9-27-2022

Pharmacological Characterization of a Novel Neuroactive Steroid Prodrug, NTS-104, and Its Neuroprotective Activity in Experimental Stroke Models

Li Zhang
Henry Ford Health, lzhang3@hfhs.org

Robin Schwartz-Byrne

Steven Gertz

Matthew McGuire

Colleen Woodhouse

See next page for additional authors

Recommended Citation

Zhang L, Schwartz-Byrne R, Gertz S, McGuire M, Woodhouse C, Powell B, Wei M, Li C, Billing CB, Verdoorn TA, Lam L, Parry TJ, Buller B, Zhang ZG, Poulsen D, and Chopp M. Pharmacological Characterization of a Novel Neuroactive Steroid Prodrug, NTS-104, and Its Neuroprotective Activity in Experimental Stroke Models. Stroke 2022.

Authors

Li Zhang, Robin Schwartz-Byrne, Steven Gertz, Matthew McGuire, Colleen Woodhouse, Brianna Powell, Min Wei, Chao Li, Clare B. Billing, Todd A. Verdoorn, Leslie Lam, Tom J. Parry, Benjamin Buller, Zhenggang Zhang, David Poulsen, and Michael Chopp

Stroke

ORIGINAL ARTICLE

Pharmacological Characterization of a Novel Neuroactive Steroid Prodrug, NTS-104, and Its Neuroprotective Activity in Experimental Stroke Models

Li Zhang, MD*; Robin Schwartz-Byrne, MS*; Steven Gertz, BA; Matthew McGuire, BA; Colleen Woodhouse, BA; Brianna Powell, BS; Min Wei, BS; Chao Li, PhD; Clare B. Billing, MS; Todd A. Verdoorn, PhD; Leslie Lam, MS; Tom J. Parry, PhD; Benjamin Buller, PhD; Zheng Gang Zhang, MD, PhD; David Poulsen, PhD; Michael Chopp, PhD

BACKGROUND: Ischemic stroke affects about 700 000 patients per year in the United States, and to date, there are no effective pharmacological agents that promote recovery. Here, we studied the pharmacokinetics, pharmacodynamics, and efficacy of NTS-105, a novel neuroactive steroid, and NTS-104, a prodrug of NTS-105, in 2 models of ischemic stroke.

METHODS: The pharmacodynamics and pharmacokinetics of NTS-104/105 were investigated in naive and stroke rats, and models of embolic and transient middle cerebral artery occlusion were used to investigate the dose-related effects of NTS-104. All rats were randomly assigned into the experimental groups, and all outcome measurements were performed blindly.

RESULTS: Blood plasma and brain pharmacokinetic analysis revealed that NTS-104 rapidly converted to NTS-105, which reached peak concentration at ≈1 hour after dosing and distributed similarly to normal and ischemic brains. NTS-104 administration 4 hours after embolic middle cerebral artery occlusion led to a dose-dependent improvement of neurological outcomes and a dose-dependent reduction of infarct volumes relative to vehicle-treated animals. A single dose level study confirmed that NTS-104 administered 4 hours after transient middle cerebral artery occlusion was also neuroprotective. Quantitative ELISA revealed that NTS-104 treatment resulted in time- and dose-dependent changes in AKT activation and cytokine levels within the ischemic brain, which included reductions of IL-6, VEGF, ICAM-1, IL-1β, MCP-1, RAGE, and GM-CSF. Time- and dose-dependent reductions in IL-6 and GM-CSF were also observed in the plasma along with an elevation of galectin-1.

CONCLUSIONS: NTS-104 is a novel prodrug that converts to a novel neuroactive steroid, NTS-105, which improves functional outcomes in experimental ischemic stroke models.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: brain ■ cytokine ■ middle cerebral artery ■ plasma ■ prodrug

Ischemic stroke occurs in ≈700 000 patients each year in the United States and is a leading cause of death and disability worldwide. Approximately 5% of patients with acute ischemic stroke receive thrombolytics or mechanical thrombectomy and of those patients, only about half experience good outcomes as defined by a score on the modified Rankin Scale score of 0 or 1.[1,2] Thus, the vast majority of patients with acute ischemic stroke are in need of an effective neuroprotective/neurorestorative therapy. Therefore, the successful pharmacological treatment of stroke remains an important clinical goal in acute ischemic stroke.

Correspondence to: Michael Chopp, PhD, Department of Neurology Henry Ford Health Education and Research Bldg, Room 3056, Detroit, MI 48202. Email mchopp1@hfhs.org
*L. Zhang and R. Schwartz-Byrne contributed equally.
The Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/STROKE2021038507.
For Sources of Funding and Disclosures, see page XXX.

Stroke is available at www.ahajournals.org/journal/str

ORIGINAL ARTICLE

Nonstandard Abbreviations and Acronyms

ECA	external carotid artery
eMCAO	embolic middle cerebral artery occlusion
GAL-1	galectin 1
mNSS	modified neurological severity score
pAKT	phosphorylated AKT
tMCAO	transient middle cerebral artery occlusion

Studies have documented the cerebroprotective nature of neuroactive steroids and that nuclear hormone receptors such as progesterone receptors represent valid therapeutic targets for neuroprotection.[3,4] The administration of neuroactive steroids has consistently limited brain damage, improved functional outcomes in preclinical stroke models,[5–7] and promoted multiple endogenous recovery and restorative processes.[8–11] Therefore, it is reasonable to hypothesize that treatment with exogenous neuroactive steroids may further enhance neuroprotection and repair after a brain insult.

Here, we present the molecular structures (Figure 1A and 1B) and pharmacokinetic and pharmacodynamic characterization of a novel neuroactive steroid, NTS-105, and its water-soluble pro-drug, NTS-104. These compounds were first described by the Emory Brain Research Laboratory and the Emory Institute for Drug Development.[12] The systemic and brain pharmacokinetics of the systemically administered pro-drug NTS-104 and parent, NTS-105 are presented herein, as are results in 2 different experimental middle cerebral artery occlusion models (one embolic [embolic middle cerebral artery occlusion (eMCAO)]) and the other transient middle cerebral artery occlusion [tMCAO]), conducted in independent studies. We report that NTS-104 treatment produces robust, dose-dependent improvements in neurological outcomes. Further, NTS-104 treatment reduced pro-inflammatory cytokines in the rat eMCAO stroke model and activated AKT within the ipsilateral hemisphere. These results suggest that NTS-104 treatment may have therapeutic benefit for the treatment of acute ischemic stroke.

METHODS

Data Availability

All data collected and analyzed for this study are archived at KCT Data (Atlanta, GA). The data that support the findings of this study are available from the corresponding author upon reasonable request. See the Supplemental Material for the preclinical checklist required by the journal.

Animal Procedures

All animals in the studies presented herein were male Wistar rats. A total of 172 rats were used in this study, of which 149 were included in the various analyses, and 23 were excluded due to early mortality or insufficient injury. All animal procedures were approved by the Henry Ford Hospital Institutional Animal Care and Use Committee under protocol number 00001003 or the University at Buffalo Institutional Animal Care and Use Committee under protocal number NSG16037N. All studies were conducted in compliance with the standards established by AAALAC-International.

Measurement of NTS-104 and NTS-105 in Plasma and Brain Samples

Male Wistar rats (N=3/group) were administered a single intramuscular (IM) dose of NTS-104 at 10 mg/kg. Stroke rats (see eMCAO model below) were dosed 6 hours after the onset of ischemia. Starting at 30 minutes after initial dosing, NTS-104 and NTS-105 concentrations in blood plasma and brain homogenates were measured at regular intervals up to 24 hours after dosing. A qualified LC-MS/MS bioanalytical method was used to quantify the levels of NTS-104 and NTS-105 in blood plasma and brain tissue samples. The internal standard used for NTS-104 quantitation was dexamethasone, and the internal standard used for NTS-105 was glyburide. These standards were chosen because they have similar physicochemical properties to NTS-104 and -105, including a similar molecular weight, reasonably similar structure, and comparable solubilities, and thus were reasonable sample handling and extraction controls.

eMCAO Model

Clot Preparation

Male Wistar rats were purchased from Charles River Laboratories and habituated for 5 days upon arrival to laboratory animal facility. A single, fibrin-rich white clot was prepared from each donor rat, which were anesthetized with 3% isoflurane. With a segment of 25 cm sterile PE-50 tubing, arterial blood was obtained from the femoral artery. The arterial blood was incubated for 2 hours at 37 °C, then stored overnight at 4 °C. Before the stroke induction surgery, ≈500 mm PE-10 tubing was connected to a 1-mL sterile saline-filled syringe. The clot-containing PE-50 tubing was connected to the PE-10 tubing and the clot was flushed out into the saline filled Petri dish. The clot was then washed by repeatedly withdrawing and flushing out 10 to 15 times with saline until it became white-pinkish in color, signifying negligible numbers of red blood cells remained. Clots were divided with a scalpel into 4 to 5 cm lengths and collected into a modified PE-50 catheter (end diameter 0.3–0.4 mm) connected to a 100-µL Hamilton microsyringe. All procedures were conducted under aseptic condition.

Stroke Induction

All surgery was conducted using aseptic technique and presterilized instruments. Rats were prepared for induced stroke using the described eMCAO model.[12,13] Rats (350–400 g; N=23 for vehicle group and N=15 for all other groups) were placed in supine position and initially anesthetized with 3.5% isoflurane following standard aseptic procedures. Anesthesia was maintained at 2.8% isoflurane and body temperature at 37 °C during procedure. Under a stereo microscope, a 20 to 30 mm midline incision was made from the jaw to the clavicle and the right

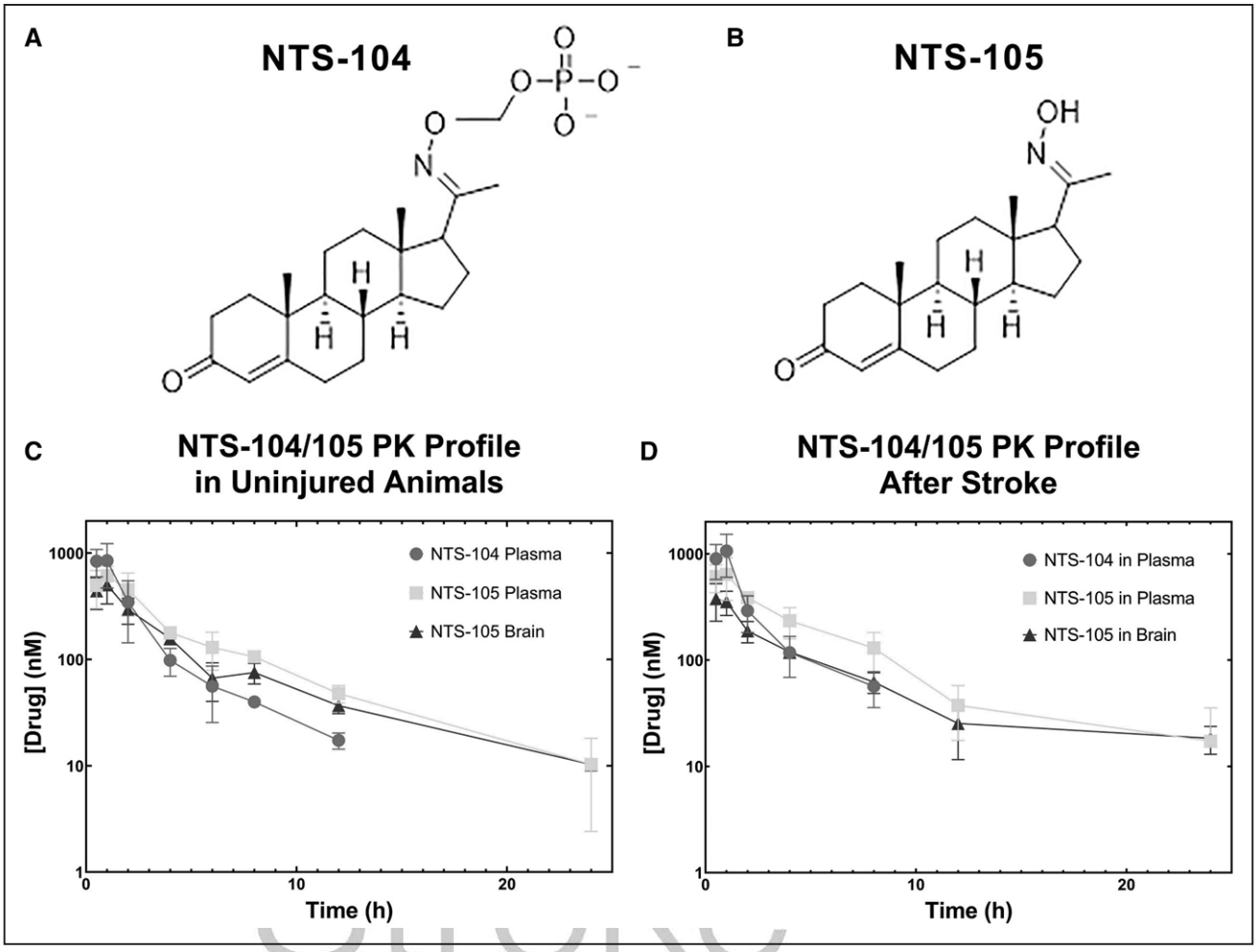

Figure 1. NTS-105 enters the brain in healthy and stroke animals, while its prodrug, NTS-104 does not cross the BBB. **A**, Structure of NTS-104, a neurosteroid backbone linked via an oxime to a phosphate. **B**, NTS-105, an oxime-containing novel neurosteroid. Plasma concentration-time profile of NTS-104 in plasma, and NTS-105 concentration in plasma and brain after administration of a single IM dose of 10 mg/kg NTS-104 in uninjured (**C**) and stroke (**D**) rats. NTS-104 was below the detection limit in brain at all timepoints. There are no statistically significant differences in plasma or brain concentrations of NTS-105 between injured and uninjured animals, and there are no statistically significant differences in any single measure from 30 to 60 min, suggesting that the true T_{max} lay somewhere between these timepoints. Data are presented as mean±SD. N=3.

common carotid artery, external carotid artery (ECA), and internal carotid artery were exposed. The right ECA was ligated with a 4-0 silk suture and the stump of the ECA was aligned with the common carotid artery. An aneurysm clip was placed around the common carotid artery and internal carotid artery to temporarily induce hemostasis. A partial arteriotomy was performed on the ECA stump and the tip of the clot filled modified PE-50 catheter was inserted into the arteriotomy. After securing the modified PE-50 catheter at the ECA stump, the aneurysm clip was removed, and the modified PE-50 catheter was advanced into the internal carotid artery rostrally for 18 to 19 mm. The clot was injected slowly in 5 to 10 µL of saline at rate of 10 µL/minute. The modified PE-50 catheter was then removed, the ECA stump ligated, the incision site was closed, and anesthesia was discontinued. Afterward, rats were placed on warming pads until recovery.

Treatment Group, Sample Size, and Dosing

Following surgery and before dosing, rats were randomized into one of the 5 groups using a randomization scheme generated by randomizer.org: vehicle (PBS, pH 8.7), NTS-104 at doses of 1, 3, 10, and 30 mg/kg (n=23 vehicle, and n=15/group for treatment arms). Sample sizes were determined based on previous unpublished pilot data. A sample size of 15 rats in each treatment group that had baseline modified neurological severity score (mNSS) values of 9 or greater was estimated to have 90% power to detect a true treatment difference of 2.6 points in the change from baseline mNSS values at the 2-sided, 0.05 significance level. This estimate assumed use of Dunnett's procedure to adjust the significance level for the 4 active dose comparisons to vehicle control, and a SD of 1.8 points in the change from baseline of the mNSS at day 28. The least significant difference for each comparison to vehicle was estimated to be 1.7.

Rats were treated with IM doses of NTS-104 at rotating sites in the hind limbs based on weight at 4, 10, 24, 48, 72, and 96 hours after injury. The NTS-104 dosage was subsequently decreased by half at 72 hours and again at 96 hours (see Figure 2A for experimental timeline). A nominal volume of 100 µL was used for injection, but the dose volume injected

ORIGINAL ARTICLE

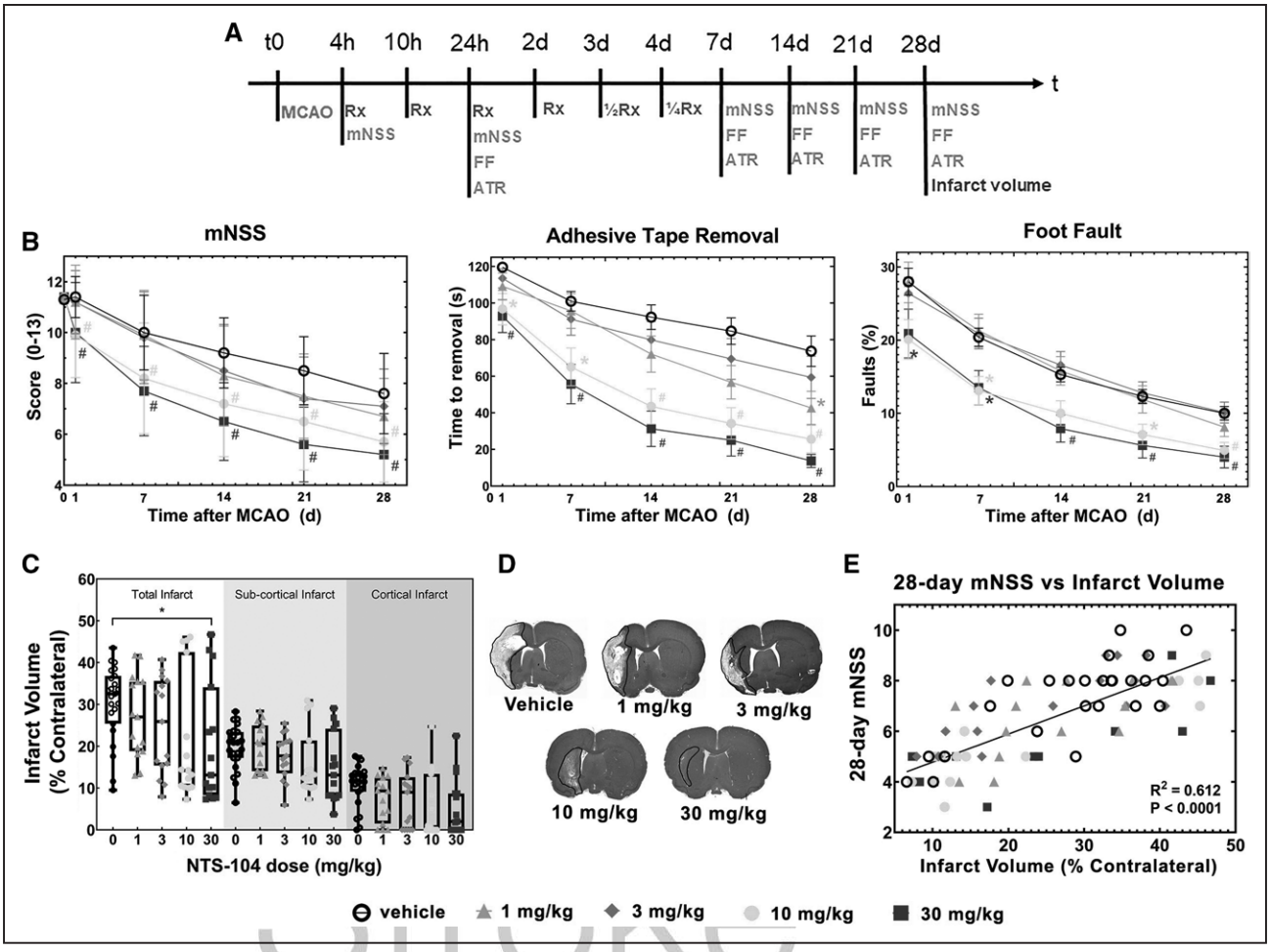

Figure 2. NTS-104 is neuroprotective after embolic middle cerebral artery occlusion (eMCAO) and improves outcomes up to 1 mo after injury.

A, Experimental design for the embolic middle cerebral artery occlusion behavior study, including drug dosing schedule and testing days for modified neurological severity score (mNSS), adhesive tape removal test (ATR), and foot fault (FF) assessment. **B**, The results of mNSS analysis, adhesive tape removal test, and foot fault assessment. mNSS data were measured starting at day 0 for baseline inclusion criteria; all other end points were measured starting on day 1. Data are presented as mean±SEM. N=23 for vehicle group. N=15 for treatment arms. **C**, A box-and-whisker plot of the distribution of infarct volumes within each treatment group; each individual's infarct volume is also displayed. Total infarction is in white, which the subcortical infarction is light shaded and the cortical infarction is dark shaded. Statistical significance was only found in the total infarction measurements, but the proportion of animals having no cortical infarct increased with dose. **D**, Representative images of the middle section of a single animal from each treatment group; infarcted region is outlined in black. **E**, Scatter plot of the mNSS on day 28 vs infarct volume showing a highly significant correlation. *P<0.05 vs vehicle. #P<0.01 vs vehicle.

was adjusted based on the weight of each rat. Rats who died during the first week of the experiment were replaced, and rats who scored 8 or lower on the mNSS at 4 hours after surgery (criteria established a priori) were excluded before randomization. Blinding codes were held by a third party who was not involved in the conduct of the study, and all personnel involved in the assessments were therefore blinded to treatment.

tMCAO

Stroke Induction

Male Wistar rats (N=15/group) were subjected to tMCAO. The tMCAO surgery was conducted using the described tMCAO model.[14,15] The surgery is substantially similar to the eMCAO model, except that the tip of a blunt-end monofilament nylon suture was inserted into the arteriotomy and advanced 18 to 19 mm, in place of the catheter described above. After 2 hours, the rats were reanesthetized and the suture was removed to reestablish perfusion.

Treatment Group, Sample Size, and Dosing

Following tMCAO and before dosing, rats were randomized into one of 2 groups: vehicle or NTS-104 at 10 mg/kg administered 4 hours after tMCAO (n=15 vehicle, and n=15/group for treatment arms). Sample sizes were determined based on data from the eMCAO study. A sample size of 15 rats in each treatment group that had baseline mNSS values of 9 or greater was estimated to have 90% power to detect a true treatment difference of 1.9 points in the change from baseline mNSS values at the 1-sided, 0.05 significance level. This estimate assumed a SD of 1.7 points in the change from baseline of the mNSS at day 14. The least significant difference for the comparison to vehicle was estimated to be 1.1. Rats were treated with IM doses of NTS-104 in the same manner as

above. Rats who died during the first week of the experiment were replaced, and rats who scored 8 or lower on the mNSS at 4 hours after tMCAO (criteria established a priori) were excluded before randomization. Blinding codes were held by a third party who was not involved in the conduct of the study, and all personnel involved in the assessments were therefore blinded to treatment.

Behavioral Analysis

Modified Neurological Severity Score

An mNSS assessment was performed on each animal within 4 hours after initiating stroke and before randomization, as previously described.[13,16] Assessments were repeated 24 hours after eMCAO injury and on days 7, 14, 21, and 28 (or days 7 and 14 for tMCAO).

Adhesive Removal Test

To evaluate sensorimotor function, an adhesive removal test (ART) was performed at 24-hour post-injury and on days 7, 14, 21, and 28 following eMCAO (or days 7 and 14 for tMCAO) to assess sensorimotor deficits, as we and others have previously described.[13,17]

Foot Fault

To evaluate ambulation/gait, the Foot fault test was performed at 24 hours post-injury and on days 7, 14, 21, and 28 (or days 7 and 14 for tMCAO) to assess motor recovery, as we and others have previously described.[13,18]

Infarct Volume

Twenty-eight (28) days after eMCAO, rats were euthanized and infarct volumes were measured as follows. Rats were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed from the skull, fixed by incubation in 4% paraformaldehyde at ambient temperature for several days. Each brain was cut into 7 coronal blocks, each with 2 mm thickness. The tissue blocks were processed and embedded, and 6-µm-thick paraffin sections from each block were cut and stained with hematoxylin and eosin for evaluation of infarct volume. The coronal sections were captured and digitized with the MCID computer imaging analysis system (Imaging Research). The area of both hemispheres and the area of infarction were measured by digital tracing the respective areas. The infarct volume was determined by multiplying the appropriate area by the section interval thickness. To reduce errors associated with tissue processing, the infarct volume is presented as the percentage of infarct volume of the contralateral hemisphere (indirect volume calculation).[12,19]

Measurement of Phosphorylated AKT, AKT, and Cytokines

Adult, male Wistar rats (350–400 g; N=6/group) underwent either sham surgery or eMCAO. At 6 hours after eMCAO, rats scoring 9 or higher on the mNSS were dosed IM with a single injection of vehicle (Tris-buffered saline, pH 8.5), or with NTS-104 at 5 or 20 mg/kg in a volume of 100 µL. At 6, 12 or 24 hours after injection, rats were deeply anesthetized and exsanguinated by cardiac puncture, at which time blood samples were collected. Rats were then perfused with 20 mL of PBS to remove blood from the cerebral vasculature. Whole blood was collected into EDTA (K3) Monoject tubes (Covidien). Plasma was prepared by centrifugation of whole blood for 10 minutes at 3000×g at 4 °C. Plasma samples were stored at -80°C. After perfusion, cortical and sub-cortical brain tissue samples from the ipsilateral hemispheres were collected on ice in chilled PBS, snap-frozen in liquid nitrogen, and then stored at −80 °C.

The levels of total AKT and phosphorylated AKT (pAKT) were determined by quantitative ELISA using the RayBiotech's Phospho AKT (Ser473) ELISA kit (PEL-AKT-S473-Q, Atlanta, GA) according to the manufacturer's instructions. Cytokine protein levels in the cortex and plasma were determined by RayBiotech via their Quantibody Rat Cytokine Array 67. One-way ANOVA was performed to compare groups within each respective time point post injection.

Statistical Analysis

Assessments repeated at multiple post-treatment time points (mNSS, foot fault, and ART) were analyzed by a linear mixed-effects model for repeated measures comparing each randomized NTS-104 treatment group to vehicle. The model included treatment, visit, and treatment by visit interaction. The model for mNSS also included the baseline assessment as a covariate. The single timepoint assessment of infarct volume was analyzed using a linear ANOVA and compared each dose group to vehicle. All statistical tests were 2-sided at the α=0.05 level for the eMCAO study, and 1-sided at the same level for the tMCAO study. Dunnett's adjustment for testing multiple dose groups versus control was used for the inferential analyses for the eMCAO study. No additional multiplicity adjustments (for the multiple end points and multiple analysis time points) were performed. Analyses of the mNSS, ART, and foot fault included data from all rats who were assigned to treatment and who had a baseline and any post-baseline assessment. This included rats that both completed the study and died (or were euthanized) during the study. The distribution properties of the data were examined, and if non-normally distributed, additional analyses were conducted by a nonparametric method based on ranks.

RESULTS

Pharmacokinetic in Normal and Stroke Animals

To determine the pharmacokinetic properties of NTS-104 and NTS-105 under normal and neuropathologic conditions, pharmacokinetic studies were performed in normal and eMCAO rats. In normal rats, IM administered NTS-104 achieved T_{max} at 1 hour after IM injection in plasma and was not measurable in plasma by 12 hours (Table; Figure 1A). NTS-105 achieved T_{max} at 1 hour after IM injection in plasma and in brain (Table; Figure 1A). For rats with eMCAO, NTS-104 achieved T_{max} at 1 hour after IM injection in plasma and was not measurable by 12 hours after injection (Table; Figure 1B). NTS-105 achieved T_{max} at 1 hour after IM injection in plasma and at 0.5 hour in the stroked brain (Table; Figure 1B). NTS-104 was not detected in brain in any time point. These

Table. Pharmacokinetic Parameters of NTS-104 and NTS-105 in Plasma and Brain of Healthy and Stroke Animals After a Single Intramuscular Injection of 10 mg/kg NTS-104

	Plasma					Brain				
	C_{max}		T_{max}	AUC		C_{max}		T_{max}	AUC	
	(ng/mL)	(nM)	(h)	(ng/mL-h)	(nM-h)	(ng/mL)	(nM)	(h)	(ng/mL-h)	(nM-h)
Healthy rats										
NTS-104	373	850	1	896	2041	NA	NA	NA	NA	NA
NTS-105	279	636	1	1214	2765	172	392	1	709	1615
Stroke rats										
NTS-104	467	1064	1	995	2267	NA	NA	NA	NA	NA
NTS-105	281	640	1	1314	2993	149	338	0.5	576	1312

data indicate that the pharmacokinetic of NTS-104 and NTS-105 was quite similar in normal and stroke rats.

Efficacy of NTS-104 in eMCAO Model

In total, sixteen (16) animals died after surgery (considered related to model injuries), five (5) before, and eleven (11) after randomization. These animals were replaced to ensure that 15 animals completed the study from each experimental arm. Additionally, four (4) animals were excluded due to a nonqualifying mNSS of 8 or lower. Before treatment, rats randomly assigned in 5 dose groups exhibited neurological deficits based on mNSS and inclusion criteria. As expected, each treatment group had lower average mNSS scores at each successive observation day, indicating spontaneous partial recovery (Figure 2B). However, treatment with NTS-104 at 4 hours after eMCAO accelerated recovery in a dose-dependent manner. While numerical improvement was observed with all 4 doses, only rats in the 10 and 30 mg/kg dose groups performed significantly better compared with vehicle-treated control animals (Figure 2B) after adjusting for Dunnett correction for multiple comparisons. Similarly, robust improvements in both ART and foot fault assessments were observed over the 28 d test period with the 10 and 30 mg/kg dose groups compared with vehicle-treated controls (Figure 2B). A significant improvement in the ART test was also observed at day 28 in the 1 mg/kg dose group.

Rats were euthanized for infarct volume analysis 28 days after eMCAO. Average infarct volumes were reduced in a dose-dependent fashion; however, the data were not normally distributed based on the Shapiro-Wilk normality test. Although Kruskal-Wallis 1-way ANOVA on ranks revealed an overall P of 0.083, post hoc Wilcoxon rank-sum test showed that the 30 mg/kg group had a significantly smaller average infarct volumes compared with vehicle treated controls (Figure 2C and 2D; $P<0.05$). The infarct volume measurements were further broken down by cortical and subcortical location, and while no statistically significant differences were found, the number of animals have no apparent cortical infarction increased with dose (Figure 2C). Because of the non-normality of the distributions, especially in the 10 and 30 mg/kg groups, 28-day mNSS was plotted versus infarct volume, and it was determined that the correlation was highly linear when individual data points were plotted (Figure 2E; $R^2=0.612$; $P<0.0001$). Notably, several animals in the 10 and 30 mg/kg group that had large infarctions also were found to have high 28-day mNSS scores.

Efficacy of NTS-104 in tMCAO

To account for model-specific effects, especially a lack of rapid reperfusion in the eMCAO model, a study in a second model (tMCAO) of experimental stroke model was conducted. The lowest effective dose from the eMCAO study (ie, 10 mg/kg) was utilized, and the observational period was shortened from 28 to 14 days. One (1) animal that was enrolled in this study died prematurely (not drug related) and was replaced, and two (2) were excluded before randomization due to nonqualifying mNSS scores. Before drug treatment, rats in both treatment groups performed equally on the mNSS. However, at 1 day after injury and thereafter, NTS-104-treated animals performed significantly better on each task than did vehicle-treated animals (Figure 3A through 3C). Infarct volume was measured at the end of the experiment, and was similar to the eMCAO results. The data were not normally distributed, so a nonparametric analysis was again performed. Although the mean infarct volume was reduced in drug- versus vehicle-treated animals, the effect approached but did not achieve statistical significance based on Wilcoxon rank-sum test ($P=0.054$; Figure 3D). These data indicate that repeat dosing of 10 mg/kg NTS-104 is effective at enhancing recovery from tMCAO.

AKT Activation and Cytokine Analysis

Quantitative ELISA was used to determine the levels of total AKT and pAKT within the ipsilateral striatum and cortex of stroke rats with and without a single IM NTS-104 treatment of 5 or 20 mg/kg. Increases in pAKT levels were detected in both the ischemic cortex and striatum at 6 hours after a single injection of NTS-104 (20 mg/kg) relative to vehicle. In contrast, the 5 mg/kg dose did not induce an increase in pAKT levels relative to vehicle treated rats

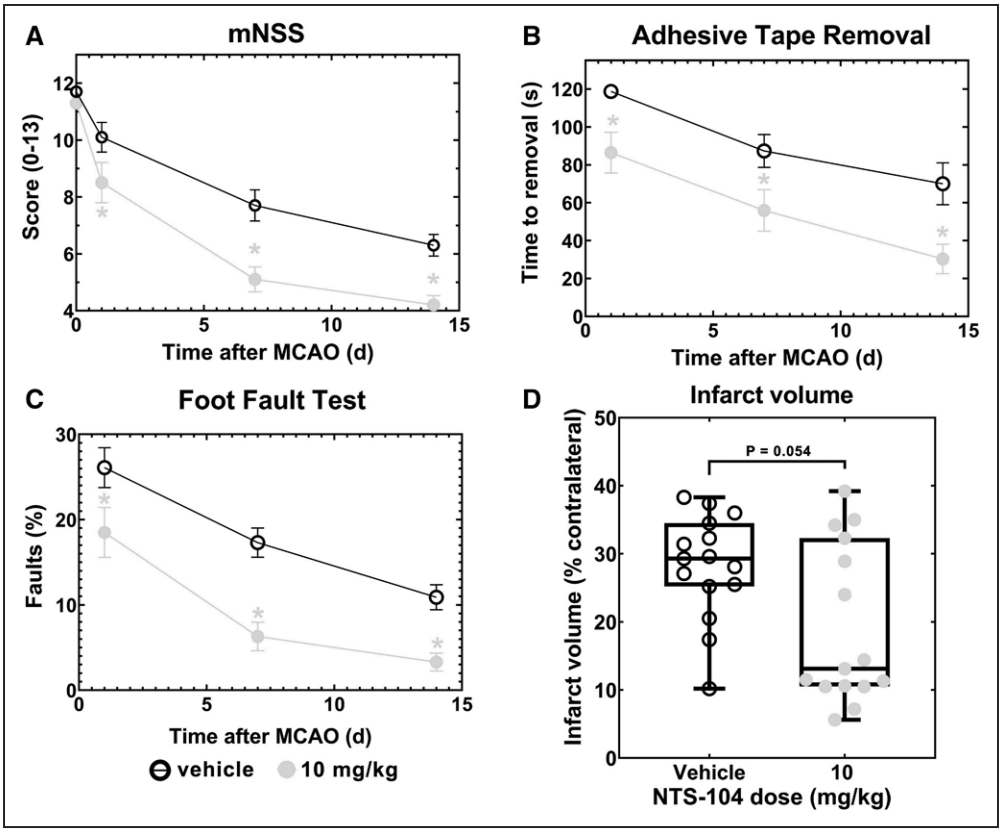

Figure 3. NTS-104 is also protective after transient middle cerebral artery occlusion (tMCAO).
Results of the behavioral outcomes of the tMCAO study for (**A**) modified neurological severity score (mNSS), (**B**) adhesive tape removal test, and (**C**) foot fault assessment of rats with tMCAO surgery. mNSS mNSS data were measured starting at day 0 for baseline inclusion criteria; all other end points were measured starting on day 1. Data are presented as mean±SEM. N=15/group. *P<0.05. Infarct volumes (**D**) were also measured to be lower in drug treated rats vs vehicle control, approaching statistical significance (P=0.054).

(Figure 4A and 4B). The 20 mg/kg NTS-104 dose also induced a decrease in pAKT levels in the striatum relative to vehicle, but not the cortex at 12 hours after treatment. A corresponding increase in total AKT was observed in both the striatum and cortex at 6 hours after dosing with 20 mg/kg of NTS-104 but not with the 5 mg/kg NTS-104 dose.

Additionally, 67 cytokines in the brain and plasma of rats were assayed using quantitative ELISA following eMCAO. We found that a single IM dose of NTS-104 at 20 mg/kg significantly reduced proinflammatory cytokines interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), receptor for advanced glycation endproducts (RAGE), vascular endothelial growth factor (VEGF), and intercellular adhesion molecule-1 within the cortex of stroked rats 6 and 12 hours after a single IM injection (Figure 4C). The reduction in the level of RAGE detected in the cortex extended further to 24 hours after dosing. A time- and dose-dependent decrease in the plasma levels of IL-6 and GM-CSF was also observed (Figure 4D). At doses of 5 or 20 mg/kg, NTS-104 increased the circulating levels of the anti-inflammatory cytokine Gal-1 (galectin-1) within the plasma at 6 and 12 hours after treatment, respectively (Figure 4D).

DISCUSSION

The data presented herein suggest that NTS-104 is a novel candidate for cerebroprotection from ischemic stroke. NTS-104 is highly water soluble relative to similarly acting neuroactive steroids, and thus it is easier to deliver than highly hydrophobic compounds. NTS-104, as a phosphate prodrug of NTS-105, is rapidly dephosphorylated to NTS-105 in the plasma. Unlike NTS-104, NTS-105 crosses the blood-brain barrier in measurable quantities, consistent with previous reports.[20] It is noteworthy that stroke did not cause a difference in the brain concentration of NTS-105 relative to healthy animals nor did it allow for successful penetration of NTS-104 into the brain parenchyma. This suggests that the opening of the blood-brain barrier is not required for NTS-105 bioavailability to the injured brain or increases brain distribution of NTS-105 following injury. Therefore, plasma levels should be sufficient to estimate the brain exposure of NTS-105 in normal volunteers (ie, in the case of early safety trials) and prospective stroke patients. Although NTS-105 was measurable in brain and plasma for the duration of the pharmacokinetic study (24 hours), it is likely not available at therapeutic levels. Progesterone is known to be bound to plasma proteins at about

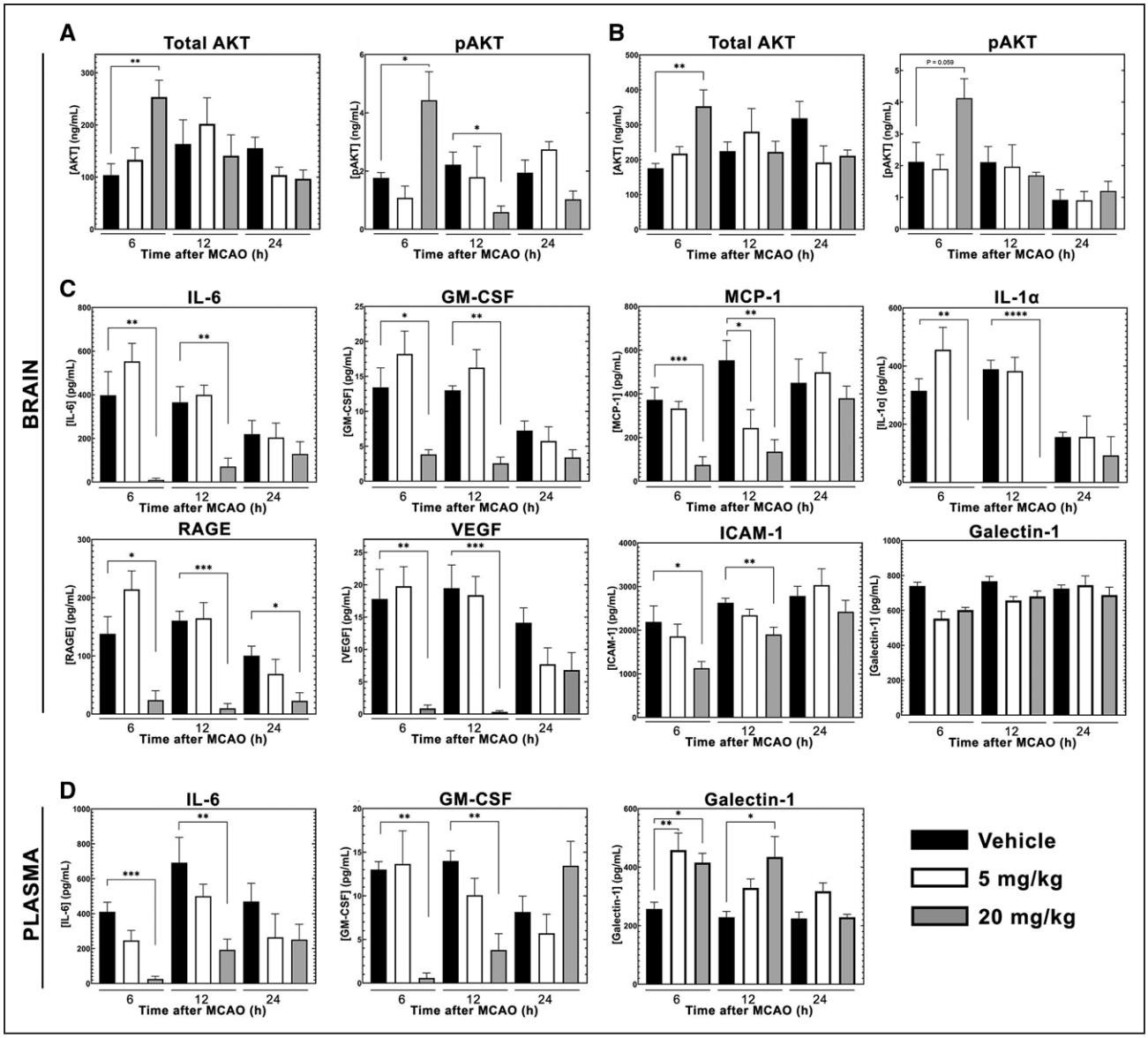

Figure 4. Several inflammation-associated proteins are altered in response to NTS-104 treatment after stroke.
AKT and pAKT were elevated in response to NTS-104 treatment (20 mg/kg) after stroke in striatum (**A**) and cortex (**B**). An ELISA-based panel revealed many pro-inflammatory cytokines to be suppressed in cortex in repose to NTS-104 treatment, whereas the anti-inflammatory galectin-1 was unaltered in cortex (**C**). Two of the pro-inflammatory cytokines, IL-6 and GM-CSF were also suppressed in plasma, whereas the anti-inflammatory galectin-1 was elevated in blood (**D**). N=6/group. Data are presented as mean±SEM. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

98% to 99%.[21] If a similar protein-binding fraction is assumed for NTS-105, and it is also assumed that NTS-105 has a similar affinity for the progesterone receptor (~1 nmol/L), then the brain concentration of NTS-105 would fall to below efficacious levels between 5 and 10 hours after delivery, potentially requiring repeat dosing or extended exposure over the acute poststroke period.

Importantly, the present work demonstrates that NTS-104 is effective at enhancing recovery in multiple experimental ischemic stroke models. Our studies measured both clinically relevant end points and histopathologic changes. In each of our 2 independent stroke studies, our prespecified primary end point was the mNSS assessment, which comprises a battery of sensorimotor tasks, under ischemic injury conditions that represent some of the pathophysiologic changes exhibited in a large percentage of stroke patients including sensory and motor deficits.

The present results suggest that NTS-104 is effective at reducing functional impairment in multiple independent studies in distinct models with unique pathophysiology. The embolic stroke model employs the placement of a fibrin-rich clot into the origin of the middle cerebral artery to induce stroke, which gradually restores perfusion over 24 to 48 hours.[22] In contrast, the transient stroke model utilizes placement of a suture to transiently occlude the middle cerebral artery, in this case for 2 hours after which the suture

is removed and perfusion is restored. These data show that the neuroprotective effect of NTS-104 is not entirely model dependent. Importantly, the improvements in neurological function and infarct volume following NTS-104 treatments initiated at 4 hours after the onset of ischemia are outside the typical neuroprotective timeframe of about 2 hours in the rat.[23,24] This suggests that NTS-104 could be delivered at clinically relevant times, potentially at or beyond thrombolytic treatment windows. An effective neuroprotectant with a therapeutic window that is long enough to capture a large percentage of patients would help to address a substantial lingering unmet medical need in stroke. However, the distribution of infarct volume measurements was apparently not normal in the 10 and 30 mg/kg cohorts (ie, the efficacious doses), which may be attributed to rats that did not exhibit reductions in infarct volume in response to treatment. Although the 28-d mNSS and infarct volumes were well correlated, the broad impact of the non-normality on functional outcomes is unclear and will be further investigated in future studies.

The cytokine measurements presented herein suggest that neuroinflammatory changes following stroke may be modulated by NTS-105, an area for future investigation. Changes in blood-borne biomarkers may offer not only a way to monitor target engagement following NTS-105 biodistribution to the injured brain but also may further inform future clinical trial designs to follow injury progression and detect a relevant response to drug treatment.

Wali et al[20] suggest that NTS-105 is primarily a progesterone receptor agonist. While this is likely true, given its structural similarity to progesterone, we cannot exclude that NTS-105 may also have novel functions that will need to be explored in future studies. The robust effect that was observed in this study on a complex neuroinflammatory cascade likely cannot be explained solely due to a single target, so it is tempting to speculate that the drug's action may be more pleiotropic than is currently known. Future investigations will hone in on the receptor binding profile and affinity thereto. As our aim in the present study was to characterize the pharmacological activity of NTS-104/105 alone, we did not attempt to assess the safety or efficacy of using NTS-104 in combination with thrombolysis (eg, tPA). However, future safety and efficacy investigations will involve the assessment of potential interactions of NTS-104/105 with approved thrombolytics.

CONCLUSIONS

In summary, the results presented herein show that NTS-104 delivers a potent neuroactive steroid, NTS-105, which penetrates the blood-brain barrier, enhances functional recovery after stroke in a dose-dependent/model-independent fashion, and produces time- and dose-dependent changes in circulating biomarkers that are consistent with the putative molecular mechanisms of action of NTS-105. Treatment with NTS-104/105 improves neurological and anatomic outcomes following experimental acute ischemic stroke in rats via multiple mechanisms, and as such, NTS-104 is undergoing nonclinical development studies required for introduction into normal volunteers and, subsequently, stroke patients. These results provide strong pharmacological evidence that NTS-104/105 could be therapeutically beneficial in the treatment of acute ischemic stroke.

ARTICLE INFORMATION

Received February 4, 2022; final revision received July 18, 2022; accepted August 11, 2022.

Affiliations

Department of Neurology, Henry Ford Hospital, Detroit, MI (L.Z., B.P., M.W., C.L., Z.G.Z., M.C.). Department of Neurosurgery, Jacobs School of Medicine and Biomedical Sciences, University at Buffalo, NY (R.S.-B., S.G., M.M., C.W., D.P.). NeuroTrauma Sciences, Alpharetta, GA (C.B.B., T.A.V., L.L., T.J.P., B.B.). Department of Physics, Oakland University, Rochester, MI (M.C.).

Acknowledgments

Dr Poulsen, Li Zhang, Zhang Gang Zhang, and Dr Chopp designed and supervised the conduct and analysis of the pharmacokinetic studies, integrated data, wrote and edited the article. R. Schwartz-Byrne and Dr Zhang performed rat eMCAO surgeries and collected all brain and plasma samples for PD analysis. S. Gertz, M. McGuire, Dr Li, and M. Wei dosed rats and assisted in tissue and plasma collection. C. Woodhouse and B. Powell performed all NSS assessments and assisted in dosing rats and collection of tissue and plasma. Drs Verdoorn, Buller, and Parry helped conceptualize studies, interpret data, and write and edit the article. C.B. Billing designed statistical methods, conducted statistical power calculations and statistical analyses, and helped write and edit the article. L. Lam helped write and edit the article and prepare figures. Dr Poulsen, an unparalleled scientist, collaborator, and friend, died during the course of this research.

Sources of Funding

This work was supported by NeuroTrauma Sciences.

Disclosures

Drs Buller, Verdoorn, and Parry are paid employees of NeuroTrauma Sciences LLC, and each owns equity in NeuroTrauma Sciences LLC. C.B. Billing is, and L. Lam was, a paid consultant of NeuroTrauma Sciences LLC. The other authors report no conflicts.

REFERENCES

1. González RG, Furie KL, Goldmacher GV, Smith WS, Kamalian S, Payabvash S, Harris GJ, Halpern EF, Koroshetz WJ, Camargo EC, et al. Good outcome rate of 35% in IV-tPA-treated patients with computed tomography angiography confirmed severe anterior circulation occlusive stroke. *Stroke*. 2013;44:3109–3113. doi: 10.1161/STROKEAHA.113.001938
2. Nagel S, Herweh C, Pfaff JAR, Schieber S, Schönenberger S, Möhlenbruch MA, Bendszus M, Ringleb PA. Simplified selection criteria for patients with longer or unknown time to treatment predict good outcome after mechanical thrombectomy. *J Neurointerv Surg*. 2019;11:559–562. doi: 10.1136/neurintsurg-2018-014347
3. Schumacher M, Mattern C, Ghoumari A, Oudinet JP, Liere P, Labombarda F, Sitruk-Ware R, De Nicola AF, Guennoun R. Revisiting the roles of progesterone and allopregnanolone in the nervous system: resurgence of the progesterone receptors. *Prog Neurobiol*. 2014;113:6–39. doi: 10.1016/j.pneurobio.2013.09.004
4. De Nicola AF, Labombarda F, Gonzalez Deniselle MC, Gonzalez SL, Garay L, Meyer M, Gargiulo G, Guennoun R, Schumacher M. Progesterone neuroprotection in traumatic CNS injury and motoneuron degeneration. *Front Neuroendocrinol*. 2009;30:173–187. doi: 10.1016/j.yfrne.2009.03.001

5. Guennoun R, Zhu X, Fréchou M, Gaignard P, Slama A, Liere P, Schumacher M. Steroids in stroke with special reference to progesterone. *Cell Mol Neurobiol*. 2019;39:551–568. doi: 10.1007/s10571-018-0627-0
6. Zhu X, Fréchou M, Liere P, Zhang S, Pianos A, Fernandez N, Denier C, Mattern C, Schumacher M, Guennoun R. A role of endogenous progesterone in stroke cerebroprotection revealed by the neural-specific deletion of its intracellular receptors. *J Neurosci*. 2017;37:10998–11020. doi: 10.1523/JNEUROSCI.3874-16.2017
7. Tanaka M, Ogaeri T, Samsonov M, Sokabe M. Nestorone exerts long-term neuroprotective effects against transient focal cerebral ischemia in adult male rats. *Brain Res*. 2019;1719:288–296. doi: 10.1016/j.brainres.2018.09.022
8. Feeser VR, Loria RM. Modulation of traumatic brain injury using progesterone and the role of glial cells on its neuroprotective actions. *J Neuroimmunol*. 2011;237:4–12. doi: 10.1016/j.jneuroim.2011.06.007
9. Gibson CL, Constantin D, Prior MJ, Bath PM, Murphy SP. Progesterone suppresses the inflammatory response and nitric oxide synthase-2 expression following cerebral ischemia. *Exp Neurol*. 2005;193:522–530. doi: 10.1016/j.expneurol.2005.01.009
10. Coughlan T, Gibson C, Murphy S. Modulatory effects of progesterone on inducible nitric oxide synthase expression in vivo and in vitro. *J Neurochem*. 2005;93:932–942. doi: 10.1111/j.1471-4159.2005.03068.x
11. Brinton RD, Thompson RF, Foy MR, Baudry M, Wang J, Finch CE, Morgan TE, Pike CJ, Mack WJ, Stanczyk FZ, et al. Progesterone receptors: form and function in brain. *Front Neuroendocrinol*. 2008;29:313–339. doi: 10.1016/j.yfrne.2008.02.001
12. Zhang RL, Chopp M, Zhang ZG, Jiang Q, Ewing JR. A rat model of focal embolic cerebral ischemia. *Brain Res*. 1997;766:83–92. doi: 10.1016/s0006-8993(97)00580-5
13. Zhang L, Zhang RL, Jiang Q, Ding G, Chopp M, Zhang ZG. Focal embolic cerebral ischemia in the rat. *Nat Protoc*. 2015;10:539–547. doi: 10.1038/nprot.2015.036
14. Li Y, Powers C, Jiang N, Chopp M. Intact, injured, necrotic and apoptotic cells after focal cerebral ischemia in the rat. *J Neurol Sci*. 1998;156:119–132. doi: 10.1016/s0022-510x(98)00036-7
15. Ye X, Yan T, Chopp M, Zacharek A, Ning R, Venkat P, Roberts C, Chen J. Combination BMSC and niaspan treatment of stroke enhances white matter remodeling and synaptic protein expression in diabetic rats. *Int J Mol Sci*. 2013;14:22221–22232. doi: 10.3390/ijms141122221
16. Chen J, Zhang ZG, Li Y, Wang Y, Wang L, Jiang H, Zhang C, Lu M, Katakowski M, Feldkamp CS, et al. Statins induce angiogenesis, neurogenesis, and synaptogenesis after stroke. *Ann Neurol*. 2003;53:743–751. doi: 10.1002/ana.10555
17. Schallert T, Upchurch M, Lobaugh N, Farrar SB, Spirduso WW, Gilliam P, Vaughn D, Wilcox RE. Tactile extinction: distinguishing between sensorimotor and motor asymmetries in rats with unilateral nigrostriatal damage. *Pharmacol Biochem Behav*. 1982;16:455–462. doi: 10.1016/0091-3057(82)90452-x
18. Hernandez TD, Schallert T. Seizures and recovery from experimental brain damage. *Exp Neurol*. 1988;102:318–324. doi: 10.1016/0014-4886(88)90226-9
19. Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR. A semiautomated method for measuring brain infarct volume. *J Cereb Blood Flow Metab*. 1990;10:290–293. doi: 10.1038/jcbfm.1990.47
20. Wali B, Sayeed I, Guthrie DB, Natchus MG, Turan N, Liotta DC, Stein DG. Evaluating the neurotherapeutic potential of a water-soluble progesterone analog after traumatic brain injury in rats. *Neuropharmacology*. 2016;109:148–158. doi: 10.1016/j.neuropharm.2016.05.017
21. Fritz MA, Speroff L. *Clinical gynecologic endocrinology and infertility*. Philadelphia, PA: Lippincott Williams & Wilkins. 2012.
22. Ding G, Jiang Q, Li L, Zhang L, Zhang ZG, Panda S, Ewing JR, Chopp M. MRI of combination treatment of embolic stroke in rat with rtPA and atorvastatin. *J Neurol Sci*. 2006;246:139–147. doi: 10.1016/j.jns.2006.02.020
23. Murata Y, Rosell A, Scannevin RH, Rhodes KJ, Wang X, Lo EH. Extension of the thrombolytic time window with minocycline in experimental stroke. *Stroke*. 2008;39:3372–3377. doi: 10.1161/STROKEAHA.108.514026
24. Zhang L, Zhang ZG, Zhang RL, Lu M, Adams J, Elliott PJ, Chopp M. Postischemic (6-Hour) treatment with recombinant human tissue plasminogen activator and proteasome inhibitor PS-519 reduces infarction in a rat model of embolic focal cerebral ischemia. *Stroke*. 2001;32:2926–2931. doi: 10.1161/hs1201.100207