal daily and the time courses

of PNU-induced benefits (**C**) vs. control baseline (**D**) were determined. The analysis of infarct volumes on day 3 (**E-F**) supported behavioral data showing a lack of statistical significance (Mann-Whitney, two-tailed) between treated and control groups on day 3 ($p=0.8878$, $n=7-8$; **B**; and $p=0.7768$, $n=8$; **E-F**), but not day 1 ($p=0.0211$, $n=9$; **A**). One control and one test animals did not survive. One treated animal refused to make contacts with the cylinder wall after day 1 and was euthanized after day 3 contributing to infarct volume data, but not behavioral data. These factors are reflected in group sample sizes. One outlier was identified by the ROUT method ($Q=1\%$) in the data pool collected on day 1 (arrowhead; **A** and **D**). Removing this outlier further strengthened statistical significance ($p=0.0091$, $n=8$; not shown) and our conclusions. Arrows indicate data points corresponding to data illustrated in **E**.

Figure 4. Therapeutic efficacy of the combined sub-chronic PNU treatment.

In this fully randomized blinded experiment, a single i.v. injection of 2.3 mg/kg PNU or vehicle was delivered immediately prior to reperfusion (i.e., 90 min after MCAO onset; day 0) followed by multiple daily s.c. PNU injections (s.c.1: 11.1 mg/kg; s.c.2–3: 5.5 mg/kg) to determine the time course of therapeutic effects elicited by the combined sub-chronic PNU regimen (Figure 7A). The animal performance in Bederson and cylinder tests was tested 72 h after MCAO immediately prior to euthanasia and brains were collected for infarct volume analysis. The combined (i.v.+s.c.) subchronic PNU treatment significantly reduced neurological deficits (cylinder test: $p=0.0003$, $n=8$; **A**; and Bederson: $p=0.0014$, $n=8$; **B**) and infarct volume ($p=0.0011$, $n=8$; **C-D**) 72 h after MCAO (Mann-Whitney, two-tailed). Arrows indicate data points corresponding to data illustrated in **E**.

Figure 5. Therapeutic efficacy of the delayed sub-chronic PNU treatment.

In this fully randomized blinded experiment, the initial i.v. injection immediately prior to reperfusion (i.e., 90 min after MCAO onset; day 0) was omitted (Figure 7B). The remaining multiple daily s.c. PNU injections (s.c.1: 11.1 mg/kg; s.c.2–3: 5.5 mg/kg) were identical to those used in the combined PNU injection regimen (Figure 7A) to determine the time course of therapeutic effects elicited by the delayed sub-chronic PNU regimen (Figure 7B). Accordingly, the first s.c. PNU injection (s.c.1; Figure 7B) was done 6.5 h after MCAO onset (i.e., 5 h after reperfusion) followed by s.c.2–3 injections 24 h and 48 h later, respectively. The animal performance in Bederson and cylinder tests was tested 72 h after MCAO immediately prior to euthanasia and brains were collected for infarct volume analysis. The delayed (s.c. only) sub-chronic PNU treatment significantly reduced neurological deficits (cylinder test: $p=0.0043$, $n=5-6$; A; and Bederson: $p=0.0122$, $n=5-6$;

B) and infarct volume ($p=0.0043$, $n=5-6$; **C-D**) 72 h after MCAO (Mann-Whitney, two-tailed). Arrows indicate data points corresponding to data illustrated in **E**.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. Correlation between behavioral and infarct parameters.

A retrospective analysis of data from 40 young adult male rats treated with PNU or vehicle daily for 72 h after MCAO revealed significant correlation ($r = -0.8373$, $p < 0.0001$) between infarct volume and performance in the cylinder test 72 h post-MCAO. Significant correlation was also detected when PNU and vehicle data were analyzed separately (PNU: $r = -0.8093$, $p = 0.0006$, $n = 14$; and vehicle: $r = -0.4982$, $p = 0.0096$, $n = 26$). Thus, our primary and secondary parameters reliably and consistently describe injury after MCAO.

Figure 7. Long-term effects from short sub-chronic PNU treatments.

Short sub-chronic PNU treatments may produce long-lasting therapeutic effects and reduce relapses. Young adult male rats were subjected to tMCAO, randomly assigned to two groups (treatment vs. control; n=6 each) and subjected to a combined sub-chronic treatment paradigm for 72 h (Figure 7A) utilizing either PNU (treatment group) or vehicle (control group). All experiments and analyses were conducted by a researcher blinded to group assignment. The effect of PNU treatment was evaluated by a cylinder test 3–4 times weekly for up to 30 days after MCAO. Based on the animal performance, 4 distinct groups of effects were identified. **A)** Group 1 was characterized by slow spontaneous recovery after tMCAO without treatment (n=5; asterisks, plus signs, grey rhombs, black dots, open squares); Group 2 did not show any recovery after tMCAO without treatment (n=1; open circles). **B)** Group 3 was characterized by sustained performance in the cylinder test for up to 30 days after

tMCAO with 4 sub-chronic PNU treatments over the first 72 h after tMCAO (n=4; grey circles, open triangles, open squares, plus signs); and Group 4 exhibited delayed relapses at around day 11 after MCAO despite 4 sub-chronic PNU treatments over the first 72 h after tMCAO (n=3; rhombs, asterisks, black dots). **C-D**) Data points in groups 1 and 3–4 were averaged for statistical analysis to evaluate the effects of time and treatment after MCAO and interactions across treatment and time factors using a repeated measures 2-way ANOVA. Open circles in **C**) indicate data from PNU treated animals shown in **D**). The statistical analysis revealed significant main effects for the factors of PNU treatment [$F(2,9)=36.27$; $p<0.0001$] and time after MCAO [$F(13,117)=5.421$; $p<0.0001$] and indicated a significant interaction of PNU treatment vs. time after MCAO [$F(26,117)=13.77$; $p<0.0001$]. The corresponding post-test multiple comparisons tests (Tukey) detected significant differences across PNU- and vehicle-treated data points spanning over 24 days of experiments despite the slow spontaneous recovery observed in five out of six control animals. This spontaneous recovery was nearly complete by days 29–30 rendering differences between PNU- and vehicle-treated groups insignificant ($p>0.05$). Between days 25 and 28, data were not collected. Several animals were subjected to a cylinder test immediately prior to MCAO. This limited data set is illustrated without analysis as black and bold symbols on the Y-axis corresponding to day 0). The following abbreviations were used: * $p<0.05$; \$ $p<0.01$; & $p<0.001$; # $p<0.0001$. Data are shown as mean $\pm$ SEM.

Figure 8. Summary of results: delayed brain tissue death and protection.

Without treatment, ischemic infarct grows and saturates within 24–72 h (red circles) after MCAO. We have shown previously that a single PNU injection 0.5 h after MCAO significantly reduced infarct volume 24 h after MCAO [5,15], but these effects vanished 48 h later resulting in a relapse [this study] (blue squares). A combination of single i.v. (90 min after MCAO) and multiple daily s.c. PNU injections produced a reliable sustained protection (green symbols). The data point labeled by a green triangle was calculated from the cylinder test data obtained 24 h after MCAO using the correlation function (Figure 1, dashed line) and the same pool of rats euthanized and tested for infarct volumes 72 h after MCAO (green rhomb). Thus, sustained treatments (green line) are required for sustained PNU efficacy because acute treatment (blue line) does not produce sustained protection. The following abbreviation was used: * $p < 0.05$. Data are shown as mean+SEM.

Table 1.
Examples of treatment durations and delays in random cytoprotective clinical trials.

Treatment duration is largely ignored in cytoprotective strategies [46–48]. We argue that both short *treatment delays* and prolonged *treatment durations* may be required to maximize sustained therapeutic efficacy after AIS as this study concludes for PNU120596, a prototypical PAM of $\alpha 7$ nAChRs.

Treatment Durations (from <24 h to 8 weeks) and Delays In Random Cytoprotective Clinical Trials				
<24 h	48–72 h	5–14 days	4 weeks	6–8 weeks
Drug/Delay to Treatment, h	Drug/Delay to Treatment, h	Drug/Delay to Treatment, h	Drug/Delay to Treatment, h	Drug/Delay to Treatment, h
Selfotel/6[87]	AR-R15896AR/12[95]	Nimodipine/6–48[106]	Nimodipine/24–48 [112,113]	Citicoline/6–24[116] *
Destorphan/48[88]	NXY-059/24[50,96]	Enlimomab/6–24 [107,108]	Cerebrolysin/12–72 [114,115]	Piracetam/7[117] *
Aptiganel/6[89]	GV150526/12[97,98]	Lubeluzole/8[109]		
Clomethiazole/12[90]	ZK200775/24[99]	Ebselen/24[110]		
UK-279,276/6[91]	Tirilazad/6[100]	ONO-2506/6		
YM872/6	IL-1 ra/6 [101]	Edaravone/24[111]		
Nalmefene/6[92]	Sipatrigine/12[102]			
Magnesium	BMS-204352/6			
Sulfate/2 [93]	Repinotan/4.5[103]			
Rhapsody/12[94]	Diazepam/12[104]			
	DCLHB/18[105]			

*) The longest treatment durations by agents stabilizing cellular membranes: citicoline (6wks) [116] and piracetam (8 wks) [118–121].