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Positive allosteric modulation of alpha7 nicotinic acetylcholine receptors as a novel approach to treatment of ischemic stroke

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Ischemic cerebral stroke is a leading cause of disability and death worldwide. Despite substantial investments in developing anti-stroke medicines, clinically effective pharmacological treatments remain inadequate. Clinical utility of tissue plasminogen activator (tPA, Alteplase), the only FDA-approved drug treatment is limited (<10%) because of the short therapeutic window and increased risk of hemorrhage. Cytoprotection is a promising stroke therapy compatible with endovascular interventions, neurogenesis and rehabilitation therapies (e.g., targeted plasticity). In this fully randomized blinded study, an effective cytoprotection strategy for ischemic stroke is proposed. In this approach, prototypical and novel Type II positive allosteric modulators (α7 PAMs) of α7 nicotinic acetylcholine receptors (nAChRs) were tested using a transient 90 min suture middle cerebral artery occlusion (MCAO) model of ischemic stroke. Acute (<24 h) and sub-chronic (>72 h) intravenous (i.v.) or subcutaneous (s.c.) administration of α7 PAMs significantly reduced brain injury and neurological deficits after MCAO. The therapeutic efficacy of α7 PAMs after stroke may arise from activation of multiple converging α7-dependent therapeutic pathways including direct cytoprotection and central/peripheral anti-inflammatory mechanisms and may hold significant translational potential. Our results may become a starting point for developing clinically efficacious therapies utilizing α7 agents and may enable health-care providers to overcome limitations linked to the lack of effective treatments after stroke.

Positive allosteric modulation of alpha7 nicotinic acetylcholine receptors as a novel approach to treatment of ischemic stroke

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List of Publications

1. Gaidhani N., Sun F., Schreihofer D., and Uteshev V.V. Duration of isoflurane-based surgical anesthesia determines severity of brain injury and neurological deficits after a transient focal ischemia in young adult rats. Brain Research Bulletin (2017) In press (PMID: 28755978).

2. Uteshev V.V., Tenovuo O. and Gaidhani N. The cholinergic potential, the vagus nerve and challenges in treatment of traumatic brain injury. Curr Pharm. Des 2016: 2083-2092 (PMID: 26818869)

3. Gaidhani N. and Uteshev V.V. (tentative title) Comparison of sub-chronic and acute efficacy of positive allosteric modulation after focal ischemia in rats. In preparation, as of 08-11-2017.

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List of Abbreviations:

α7 nAChRs – Alpha 7 nicotinic acetylcholine receptors

ACh – Acetyl choline

CCA – Common carotid artery

CCAO – Common carotid artery occlusion

DMV – Dorsal motor nucleus of the vagus nerve

ECA – External carotid artery

EC_{50} – Half maximal effective concentration

EPGN – Epigen

FAST-MAG – Field Administration of Stroke Therapy–Magnesium Phase 3 Clinical Trial

ICA – Internal carotid artery

IC_{50} – Half maximal inhibitory concentration

IV – Intravenous

LDF – Laser doppler flowmetry

NAb – Nucleus Ambiguus

NTS – Nucleus of the solitary tract

PAM – Positive allosteric modulators

rCBF – Regional cerebral blood flow

S.C – Subcutaneous

STAIR – Stroke Therapy Academic Industry Roundtable

TBI – Traumatic brain injury

tMCAO – transient middle cerebral artery occlusion

tPA – Tissue plasminogen activator

TTC – 2,3,5-triphenyl-2H-tetrazolium chloride

VNS – Vagus nerve stimulation

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Chapter1

INTRODUCTION

Ischemic cerebral stroke is a leading cause of disability and death worldwide. Despite substantial investments in developing anti-stroke medicines, clinically effective pharmacological treatments remain inadequate.[1] Clinical utility of tissue plasminogen activator (tPA, Alteplase), the only FDA-approved drug treatment is limited (<10%)[2] because of the short therapeutic window and a risk of increased hemorrhagic response. Cytoprotection has been proposed as stroke therapy complimentary to reperfusion,[3] yet multiple approaches have failed.[3,4] Reasons for these failures are diverse[3,4] and include lack of reproducibility and translation. However, cytoprotection remains a promising therapeutic strategy in stroke in a characterized clinical setting.[5] In contrast to tPA, cytoprotectants can be effective in both ischemic and hemorrhagic strokes and thus, can be given prior to brain imaging.[6] As a result, in an event of ischemic stroke, cytoprotectants can serve as an important bridge to endovascular interventions (e.g., thrombectomy).[6] A potent cytoprotectant could raise multifold the percent of patients successfully treated with thrombolysis (tPA) and/or thrombectomy providing a tremendous benefit to stroke patients. Ambulance practitioners can rapidly deliver IV Rx. In the FAST-MAG study,[3] IV medications were successfully delivered in the field within 1 hr (73.4%) and or 2 hrs (99%) of stroke onset. Thus, a concomitant use of cytoprotectants with thrombolysis and/or thrombectomy offers a valuable clinical advantage.[6] Furthermore, cytoprotection is expected to be compatible with rehabilitation treatments (e.g., targeted plasticity[7] and neurogenesis through conversion of stem[8] or glial[9] cells into mature neurons).[6]

1.1 General overview. The cholinergic system is essential for maintenance of cognitive, autonomic, and immune homeostasis in mammals.[10-22] Endogenous cholinergic tone elevated by

ischemic or traumatic brain injury may serve as a combination auto-therapy targeting multiple distinct physiological pathways generating cytoprotective anti-inflammatory efficacy.[6,23] These endogenous protective mechanisms can be augmented by cholinergic treatments including nicotinic receptor agonists, positive allosteric modulators, and vagus nerve stimulation[7,10,12,23]. The α7 subtype of nicotinic acetylcholine receptors (nAChRs) is uniquely positioned as a promising therapeutic target in stroke and traumatic brain injury because of the cytoprotective anti-inflammatory efficacy of α7 nAChR activation and the ubiquitous expression of α7 nAChRs in mammalian neuronal, glial, and immune tissues.[6,10,12] Furthermore, there is a substantial body of evidence linking the age- and trauma-related reduction in the expression and function of α7 nAChRs to neurodegenerative, sensorimotor and psychiatric disorders associated with cognitive decline and attention deficits.[24-37] By contrast, activation of α7 nAChRs enhances neuronal resistance to ischemia and other insults *in vivo* and *in vitro*[20,38-51] as well as improves neurocognitive functions in patients and animal models of various neuropathologies: e.g., dementias, schizophrenia and brain trauma.[28,43,49,50,52-67] An important rationale for the therapeutic use of α7 agents arises from the fact that α7 nAChRs are ubiquitously expressed throughout the brain [68] including brain regions highly vulnerable to ischemia, such as cortex, striatum and hippocampus.[69-72] Thus, therapeutic strategies that are based on the use of α7 nAChR agents may benefit stroke patients via multiple mechanisms and routes of action.[6]

1.2 Therapeutics of α7 nAChRs.[23] ***(Published with permission from Bentham Science Publishers)*** Age- and trauma-related alterations in the expression and function of α7 nAChRs correlate with neurodegenerative disorders associated with cognitive decline and attention deficits.[24,25,27-31,33,34,36,37] By contrast, activation of α7 nAChRs by endogenous (e.g., choline, ACh, vagus nerve stimulation) or exogenous (e.g., nicotine) nicotinic agents is cytoprotective[38,40-

[42,44,45,48,67,73-76] and anti-inflammatory.[12,77-80] Because functional α7 nAChRs are ubiquitously expressed in neuronal, glial and immune tissues,[70,71,81-84] activation of α7 nAChRs may benefit neuronal survival and function via multiple mechanisms and routes of action (reviewed in [10-12]). Activation of α7 AChRs directly enhances neuronal resistance to injury[38-40,73,74,79] and inhibits inflammatory responses both centrally[79,85-89] and peripherally.[84,90,91] The rationale for therapeutic use of α7 nAChR agents is based on the fact that expression and activation of α7 nAChRs persist following stroke and brain trauma.[24,25,29,34,35,37,92-94] Thus, activation of α7 nAChRs augmented by biologically active compounds or vagus nerve stimulation may provide therapeutic benefits after stroke and TBI.[6,12,23]

1.3 Choline and ACh as endogenous agonists of α7 nAChRs. Although choline is a selective agonist of α7 nAChRs,[95,96] a ubiquitous cell membrane-building material and a precursor-metabolite of ACh, at physiological levels[26,97] choline alone is ineffective as an α7 nAChR agonist because of its low potency for α7 activation (EC_{50}~0.5 mM)[98] and tendency to induce α7 desensitization (IC_{50}~40 μM).[99] Endogenous ACh may also activate α7 nAChRs and enhance resistance to ischemic injury. However, extracellular levels of ACh are very low (<10 nM) due to ACh hydrolysis[100] and thus, only synaptic ACh may be primarily effective. As a result, endogenous choline/ACh have not been regarded as potent therapeutic agents because endogenous levels of choline/ACh do not produce therapeutic levels of α7 activation. We have proposed that these limitations can be overcome by the use of selective α7 agonists or Type-II positive allosteric modulators (PAMs) of α7 nAChRs ([11,38,51]). In addition to endogenous choline/ACh, activation of α7 nAChRs can also be achieved by exogenous α7 agonists.[101] However, it has been also proposed[10,12] that without α7 PAMs, α7 agonists result in α7 desensitization and reduced therapeutic efficacy. By contrast, inhibition of α7 desensitization can augment α7 function and

enhance the therapeutic efficacy of α7 activation.[14,18,102-105] We proposed that these conditions can be achieved by the use of α7 PAMs, such as PNU-120596 (referred thereafter as PNU). Moreover, energy deprivation and cell death elevate extracellular levels of choline[106-108] providing a large source of this endogenous α7 agonist as has been shown by direct measurements in the ischemic core/penumbra in a rat middle cerebral artery occlusion (MCAO) model of cerebral ischemic stroke.[109] Thus, α7 PAM-based treatments may enhance ischemia-activated auto-therapies by converting endogenous α7 agonists into potent therapeutic agents where/when they are most needed (i.e., in the ischemic penumbra post-ischemia). In addition to direct neuroprotection by α7 nAChR activation, both central[77,85,110] and peripheral[78,91] anti-inflammatory efficacies arise from activation of the cholinergic anti-inflammatory pathways.[6] Taken together these data suggest that α7 nAChRs are involved in multiple diverse central and peripheral cellular and molecular mechanisms with converging cytoprotective anti-inflammatory efficacies.[6]

1.4 α7 PAMs as highly selective α7 agents. As an alternative to somewhat indiscriminate action of exogenous α7 agonists, α7 PAMs are proposed[12] (aim 2) as a promising approach to counteracting neurocognitive deficits [14,18], nociception [103,111,112] and cerebral ischemia.[38,51] α7 PAMs can be Type I and II. Both types potentiate α7 responses, but only Type-II PAMs (referred thereafter as simply PAMs) inhibit α7 desensitization. The rationale for use of PAMs after stroke (aim 2) arises from:[6,12] 1) cytoprotective anti-inflammatory effects resulting from α7 activation;[41,44,48,50,57,69,71-73,84-87,113-115] 2) naturally elevated levels of α7 agonist, choline, in the peri-infarct area;[26,97,108,109,116-119] and 3) ubiquitous α7 nAChR expression in brain regions vulnerable to ischemia[69-72,120] and inflammation.[91,121,122] Because physiological levels of extracellular choline (~5 μM)[26,97,116] are insufficient for α7 activation (EC_{50}~0.5 mM)[98] and conventional agonism induces α7 desensitization (IC_{50}~40 μM)[99] limiting utility of this

mechanism due to tolerance.[14,102,103,123] Our approach uses PAMs to enhance activation and reactivation of pre-desensitized α7 nAChRs to amplify the brain's capacity to resist injury by both augmenting cytoprotection[12,38] and suppressing inflammation.[6]

PAMs offer at least three important advantages over exogenous α7 agonists. 1) PAMs alone do not activate α7 nAChRs, but increase the activation efficacy/potency of α7 agonists[124,125] including endogenous α7 agonists [59,124,125]. Thus, PAMs only amplify activation of α7 nAChRs by endogenous choline and ACh which are released naturally as needed.[6,10,12]. 2) PAMs are extremely selective for α7 nAChRs[12] because allosteric binding sites are less conserved; exhibit a greater structural diversity than orthosteric sites[126] and thus, allosteric sites are more likely to be selectively targeted by synthetic compounds[127]; 3) Without α7 PAMs, nicotinic agonists desensitize α7 nAChRs. As a result, therapeutic effects of nAChR agonists develop tolerance.[14,102-104] PAMs inhibit α7 desensitization[59,128,129] and thus, may reduce tolerance and increase the therapeutic efficacy of α7 activation.[38,51,111] The half-life time of PNU120596, a prototypical PAM synthesized by Pfizer, is ~10 hrs,[130] thus, a daily regimen is appropriate for multiple sub-chronic PNU injections.

Our preliminary and published studies have demonstrate that 1 mg/kg PNU administered i.v. up to 6 hours after MCAO significantly reduces brain injury and neurological deficits in rats.[38,51] By contrast, the therapeutic efficacy of donepezil, an inhibitor of ACh hydrolysis, has been reported to cease within the first 2 hrs post-MCAO.[131] Moreover, complementary results obtained by our lab in experiments with acute hippocampal slices demonstrate that 1 μM PNU co-administered with 20-200 μM choline significantly delay neuronal anoxic depolarization and injury induced by oxygen-glucose deprivation and that these effects of PNU require activation of α7 nAChRs.[38] However, while PNU is efficacious, both its pharmacological and biochemical

properties can be significantly improved. In addition, PNU is only a research tool and has not been developed. Thus, in aim 2, we tested novel PAMs superior to PNU and suitable for post-stroke treatment and new intellectual property. The novel PAMs were synthesized by our research partners at Epigen Biosciences Inc. (San Diego, CA). Thus, we proposed to identify novel and highly potent α7-PAMs with improved in vivo efficacy. Other chemical and physiological characteristics such as solubility, toxicity and ADME profile will be conducted outside of this study. The *rationale* for this approach is that *novel, potent agonists and PAMs with improved pharmacological and biochemical properties are expected to demonstrate efficacy in in vivo stroke model and represent a possible treatment option in stroke-induced brain injury.* This contribution is expected to generate a novel clinically-suitable treatment option not currently available to health care providers by enhancing α7-dependent cholinergic tone using selective α7 agonists and/or PAMs as potent therapeutic agents.[6,10,12]

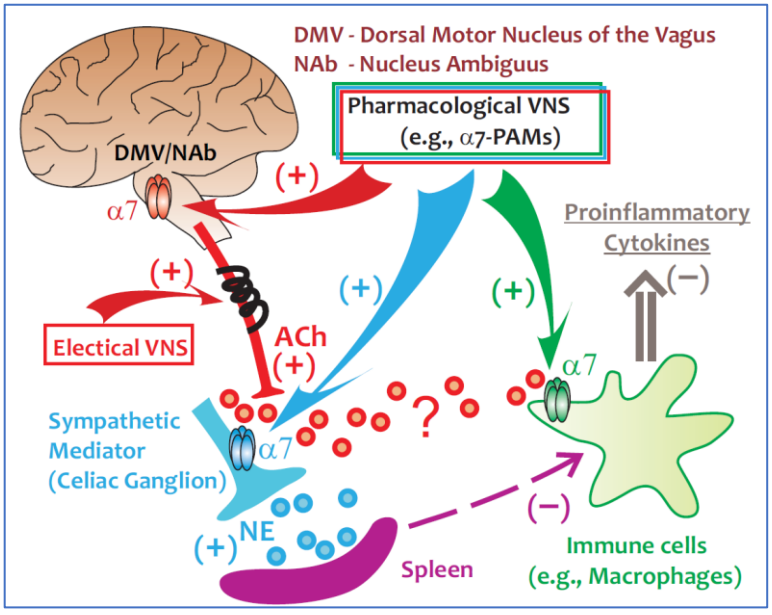

Figure 1.1. Functional α7 nAChRs are broadly expressed both centrally and peripherally.[23] ***(Published with permission from Bentham Science Publishers)***

At least three groups of vagal-related neuroimmune tissues can be identified as potential pharmacological targets for anti-inflammatory therapies after stroke and TBI: 1) *central targets*: vagal premotor (i.e., NTS) and motor (i.e., DMV, NAb) neurons expressing functional α7 nAChRs;[132,133] 2) *peripheral targets*: sympathetic α7-expressing noradrenergic neurons and terminals innervating the spleen;[90,134] and 3) mixed *central-peripheral targets*: α7-expressing immune cells.[85,91] Electrical stimulation of the motor vagus nerve has been proposed as one of the promising anti-inflammatory treatments after TBI.[24] Pharmacological agents (e.g., by α7-PAMs) could be used to modulate vagal nerve activity via multiple mechanisms to optimize its anti-inflammatory efficacy.[13]

1.5 Vagus nerve stimulation.[23] ***(Published with permission from Bentham Science Publishers)***

Despite the current lack of mechanistic insights into how exactly vagal tone translates to peripheral anti-inflammatory efficacy, it is now clear that the α7 subtype of nAChRs plays a critical role in the vagal parasympatho-sympathetic modulation of inflammation as evidenced by the absence of vagal anti-inflammatory efficacy in α7 knock-out mice[84,90] and the ability of splenic nerve stimulation to restore anti-inflammatory efficacy in these mice.[90] Among neuronal nAChRs, the α7 subtype is the most ubiquitous: functional α7 nAChRs are commonly expressed in both neuronal and non-neuronal tissues including glial and immune cells.[71,81,84,135-137] These receptor-channel complexes are highly permeable to Ca^{2+} ions,[138-140] a property that allows α7 nAChRs modulate synaptic neurotransmission, intracellular biochemistry and gene expression.[28,141,142] In fact, Ca^{2+} influx mediated by presynaptic α7 nAChRs is sufficiently strong to trigger synaptic neurotransmitter release in the absence of action potentials and voltage-gated calcium ion

channels[143] (a plausible mechanism that may preserve anti-inflammatory cholinergic efficacy even in the absence of sympathetic inputs to the spleen). Because functional α7 nAChRs are broadly expressed both centrally and peripherally, at least three groups of vagal-related neuroimmune tissues can be identified as potential pharmacological targets for anti-inflammatory therapies after stroke and TBI (Figure 1.1.; from [23]: 1) central targets: vagal premotor (i.e., NTS) and motor (i.e., DMV, NAb) neurons expressing functional α7 nAChRs;[132,133] 2) peripheral targets: sympathetic α7-expressing noradrenergic neurons and terminals innervating the spleen;[90,134] and 3) mixed central-peripheral targets: α7-expressing immune cells.[85,91] Accordingly, treatments that enhance α7 nAChR activation would be expected to enhance α7-dependent anti-inflammatory efficacy. Electrical stimulation of the motor vagus nerve has been proposed as one of the promising anti-inflammatory treatments after stroke[7] and TBI[144]. Accordingly, pharmacological agents (e.g., by α7-PAMs) could be used to modulate vagal nerve activity via multiple mechanisms to optimize its anti-inflammatory efficacy.[6,10,12,145]

Vagus nerve stimulation (VNS) is FDA-approved for treatment of drug-resistant epilepsy (www.accessdata.fda.gov/cdrh_docs/pdf/p970003.pdf; accessed October 29, 2017) and depression (www.accessdata.fda.gov/cdrh_docs/pdf/P970003S050b.pdf; accessed October 29, 2017). In animal studies, VNS has been found to benefit cognitive and motor functions after stroke[7,146,147] and TBI.[148,149] Animal studies have suggested that the beneficial effects of VNS on cognitive and motor recovery after TBI may originate from multiple sources [144] including anti-inflammatory and neuroprotective effects [148,150-152], elevated levels of neuroplasticity-enhancing neurotransmitters in the cortex and hippocampus (e.g., acetylcholine and norepinephrine),[7,146,149,152-158] inhibition of excitotoxicity by normalization of glutamate-GABA imbalance,[152,159,160] prevention of blood-brain-barrier disruption[161] and ghrelin-mediated

neuroprotection.[161,162] Similar mechanisms may be effective after stroke.[6,10,122] These animal studies suggest that electrical stimulation of the vagus nerve alone or in combination with sensorimotor training can potentially improve clinical outcomes after stroke[7,146,147] and TBI.[163] These concepts are likely extendable to patients, however, the effects of electrical VNS in individuals without epilepsy or depression have not been reported because of its invasive nature. Although in clinical practice the parameters of VNS are optimized to preferentially stimulate smaller afferent vagal fibers, the potential for therapeutic efficacy of efferent vagal activity cannot be underestimated because of its systemic anti-inflammatory efficacy associated with inhibition of edema and preservation of brain-blood-barrier integrity after stroke and TBI.[149,151,161,164-167]

In effect, the subdivision of the vagus nerve into afferent and efferent branches and thus, the presence of afferent and efferent VNS components replaces the original puzzle of VNS efficacy with two somewhat independent but no less challenging puzzles. On the one hand, the mechanism underlying restoration of cognitive and sensori-motor functions by afferent VNS are far from being understood because of its intrinsic complexity: afferent VNS translates into a simultaneous stimulation of multiple brain nuclei including the NTS, the noradrenergic locus coeruleus and the cholinergic basal forebrain (see above)[149,168,169] followed by a release of multiple neurotransmitters (e.g., ACh, norepinephrine, serotonin, GABA, glutamate; for a comprehensive review see [170] and [144]) in various higher brain centers (e.g., amygdala,[171] hippocampus[172]). VNS also induces a strong c-fos expression in many brain regions[173] and enhances plasticity in the sensorimotor cortex.[7] On the other hand, the impact of efferent vagal activity on post-injury brain edema, neuroinflammation and blood-brain-barrier integrity remains to be elucidated and so is the contribution of VNS-mediated α7 nAChR activation and its central vs. peripheral components.[84,91,151,167,174-177] Still a third arm to this puzzle has been presented from observations of the central α7-dependent anti-

inflammatory efficacy[79,85] which prompted some researchers to propose the existence of central anti-inflammatory pathways.[77,87] An important unknown here is the relationship between the inflammatory reflex[80] and the anti-inflammatory cholinergic activity[176] as multiple vagus dependent and independent anti-inflammatory pathways may be activated by the same blood-borne pathogens. Although dividing VNS activity into afferent and efferent components is perhaps, a colossal simplification, it is nevertheless meaningful because of the distinct physiological structures (central vs. peripheral) that support the relatively independent action of afferent vs. efferent vagal branches and because such a subdivision highlights the involvement of relatively independent physiological and pharmacological principles: central (α7-independent?) plasticity vs. centrally- and peripherally-originating α7-dependent anti-inflammatory efficacy.

1.6 Cytoprotection by isoflurane anesthesia. Animal models of ischemic brain injury are critical for determining initial *in vivo* efficacies and adverse reactions of novel drugs for ischemic stroke. Anesthesia is an important required component of these models (Animal Welfare Information Center; https://www.nal.usda.gov/; accessed October 29, 2017). However, anesthesia may protect mammalian brain from ischemic injury [178] and anesthetic agents used for surgical anesthesia may confound a reliable evaluation of therapeutic efficacy of novel drugs by unpredictably altering the severity of experimental injury. This concern is evaluated in the present study using a transient 90 min suture middle cerebral artery occlusion (MCAO) model of ischemic stroke in young adult male rats and FDA-approved inhalation anesthetics, isoflurane and nitrous oxide (N_2O). Note that to initiate transient focal ischemia, a standard suture occlusion technique commonly referred to as MCAO is used (see *Methods*). This technique may not specifically target the MCA and thus, vascular territories other than the MCA may also be occluded.[179-181] Nevertheless, the term MCAO

is commonly used in the literature to describe procedures used in this study to model ischemic stroke and adopted here within the limitations referenced above.

Although isoflurane and N_2O have had a long and safe record in medical, dental and experimental anesthesia, both of these inhalation agents are being phased out of medical practice and replaced with superior anesthetic agents and approaches [182-185]. Nevertheless, isoflurane and N_2O are routinely used to induce and maintain anesthesia during animal surgeries in pre-clinical studies. Both isoflurane and N_2O are effective neuroprotectants [178] and may unpredictably interfere with outcomes of neurobehavioral assays leading to data misinterpretation and erroneous conclusions.[186] Both agents have demonstrated neuroprotective efficacy in treatments in experimental models of ischemia.[187-195] Although some ischemic preparations do not require anesthesia,[186,196] highly invasive clinically-relevant models of ischemic stroke (e.g., MCAO) require deep anesthesia.

The duration of MCAO is a key experimental parameter precisely described in scientific reports because it defines the severity of brain injury and the efficacy of neuroprotective mechanisms or treatments.[122] However, the duration of surgical anesthesia is not a reportable parameter and does not directly correlate with the duration of MCAO. In our preliminary experiments that served as a graduate project for one of us (NG), we have detected a high variability in ischemic volume and neurological deficits in assays conducted 24 hrs after a 90 min MCAO. That observation could not be explained by the variability in duration or quality of occlusion because it persisted in the absence of treatments and co-existed with a reliable, stable decrease in cerebral blood flow confirmed by Laser Doppler Flowmetry (LDF). Thus, a confounding effect of anesthetics is suspected. We hypothesize in aim 1 that a variable duration

of isoflurane$\pm N_2O$ anesthesia could translate into variable degrees of ischemic injury and neurological deficits after MCAO.

1.7 Mechanism of isoflurane action. Although the mechanism of action of isoflurane is not fully understood, several lines of research suggest that isoflurane produces its anesthetic effect by modulating central $GABA_A$, glycine and glutamate receptors [197,198]. Isoflurane has also been shown to modulate activity of thalamocortical neurons in a receptor-independent manner [199,200]. Isoflurane enhances the GABAergic function by increasing the probability of opening of $GABA_AR$-mediated channels and thus, enhancing inhibition of both synaptic and extrasynaptic $GABA_ARs$. Isoflurane may also inhibit NMDA and glutamate receptors [197,198]. In thalamocortical neurons essential for relaying sensorimotor modalities and in mediating consciousness [199,200], isoflurane activates certain 'leak' potassium channels resulting in hyperpolarization and shunted voltage-dependent sodium and calcium currents [200].

1.8 Pharmacokinetics of Isoflurane. Under constant-volume ventilation and well-maintained cardiac output, the body uptake of isoflurane rises to maximum at the 2nd or 3rd minute and then, remains stable [199,200]. However, the brain uptake of isoflurane is delayed due to the blood brain barrier because isoflurane is relatively poorly soluble in lipids (the oil/gas coefficient is 98) [199,201]. Isoflurane metabolism is very limited and the primary route of elimination from the body is expiration from lungs [202]: ~95% of inhaled isoflurane is eliminated unchanged by expiration through lungs. Only 0.17% of systemic isoflurane can be detected in urine [203]. Hepatic biotransformation of isoflurane results in defluorination by cytochrome P450 2E1. Due to very limited hepatic metabolism of isoflurane, biotoxicity due to fluoride metabolites is minimal [204]. The elimination process is divided in to three phases. The first phase of elimination is through the lungs via the alveolar space and the half-time of elimination is less than 4 minutes. The second

phase of elimination is in well-perfused organs like brain, liver, heart and kidneys with the half-time of elimination of about 12 – 27 minutes. The third phase of elimination is through muscles and adipose tissue, which has the half time of elimination of about 2 – 6 hours [205]. Additionally, very small quantity of absorbed isoflurane (<0.5%) is also eliminated via skin [206].

1.9 Specific Aims. The Stroke Treatment Academia Industry Roundtable (STAIR) criteria[207,208] give guidelines for the rigorous objective evaluation of new chemical agents in pre-clinical models before translation to the clinic. Accordingly, the goal of this project is dual: 1) to increase the rigor and reproducibility of our preclinical studies by identifying and reducing sources of data variability in a transient suture middle cerebral artery occlusion (MCAO) model of ischemic stroke in young adult rats (Aim 1); and 2) to apply the optimized MCAO model developed in aim 1 to fully randomized blinded studies to evaluate the efficacy of prototypical and novel positive allosteric modulators (Aim 2) of α7 nicotinic acetylcholine receptors (nAChRs) as candidates for development for the treatment of stroke, a high unmet need.

Aim 1: To increase the rigor and reproducibility of pre-clinical ischemic stroke studies by reducing data variability and increasing research transparency. A transient suture middle cerebral artery occlusion (MCAO) model of ischemic stroke is used in randomized blinded experiments in young adult rats. We hypothesize that isoflurane anesthesia duration and room temperature contribute to data variability. These sources of data variability will be identified and/or reduced to enhance the rigor and reproducibility of stroke research.

Aim 2: To determine the efficacy of prototypical and novel selective α7 PAMs after MCAO in young adult rats. Activation of α7 nAChRs by endogenous α7 agonists (i.e., choline and ACh) increases brain resistance to ischemia and can be augmented by prototypical α7 PAMs.[38,51,122].

However, the existing studies have not tested the efficacy of delayed (>4 hrs after ischemia) multiple (daily) sub-chronic (72 hrs) treatments with α7 PAMs. We hypothesize that delayed sub-chronic treatments with prototypical and novel α7 PAMs will reduce brain injury and neurological deficits after ischemic stroke in a challenging experimental paradigm characterized by delayed (4 hrs after MCAO) multiple (daily) sub-chronic (72 hrs) treatments in the MCAO model optimized in Aim 1.

1.10 Significance and Rationale. Stroke is a leading cause of disability and death in the United States [209,210]. Substantial efforts invested in developing anti-ischemic medicine have not produced clinically-efficacious therapies1 and clinical management of brain injury resulting from ischemic stroke generally involves only palliative treatments. These failures highlight the need for development of new therapeutic approaches to prevention of ischemic brain injury. While exogenous α7 agonists are valuable assets in treatment of neurological deficits [14,18,111,114] therapeutic interventions that could augment capacity of the brain to protect itself from ischemic damage present an intriguing and potentially powerful therapeutic alternative [11,211] and have not been thoroughly explored. The existing literature [14,18,111,112,114] supports the high therapeutic potential of α7 PAMs and shows that PAM-based treatments can rescue neurons after focal cerebral ischemia by selectively augmenting α7 nAChR activation[38,51,122] in *in vivo* and *in vitro* models of cerebral ischemic stroke. PAMs only amplify the α7-dependent endogenous cholinergic tone in a spatially and temporally restricted manner [212] creating a potential for differential efficacy and improved safety as compared to α7 agonists that activate α7 nAChRs indiscriminately[6,10,12]. Thus, α7 PAM-based treatments in cerebral ischemia may enhance injury- and/or inflammation-activated auto-therapies by converting endogenous cholinergic tone into potent therapeutic action where/when it is most needed (i.e., in the ischemic penumbra[15] and/or along the cholinergic anti-

inflammatory pathway[6]). The anticipated clinical utility of α7 PAMs is likely to extend beyond stroke to certain neurodegenerative and psychiatric disorders linked to cognitive decline and attention deficits (e.g., dementias, schizophrenia) because these conditions are associated with decreased cholinergic tone and a decrease, but not disappearance, of functional α7 nAChRs [213]. Thus, besides cytoprotective anti-inflammatory effects, α7 PAMs are expected to improve cognitive function and attention by increasing cholinergic tone via potentiating α7 activity [49,54,56,61]. In this regard, α7 PAM-based treatments are expected to benefit stroke patients via multiple mechanisms and routes of action. This project further explores the therapeutic promise of α7-dependent cytoprotective anti-inflammatory therapy. The endogenous cytoprotective anti-inflammatory efficacy may act as an important physiological function of these ubiquitous receptors and may hold significant translational potential.

Clinical management of ischemic brain injury involves only palliative treatments and effective drug therapies are unavailable. While endogenous α7-dependent protection of brain tissues is evident, its efficacy may be limited to less severe injuries [214]. Our data suggest that the efficacy of endogenous α7-dependent protection can be significantly enhanced by PAMs that inhibit α7 desensitization and enhance α7 activation by agonists such choline and ACh [12,38,51,59,124,125]. Currently, PAMs are not in clinical trials for any indication (see www.clinicaltrials.gov). Thus, this novel and substantively different approach to managing brain injury and neurological deficits after stroke holds significant promise and presents an exciting therapeutic opportunity in drug discovery/development and may enable health care providers to overcome current limitations associated with the lack of effective treatments after cerebral stroke.

1.11 Innovation (Aim 1). Neuroprotective effects of isoflurane have been extensively investigated and reported previously[185,187,189,191,192]. Nevertheless, isoflurane is routinely used for induction and

maintenance of surgical anesthesia in preclinical stroke studies. It is common however in stroke studies to not monitor and report anesthesia durations. This practice contradicts the STAIR guidelines aimed at elevation of transparency, rigor and reproducibility of stroke research. The studies pertinent to aim 1 are innovative in that we used a common tMCAO model of ischemic stroke to demonstrate that a typical range of anesthesia durations required for this model and related experimental or surgical procedures (e.g., Laser Doppler flowmetry) acts as a significant source of data variability evidenced by measurements of infarct volume and neurological deficits. These studies thus conclude that variability in anesthesia durations across experimental groups should be monitored, minimized, standardized and become a required reportable parameter in studies utilizing tMCAO models of stroke.

Innovation (Aim 2). Selective α7 PAMs offer the opportunity for a multi-faceted therapy that affords persistent direct cytoprotection and management of central and peripheral anti-inflammatory pathways (Figure 1.1.). This concept is novel, unconventional and substantively different from previous strategies with α7 agonists (e.g., 4OH-GTS-21, EVP-6124, ABT-126). PAMs are not in clinical trials (www.clinicaltrials.gov) for stroke and may provide health care providers with a new option to overcome limitations of available therapies.

Chapter 2

Duration of isoflurane-based surgical anesthesia determines severity of brain injury and neurological deficits after a transient focal ischemia in young adult rats

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NG: study design, data collection, analysis, interpretation; **FS**: data collection, analysis, discussion; **DS**: data collection, analysis, discussion; **VVU**: study design, data analysis, interpretation, figures, writing

2.1. ABSTRACT

Tremendous efforts and funds invested in discovery of novel drug treatments for ischemic stroke have so far failed to deliver clinically efficacious therapies. The reasons for these failures are not fully understood. An indiscriminate use of isoflurane-based surgical anesthesia with or without nitrous oxide may act as an unconstrained, untraceable source of data variability, potentially causing false-positive or false-negative results. To test this hypothesis, a common transient suture middle cerebral artery occlusion (tMCAO) model of ischemic stroke in young adult male rats was used to determine the impact of a typical range of anesthesia durations required for this model on data variability (i.e., infarct volume and neurological deficits). The animals were maintained on spontaneous ventilation. The study results indicated that: 1) Variable duration of isoflurane anesthesia prior, during and after tMCAO is a significant source of data variability as evidenced by measurements of infarct volume and neurological deficits; and 2) Severity of brain injury and neurological deficits after tMCAO is inversely related to the duration of isoflurane anesthesia: e.g., in our study, a 90 min isoflurane anesthesia nearly completely protected brain tissues from tMCAO-induced injury and thus, would be expected to obscure the effects of stroke treatments in pre-clinical trials. To elevate transparency, rigor and reproducibility of stroke research and minimize undesirable effects of isoflurane on the outcome of novel drug testing, we propose to monitor, minimize and standardize isoflurane anesthesia and make anesthesia duration a required reportable parameter in pre-clinical studies. We propose to adopt 20 min as an optimal anesthesia duration that minimizes neuroprotective effects of isoflurane and permits a successful completion of tMCAO surgical procedures in rodents. As the mechanisms and neuroprotective, metabolic and immune effects of general anesthesia are not understood, the results of this study cannot be generalized to other anesthetics, species and experimental models.

2.2. INTRODUCTION

Animal models of ischemic brain injury are critical for determining initial *in vivo* efficacies and adverse reactions of novel drugs for ischemic stroke. Anesthesia is an important required component of these models (Animal Welfare Information Center; https://www.nal.usda.gov/; accessed October 1, 2016). However, anesthesia may protect mammalian brain from ischemic injury [184] and anesthetic agents used for surgical anesthesia may confound a reliable evaluation of therapeutic efficacy of novel drugs by unpredictably altering the severity of experimental injury [178]. This concern is evaluated in the present study using a transient 90 min suture middle cerebral artery occlusion (tMCAO) model of ischemic stroke in young adult male rats and FDA-approved inhalation anesthetics, isoflurane and nitrous oxide (N2O). Note that to initiate transient focal ischemia, a standard suture occlusion technique commonly referred to as tMCAO is used (see Methods). This technique may not specifically target the MCA and thus, vascular territories other than the MCA may also be occluded [179,180,183]. Nevertheless, the term tMCAO is commonly used in the literature to describe procedures used in this study to model ischemic stroke and adopted here within the limitations referenced above.

Although isoflurane and N2O have had a long and safe record in medical, dental and experimental anesthesia, both inhalation agents are being phased out of medical practice and replaced with superior anesthetic agents and approaches [178,190,214,215]. Nevertheless, isoflurane and N2O are routinely used to induce and maintain anesthesia during animal surgeries in pre-clinical studies. Both isoflurane and N2O are effective neuroprotectants [184] and may unpredictably interfere with outcomes of neurobehavioral assays leading to data misinterpretation and erroneous conclusions[217]. Both agents have demonstrated neuroprotective efficacy in treatments in experimental models of ischemia [185,187-189,191-195]. Although some ischemic preparations do not

require anesthesia[217,235], highly invasive clinically-relevant models of ischemic stroke (e.g., tMCAO) require deep anesthesia.

The tMCAO model is common in studies of ischemic stroke [72,188]. The duration of tMCAO is a key experimental parameter precisely described in scientific reports because it defines the severity of brain injury and the efficacy of neuroprotective mechanisms or treatments [181]. However, the duration of surgical anesthesia is not a reportable parameter and does not directly correlate with the duration of tMCAO. The practice to not monitor and report anesthesia durations across experimental groups contradicts the Stroke Treatment Academic Industry Roundtable (STAIR) recommendations aimed at elevation of transparency, rigor and reproducibility of stroke research [208]. In our preliminary experiments that served as a graduate project for one of us (NG), we have detected a high variability in ischemic volume and neurological deficits in assays conducted 24 h after a 90 min tMCAO. That observation could not be explained by the variability in duration or quality of occlusion because it persisted in the absence of treatments and co-existed with a reliable, stable decrease in cerebral blood flow confirmed by Laser Doppler Flowmetry (LDF). Thus, a confounding effect of anesthetics was suspected. Our hypothesis was that a variable duration of isoflurane + N2O anesthesia could translate into variable degrees of ischemic injury and neurological deficits after tMCAO.

In this study, we used a common tMCAO model of ischemic stroke to demonstrate that a typical range of anesthesia durations required for this model and related experimental or surgical procedures (e.g., Laser Doppler flowmetry) acts as a significant source of data variability, potentially causing false-positive or false-negative results in preclinical trials and interfering with development of novel stroke therapies. To minimize the undesirable impact of isoflurane on the outcome of novel drug testing, we propose to standardize anesthesia procedures whenever possible

and make anesthesia duration a required reportable parameter in relevant pre-clinical studies of stroke. Based on the presented data, when practically appropriate, we propose to adopt 20–30 min as an optimal anesthesia duration in rats that both minimizes adverse neuroprotection by isoflurane and permits a successful completion of tMCAO.

2.3. MATERIAL AND METHODS

Animals were randomly assigned to groups. Analysis of infarct volumes was blind. Behavioral assays were conducted by the same researcher who conducted MCAO surgeries.

2.3.1. Animals

Young adult male Sprague Dawley (SD) rats (∼280 g) were purchased from Charles River (Wilmington, MA, USA) and used in accordance with the Guide for the Care and Use of Laboratory Animals (NIH 865-23, Bethesda, MD, USA). All experimental protocols were approved by the UNTHSC Institutional Animal Care and Use Committee. All animal protocols comply with the ARRIVE guidelines.

2.3.2. Anesthesia

Isoflurane was used to induce and maintain surgical anesthesia for a defined duration during a 90-min suture tMCAO. Rectal temperature was maintained at∼37 °C using a heating pad. The animals were maintained on spontaneous ventilation because intubation and mechanical ventilation introduce variable delays to both MCAO and reperfusion surgeries while advantages are obscure [216], especially for short (e.g., 20–40 min) surgeries, as cardiorespiratory reflexes are bypassed while hyperventilation may result in hypocapnia-induced vasoconstriction. Physiological parameters (e.g., blood pressure, PCO2) were not monitored due to the absence of reliable non-invasive measuring tools for anesthetized rodents. These parameters were not

controlled to avoid potential sources of data variability as pharmacological interventions (e.g., phenylephrine as a blood pressure stabilizing agent) may interfere with novel drug testing defeating the purpose of this study which is to increase data reproducibility and eliminate sources of data variability in drug discovery/development. Because the intragroup variability of infarct volumes and neurological deficits was smaller than differences across groups, the possible intragroup spontaneous deviations in respiratory rates, blood pressure and other physiological parameters did not significantly impact stroke outcomes as long as anesthesia duration stayed constant. Isoflurane (4% induction; 1.8% maintenance) was purchased from Henry Schein Animal Health (Dublin, OH) and delivered by a mask as a gaseous mixture with one of the following agents: 1) 100% O2; 2) 70% N2O +30% O2; or 3) 100% Air. In experiments where regional cerebral blood flow (rCBF) was measured, anesthesia duration was variable because the time required for rCBF measurements was variable across animals. However, anesthesia duration was precisely measured in each of these experiments. To better quantify the relationship between anesthesia duration and outcomes of tMCAO, a separate set of experiments was conducted where anesthesia was initiated for the first 20, 40 or 90 min of a 90 min tMCAO. To initiate re-perfusion at the end of tMCAO, the animals were sedated again for 5 min using the same anesthetic mixture and parameters.

2.3.3. Regional cerebral blood flow (rCBF) measurements

The rCBF measurements were conducted to confirm the timing and stability of tMCAO and should not be viewed as quantitative measures of the degree of tMCAO. For rCBF measurements, rats were anesthetized with isoflurane (4% induction; 1.8% maintenance) +O2 (100%), delivered by a mask. A skin incision (~1 cm long) was made in the central area of the shaved skull, and a probe holder with a Laser-Doppler Flowmeter (LDF) probe (Periflux system 5000; Perimed,

Stockholm, Sweden) was attached to the skull (1 mm posterior to Bregma and 5 mm lateral to Midline) in the left hemisphere ipsilateral to tMCAO using superglue. The rCBF was recorded before and during common carotid artery occlusion (CCAO) and then, during and after tMCAO as a percent of the mean baseline value recorded over the last 5 min before CCAO. A successful MCAO was defined as an abrupt reduction in rCBF by >70% followed by a recovery to the flow level corresponding to CCAO (Figure 2.1).

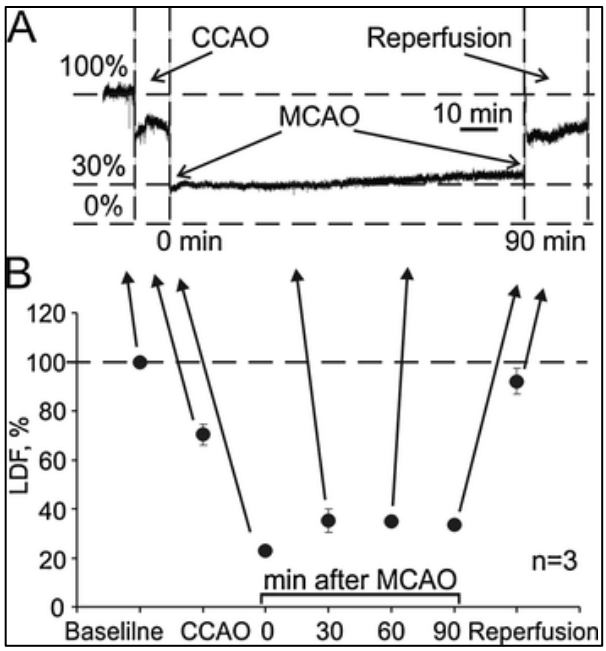

Figure. 2.1. rCBF measurements. A typical example of LDF recording from an individual rat is shown in the top panel. The averaged LDF profile from n = 3 rats is shown in the bottom panel. In these experiments, the rCBF was continuously recorded and evaluated using a LDF before and during occlusion of the CCAO and then, during and after tMCAO as a percent of the mean baseline value recorded over the last 5 min before CCAO (i.e., 100%). A successful tMCAO was defined as an abrupt sustained reduction in rCBF by >70% followed by a recovery to the flow level corresponding to CCAO. rCBF measurements were done under continuous anesthesia.

2.3.4. Transient middle cerebral artery occlusion (tMCAO)

Our approach was to conduct a tMCAO within a narrow window of experimental parameters to ensure stable ischemic insult and injury [181]. After the effective experimental parameters were established and confirmed by rCBF measurements (Figure. 2.1.), these parameters were kept constant in subsequent experiments supported by intermittent CBF measurements. Specifically, we used only 285 + 5 g SD rats purchased from Charles River and accommodated in our animal

facility for 5–7 days after arrival. To initiate tMCAO, 4-0 monofilament nylon suture having a coating of silicon rubber on tip (diameter with coating 0.39+/- 0.02 mm; coating length 4-5 mm) (Doccol Corporation, Sharon, MA, USA) was used to advance from bifurcation of carotid (i.e., 19-mm). These restrictions ensured stability and reproducibility of experimental parameters across groups as confirmed by rCBF measurements. Animals were anesthetized with an isoflurane-based mixture (see *Anesthesia*), delivered by a mask. After a midline incision in the neck, the left common carotid artery (CCA) was exposed, dissected and permanently ligated. A 4-0 monofilament nylon suture (19-mm) was inserted from the CCA into the left internal carotid artery to occlude the origin of the left MCA. Rats were allowed to regain consciousness in a warmed recovery cage for the rest of MCAO. After 90 min of MCAO, the thread was removed for re-perfusion. The CCA was permanently ligated, and the wound was closed. Rectal temperature was maintained at∼37 °C using a heating pad. A total of 63 animals were used. Of these, 5 animals (i.e., 7.9%) did not survive the procedures which is reflected by differences in the sample size across groups. Animals were randomly assigned to groups prior to group labeling. All data were reported.

2.3.5. Measurements

2.3.5. 1 Infarct Volume measurement

Immediately prior to euthanasia by decapitation 24 h after tMCAO, animals were anesthetized for <1 min with the same anesthetic mixture used for surgical anesthesia. The brains were removed. Coronal sections (2-mm thickness) immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline for 20 min at 37 °C then, fixed for 2 h in 4% paraformaldehyde. The infarct and contralateral brain section areas were measured using ImageJ. 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 (NIIA) divided by the TCA: i.e., IA = 100% *x* (TCA–NIIA)/TCA.

2.3.5. 2 Neurobehavioral testing

Neurobehavioral tests were done 15 min prior to euthanasia. The order of testing (Bederson → cylinder) was always the same for data consistency.

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. A mirror behind the cylinder allowed observing forelimb movements when the rat faced away from the researcher. 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. Stroked 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% x (RL + ½ 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.

2.3.6. Statistical analysis

2.3.6. 1 Anesthesia during and after tMCAO (i.e., post-conditioning)

The sample size n = 6 yields 1-β > 0.8 (α=0.05) and was estimated from a power analysis of extrapolated preliminary (Figure 2.2) and published [181] data. This sample size was then used in all experiments except for tests with pre-conditioning (Figure. 2.5; see the Section 3.6.2 below). However, a larger samples size (n = 7-10) was accumulated in experiments pertaining to Figure. 2.2 (n = 9) and Figure. 2.3 (n = 9-10). These additional data points were collected during preliminary tests related to rCBF measurements (Figure. 2.2) and establishing experimental procedures where neurological deficits were not tested in 11 rats (Figure.2.3). In addition, in the analysis of infarct volume, the group sample size had to be increased to at least 7 to enable tests for data normality and homogeneity of variances. Two animals in the 90-min anesthesia group did not survive MCAO in experiments with isoflurane + N2O (Figure. 2.3) resulting in the reduced sample size (n = 8) in the corresponding group. Two other animals (one per group in the 20 min and 90 min anesthesia groups) did not survive MCAO in experiments with isoflurane + air (Figure. 2.4). In one experiment pertaining to 40 min anesthesia, infarct volume (77%) was identified as an outlier (studentized residual = 5.32; data-mean >6.7 standard deviations). The same animal also demonstrated uncharacteristically elevated neurological deficits as compared to the rest of the group (Bedersons' score: 3; cylinder's % use: 15). The reason for these deviations was unclear and this outlier was excluded from the analysis. The results are presented as mean ± S.E.M. Statistical significance of continuous data (i.e., ischemic infarct volumes) was determined by a one-way-ANOVA followed by either the Tukey's (for groups with equal variances) or the Dunnett's T3 (for groups with unequal variances) post-test multiple comparisons tests. The infarct volume data were tested for normality (Anderson-Darling test) and homogeneity of variances (Levene's test). These

tests have confirmed the Gaussian distribution of infarct volumes in all experiments (Anderson-Darling test, $p > 0.05$). However, the test for homogeneity of variances supported equal variances across the infarct volume groups only in experiments utilizing isoflurane + N2O (Levene's test, $p > 0.05$). In experiments utilizing isoflurane + air, the infarct volumes were sampled from apparently different populations characterized by significantly different variances (Levene's test, $p < 0.01$). Thus, in experiments utilizing anesthesia + air, a one-way ANOVA with the Dunnett's T3 post-test multiple comparison test was used applicable to data with unequal variances. By contrast, data based on categorical scoring systems (i.e., Bederson and cylinder tests) were analyzed using a non-parametric Kruskal-Wallis test with the Dunn's post-test multiple comparison test. Statistical analysis was done using SyStat 13 (Systat Software, Inc., San Jose, CA) and Prism 6 (GraphPad Software Inc., La Jolla, CA). Analysis of linear regression was done using Mathematica-2.2.3 (Wolfram-Research, Champaign, IL). The t-/p-values were calculated from the coefficient of determination, as published[185]. Statistical significance was defined by p-values (* ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001).

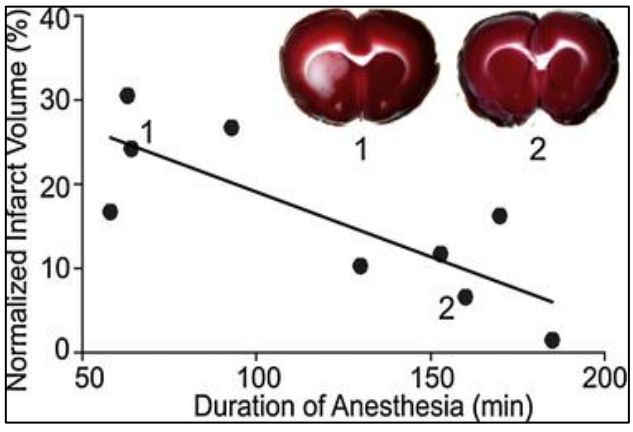

Figure.2.2. Ischemic infarct volume inversely correlates with duration of isoflurane + O2 surgical anesthesia during continuous rCBF measurements. In preliminary experiments, rCBF measurements inadvertently resulted in variable anesthesia durations because the time required for rCBF measurements was variable across animals. Analysis of data points from nine animals (including three animals used for Figure. 2.1) were analyzed using a linear regression analysis. These data revealed a significant inverse correlation between infarct volume and anesthesia duration as a significant trend ($y = -0.15x + 34.34$) was detected: $R^2 = 0.6436$; $p \leq$

0.01, t = 3.555, DF = 8. Given the confirmed drop in the rCBF in our experiments (Figure. 2.1), these data suggested that anesthesia duration inversely correlates with severity of ischemic brain injury after a 90 min tMCAO. The delay between the onset of anesthesia and MCAO was always 12–15 min. Infarct volume was measured 24 h after tMCAO using a TTC staining method. Animals were anesthetized with isoflurane (4% induction; 1.8% maintenance) +100% O2. For definition of infarct volume measurements and % use in cylinder tests see Section 2.3.5 in Methods. Note that examples of individual brain sections labeled **1** and **2** in the insert deviate from the mean infarct volume data points each averaged over 5 sections obtained from the same rats.

2.3.6. 2 Anesthesia before and during tMCAO (i.e., pre-conditioning).

Ten randomly selected animals were used in experiments with pre-conditioning (Figure.2.5). The sample size n = 5 was estimated from the power analysis to yield $1-\beta>0.8$ ($\alpha=0.05$). However, one control animal did not survive the first 24 h after tMCAO surgery and thus, an additional (eleventh) animal was used to maintain the sufficient power and match the group sizes. A non-parametric two-tailed unpaired Mann-Whitney U test was used in all statistical assays because the data exhibited homogeneous variance but were not confirmed to be normally distributed. Statistical significance was defined by p-values (*≤0.05; **≤0.01; ***≤0.001; ****≤0.0001).

2.4. RESULTS

In preliminary experiments, a Laser Doppler Flowmetry (LDF) was used to measure regional cerebral blood flow (rCBF) during isoflurane anesthesia before, during and after a 90 min tMCAO. In these experiments, anesthesia was induced and maintained by a mixture of isoflurane (4% induction; 1.8% maintenance) and O2 (100%). A successful tMCAO was defined as an abrupt reduction in the rCBF by >70% followed by a recovery to the flow level corresponding to CCAO (Figure. 2.1) (see Methods). The use of LDF caused an unavoidable variability in anesthesia

duration because the time required for rCBF measurements was variable across animals. Infarct volume was analyzed 24 h after tMCAO. These experiments illustrate a realistic challenge where experimental procedure with variable durations (i.e., rCBF measurement) result in matching variable durations of anesthesia causing variable infarct volumes. When data points from nine animals (including three animals used for Figure. 2.1) were analyzed using a linear regression analysis, a significant trend ($y = -0.15x + 34.34$) was detected (Figure. 2.2): $R^2 = 0.6436$; $p \leq 0.01$, $t = 3.555$, $DF = 8$. Given the confirmed drop in the rCBF in our experiments (Figure. 2.1), these data suggested that anesthesia duration inversely correlates with severity of ischemic brain injury after a 90 min tMCAO. In these experiments the delay between the onset of anesthesia and MCAO was always 12–15 min. One animal did not survive the surgery with isoflurane + O2 anesthesia.

To further quantify this relationship and determine the optimal anesthesia duration, young adult SD male rats were randomly assigned to three groups and subjected to a 90 min tMCAO with anesthesia duration in each group limited to 20, 40 or 90 min, respectively. The animals were evaluated for neurological deficits 24 h later immediately prior to euthanasia and collection of brain tissues for evaluation of infarct volumes. Two sets of identical experiments were conducted using isoflurane (4% induction; 1.8% maintenance) mixed with two commonly used vehicles: (1) 70% N2O +30% O2 (Figure. 2.3); or (2) 100% Air (Figure. 2.4). The results of both studies were similar and demonstrated that infarct volume (Figures. 2.3A, B and 2.4A, B) and neurological deficits evaluated in Bederson and cylinder tests (Figure. 2.4C-D) inversely correlate with anesthesia duration regardless of the exact anesthetic mixture used.

In experiments utilizing isoflurane + N2O, the infarct volumes were normally distributed with equal variances (see *Methods*) thus, one-way ANOVA was used for statistical analysis. The analysis demonstrated that increasing anesthesia duration from 20 min (n = 10) to 40 min (n = 9)

and 90 min (n = 8) significantly reduced infarct volume (one-way ANOVA, Tukey's multiple comparisons test; $F(2,24) = 75.42$, $p \leq 0.0001$; Figure. 2.3A, B) and neurological deficits (Kruskal-Wallis, Dunn's multiple comparisons test; Figure. 2.3C, D) in Bederson ($H = 13.85$, $p \leq 0.0001$; Figure. 2.3C) and cylinder ($H = 13.17$, $p \leq 0.0001$; Figure. 2.3D) tests. The results of the corresponding post-hoc multiple comparisons tests are summarized in Table 2.1. These results support significant inverse relationship between the duration of isoflurane + N2O surgical anesthesia and the severity of ischemic brain injury and neurological deficits after tMCAO. In these tests, the analysis of infarct volume included eleven animals from the initial set of experiments where neurological deficits were not tested. Two animals did not survive the surgery with isoflurane + N2O anesthesia.

Table 2.1: Dependence of infarct volume and neurological deficits on isoflurane + N2O anesthesia duration

	20 Min	**40 Min**	**90 Min**
Infarct Volume (%)	60.00 ± 3.75###	31.73 ± 5.50****	9.26 ± 2.10****
Bederson Test Score	3.00 ± 0.00	1.83 ± 0.31	0.17 ± 0.18***
Cylinder Test (% use of injured limb)	22.20 ± 1.56	35.00 ± 4.26	53.00 ± 2.19***

Increasing anesthesia duration from 20 min (n = 10) to 40 min (n = 9) and 90 min (n = 8) significantly reduces infarct volume (one-way ANOVA, Tukey's multiple comparisons test; $F(2,24) = 75.42$, $p \leq 0.0001$; Figure. 2.3A, B) and neurological deficits (Kruskal-Wallis, Dunn's multiple comparisons test; Figure. 2.3C, D) in Bederson ($H = 13.85$, $p \leq 0.0001$; Figure. 2.3C) and cylinder ($H = 13.17$, $p \leq 0.0001$; Figure. 2.3D) tests. The corresponding post-hoc multiple comparisons tests detected the following levels of significance for: infarct volume [$p \leq 0.0001$, 20

min vs. 40 min; $p \leq 0.0001$, 20 min vs. 90 min; and $p \leq 0.001$, 40 min vs. 90 min]; Bederson [$p > 0.05$, 20 min vs. 40 min; $p < 0.001$, 20 min vs. 90 min; and $p > 0.05$, 40 min vs. 90 min] and cylinder [$p > 0.05$, 20 min vs. 40 min; $p \leq 0.001$, 20 min vs. 90 min; and $p > 0.05$, 40 min vs. 90 min] tests. Statistical significance was defined by p-values (###≤0.001; ***≤0.001; ****≤0.0001). Symbols *) and #) are referred to comparison with 20 and 40 min, respectively.

Table 2.2: Dependence of infarct volume and neurological deficits on isoflurane + air anesthesia duration.

	20 Min	**40 Min**	**90 Min**
Infarct Volume (%)	53.78 + 7.65##	24.48 + 1.42**	9.11 + 4.06****
Bederson Test Score	3.00 + 0.00	1.71 + 0.20	0.29 + 0.22****
Cylinder Test (% use of injured limb)	18.71 + 3.01	36.29 + 1.88	53 + 2.28****

Increasing anesthesia duration from 20 min (n=7) to 40 min (n=7) and 90 min (n=7) significantly reduced infarct volume (one-way ANOVA, Dunnett's T3 test; $F(2,18)=27.90$, $p \leq 0.0001$; Figure. 2.4A,B) and neurological deficits (Kruskal-Wallis, Dunn's multiple comparisons test; Figure.2.4C,D) in Bederson ($H=18.39$, $p \leq 0.0001$; Figure.2.4C) and cylinder ($H=17.84$, $p \leq 0.001$; Figure.2.4D) tests. The corresponding post-hoc multiple comparisons tests detected the following levels of significance for: infarct volume [$p \leq 0.01$, 20 min vs. 40 min; $p \leq 0.0001$, 20 min vs. 90 min; and $p \leq 0.01$, 40 min vs. 90 min]; Bederson [$p > 0.05$, 20 min vs. 40 min; $p \leq 0.0001$, 20 min vs. 90 min; and $p > 0.05$, 40 min vs. 90 min] and cylinder [$p > 0.05$, 20 min vs. 40 min; $p \leq 0.0001$, 20 min vs. 90 min; and $p > 0.05$, 40 min vs. 90 min] tests. Statistical significance was defined by p-values (##≤0.01; **≤0.01; ****≤0.0001). Symbols *) and #) are referred to comparison with 20 and 40 min, respectively.

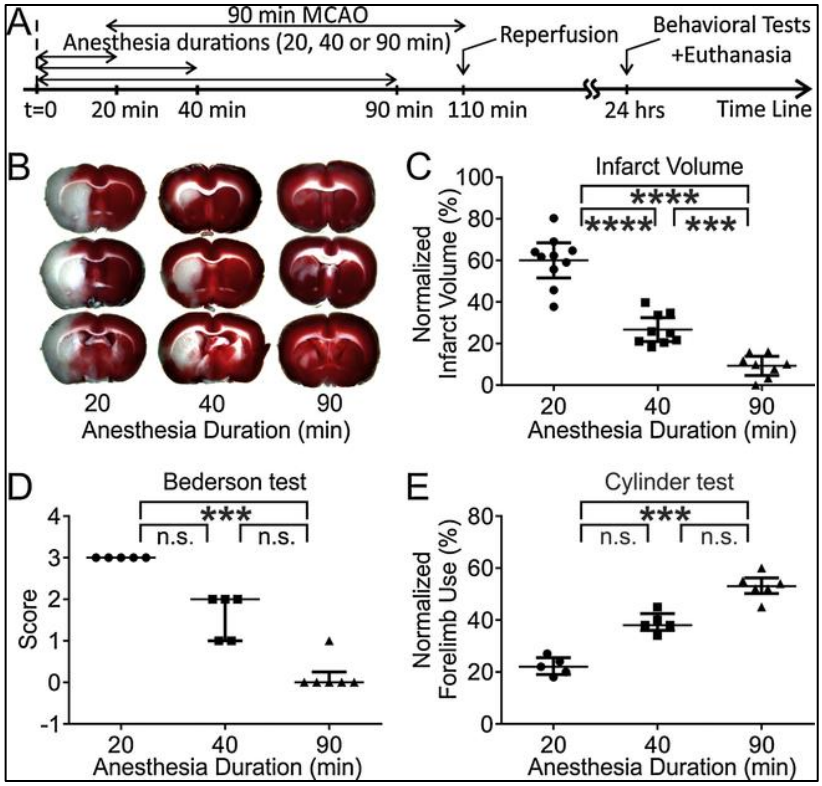

Figure. 2.3. Duration of isoflurane + N2O surgical anesthesia defines severity of ischemic injury and neurological deficits after a 90 min tMCAO: A variable exposure of animals to isoflurane + N2O anesthesia acts as a significant source of variability in infarct volume and neurological deficits. (A) A schematic representation of experimental timeline. (B) Representative images of ischemic infarcts detected 24 h after tMCAO across groups of animals anesthetized for various durations: 20 min (left), 40 min (middle) and 90 min (right). (C) Dependence of infarct volume on anesthesia duration. (D, E) Results of Bederson (D) and cylinder (E) tests as a function of anesthesia duration. Ischemic injury and neurological deficits inversely correlate with anesthesia duration. The data in panel B are illustrated as the mean with 95% confidence interval (CI). The data in panels (D, E) are illustrated as the median with interquartile range. Animals were anesthetized with isoflurane (4% induction; 1.8% maintenance) +70% N2O +30% O2. For definition of infarct volume and neurological measurements and % use in cylinder tests see Section 2.3.5 in Methods.

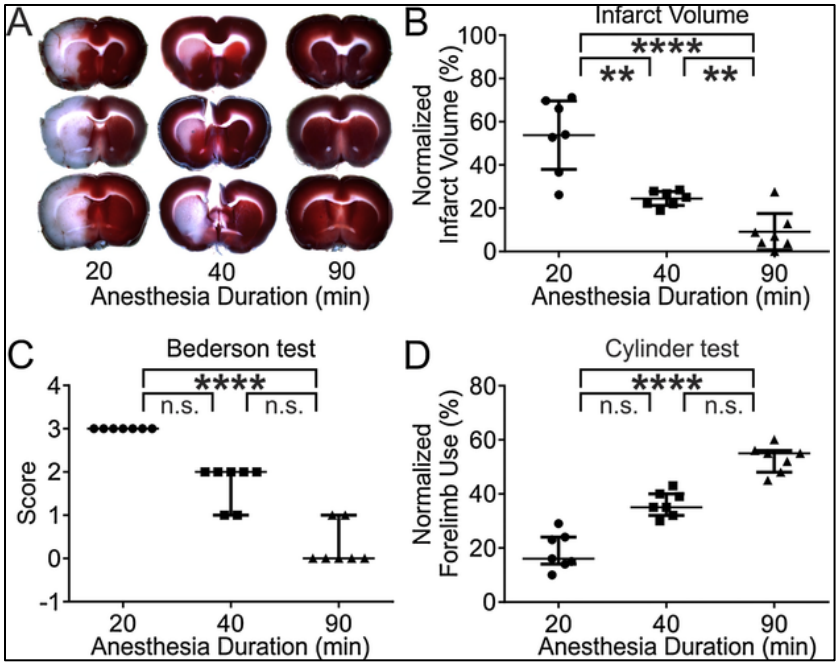

Figure. 2.4. Duration of isoflurane + air surgical anesthesia defines severity of ischemic injury and neurological deficits after a 90 min tMCAO: A variable exposure of animals to isoflurane + air anesthesia acts as a significant source of variability in infract volume and neurological deficits. Experiments with isoflurane + N2O and isoflurane + air anesthesia were conducted using the same timeline (see Figure. 3A). (A) Representative images of ischemic infarcts detected 24 h after tMCAO across groups of animals anesthetized for various durations: 20 min (left), 40 min (middle) and 90 min (right). (B) Dependence of infarct volume on anesthesia duration. (C, D) Results of Bederson (C) and cylinder (D) tests as a function of anesthesia duration. Ischemic injury and neurological deficits inversely correlate with anesthesia duration. The data in panel B are illustrated as the mean with 95% confidence interval (CI). The data in panels C, D are illustrated as the median with interquartile range. Animals were anesthetized with isoflurane (4% induction; 1.8% maintenance) +100% air. For definition of infarct volume measurements and % use in cylinder tests see Section 2.3.5 in Methods

In experiments utilizing isoflurane + air (n = 7), the infarct volumes although normally distributed were sampled from apparently different populations characterized by significantly

different variances (see *Methods*) supporting significant effects of anesthesia duration on the infarct volume variance. When the infarct volume means were evaluated using a one-way ANOVA with the Dunnett's T3 multiple comparison test for unequal variances, increasing anesthesia duration from 20 min to 40 min and 90 min significantly reduced infarct volume (one-way ANOVA, Dunnett's T3 test; $F(2,18) = 27.90$, $p \leq 0.0001$; Figure. 2.4A,B) and neurological deficits (Kruskal-Wallis, Dunn's multiple comparisons test; Figure. 2.4C,D) in Bederson ($H = 18.39$, $p \leq 0.0001$; Figure. 2.4C) and cylinder ($H = 17.84$, $p \leq 0.001$; Figure. 2.4D) tests. The results of the corresponding post-hoc multiple comparisons tests are summarized in Table 2.2. These results support significant inverse relationship between the duration of isoflurane + air surgical anesthesia and the severity of ischemic brain injury and neurological deficits after tMCAO. Two animals did not survive the surgery with isoflurane + air anesthesia. Figures. 2.3 and 2.4 illustrate an intriguing trend: in experiments utilizing 20 min isoflurane anesthesia, severe cortical and sub-cortical brain injuries were observed in all animals (i.e., 10 anesthetized with isoflurane + N2O and 7 anesthetized with isoflurane + air; Figures. 2.3A and 2.4A, left most columns), while most animals subjected to 40 min isoflurane anesthesia (i.e., 8 out of 9 anesthetized with isoflurane + N2O and 6 out of 7 anesthetized with isoflurane + air) exhibited mostly subcortical injury i.e., injury containing either no cortical component (middle column, Figure. 2.4A) or only a minimal (defined here as <10%) cortical component (middle column, Figure. 2.3A). The chi-square analysis of cortical vs. subcortical injuries determined that the injury phenotype was not random and cortical injury occurred significantly less frequently in animals subjected to 40 min isoflurane + N2O anesthesia than would be expected by chance ($\chi^2 = 4.59$, $df = 3$, $p = 0.0321$, $n = 9–10$). By contrast, statistical significance was not achieved in animals subjected to isoflurane + air ($\chi^2 = 2.52$, $df = 3$, $p = 0.1121$, $n = 7$). When animals subjected to isoflurane + N2O and isoflurane + air were

combined and analyzed together, the injury phenotype was still not random (χ^2 = 6.49, df = 3, p = 0.0108, n = 16–17). Combining isoflurane + N2O and isoflurane + air conditions is justified because the effects of isoflurane + N2O and isoflurane + air were remarkably similar (Figures. 2.3 and 2.4). These data are consistent with greater vulnerability of subcortical tissues to tMCAO and/or greater protection of cortical tissues by isoflurane.

Extended anesthesia after the onset of tMCAO allows conducting various surgical procedures and measurements such as measurements of rCBF (Figure. 2.2) or other physiological parameters during tMCAO and reperfusion, i.v. injections of drugs with various delays after tMCAO and installation of infusion pumps and other (sub)chronic techniques. However, these and other procedures, including tMCAO itself, may also require extended anesthesia prior to the onset of tMCAO (see Figure. 2.5A for anesthesia pre-conditioning timeline). The effects of variable durations of pre-tMCAO anesthesia on data variability after tMCAO are not known and were tested in a separate set of experiments. The results of these experiments are shown in Figure. 2.5. Extended anesthesia (i.e., 70 min prior to tMCAO + 20 min tMCAO; Figure. 2.5) significantly reduced infarct volume and neurological deficits in a way similar to extended anesthesia after tMCAO (i.e., 20 min tMCAO + 70 min after tMCAO onset; Figures. 2.3 and 2.4). These results further support and expand our conclusions that variable anesthesia durations including both isoflurane pre- and post-tMCAO conditioning (See Section 3.6.1–3.6.2) are significant sources of data variability in this common *in vivo* model of ischemic stroke (Tables 2.3 and 2.4).

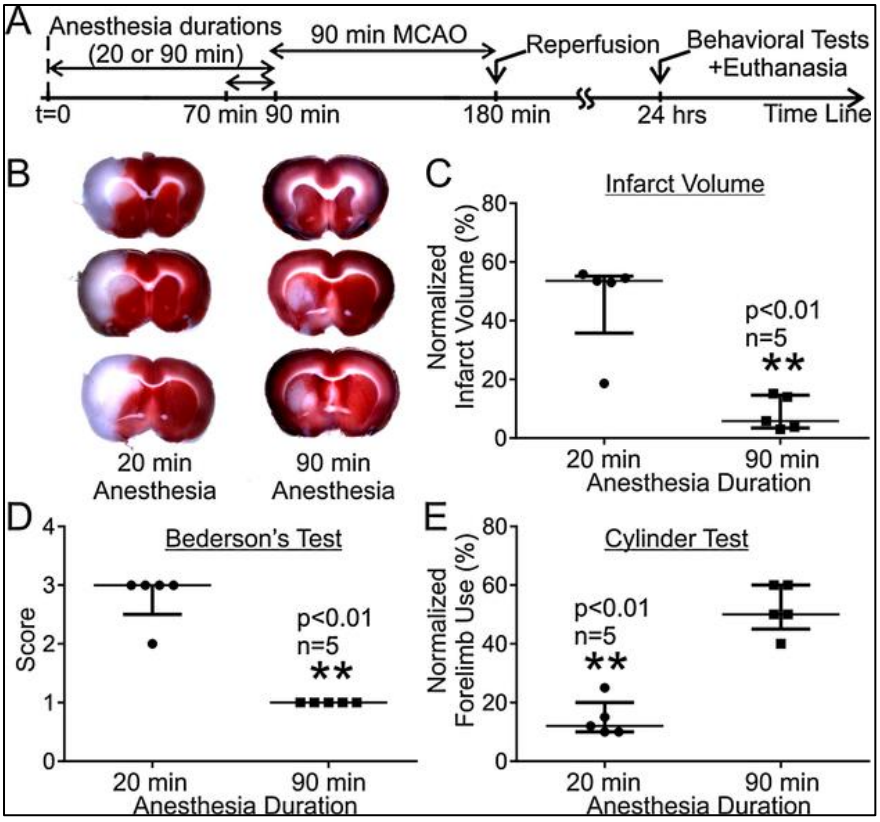

Figure.2.5. Isoflurane + N2O surgical anesthesia pre-conditioning reduces ischemic injury and neurological deficits after a 90 min tMCAO: A variable exposure of animals to isoflurane + air anesthesia acts as a significant source of variability in infract volume and neurological deficits. (A) A schematic representation of experimental timeline. (B) Representative images of ischemic infarcts detected 24 h after tMCAO across groups of animals anesthetized for various durations: 20 min (left), 40 min (middle) and 90 min (right). (C) Dependence of infarct volume on anesthesia duration. (D, E) Results of Bederson (D) and cylinder (E) tests as a function of anesthesia duration. Ischemic injury and neurological deficits inversely correlate with anesthesia duration. The data in panels (C–E) are illustrated as the median with interquartile range. Animals were anesthetized with isoflurane (4% induction; 1.8% maintenance) +70% N2O +30% O2. For definition of infarct volume measurements and % use in cylinder tests see Section 2.3.5 in Methods.

Table 2.3: Prevalence of cortical vs. subcortical ischemic infarcts after isoflurane+N2O anesthesia

	20 Min	**40 Min**
Cortical Infarct Volume (#)	10	1
Subcortical Infarct Volume (#)	10	8

Analysis of cortical vs. subcortical ischemic infarcts demonstrated severe cortical and sub-cortical brain injuries in all animals subjected to 20 min isoflurane+N2O anesthesia (i.e., 10 out of 10); while most animals subjected to 40 min isoflurane+N2O anesthesia (i.e., 8 out of 9) exhibited mostly subcortical injury defined as injury containing either no cortical component or only a minimal (<10%) cortical component. The χ^2 analysis of cortical vs. subcortical injuries determined that the injury phenotype was not random and cortical injury occurred significantly less frequently in animals subjected to 40 min isoflurane+N2O anesthesia than would be expected by chance ($\chi 2=4.59$, df=3, p=0.0321, n=9–10).

Table 2.4: Prevalence of cortical vs. subcortical ischemic infarcts after isoflurane + air anesthesia

	20 Min	**40 Min**
Cortical Infarct Volume (#)	7	1
Subcortical Infarct Volume (#)	7	6

Analysis of cortical vs. subcortical ischemic infarcts demonstrated severe cortical and sub-cortical brain injuries in all animals subjected to 20 min isoflurane + air anesthesia (i.e., 7 out of 7); while most animals subjected to 40 min isoflurane + air anesthesia (i.e., 6 out of 7) exhibited mostly subcortical injury defined as injury containing either no cortical component or only a minimal (<10%) cortical component. While cortical injury occurred less frequently in animals subjected to 40 min isoflurane + air anesthesia, the trend was not statistically significant given the existing sample size ($\chi^2=2.52$, df=3, p=0.1121, n=7)

2.5. ROOM TEMPERATURE AS A SOURCE OF VARAIBILITY

Ambient temperature of animal housing room may also affect stroke outcome. IACUC guidelines recommend housing animals between 69–79 °F. Our preliminary data suggested that animals after 90 min MCAO when housed at 69°F show less neurological damage as compared to those housed at 79°F. This data variability is in accordance with the hypothesis that lower temperature reduces ischemic stroke-induced brain damage. Failure to report these parameters may result in lack of reproducibility within different laboratories. In our study, we tested the hypothesis that stroke-animals housed at 69°F show better recovery than animals housed at 79°F.

Animals were divided randomly in either hot (79 °F) or cold (69 °F) groups. A transient (90 min) MCAO procedure was performed on each animal, as previously described. Animals were then housed for 72 hours either in hot room (79 °F) or cold room (69 °F) respectively. Behavioral tests, in the order of bederson and cylinder, were performed in their respective rooms for both groups. Infarct volume measurement was done after 72 hours of the onset of stroke using TTC staining method (see Methods)

2.5.1. Results

Figure 2.6 shows there is no significant difference in both the infarct volume and cylinder test between the animals housed at 69°F and 79°F. The intragroup data variability observed cannot be explained by temperature difference. One possibility that could have resulted in such a variability is variable endogenous cytoprotection within the animals. The effect of animal housing temperature on data variability can neither be confirmed nor rejected based on current data. A larger animal sample will be required for a conclusive data.

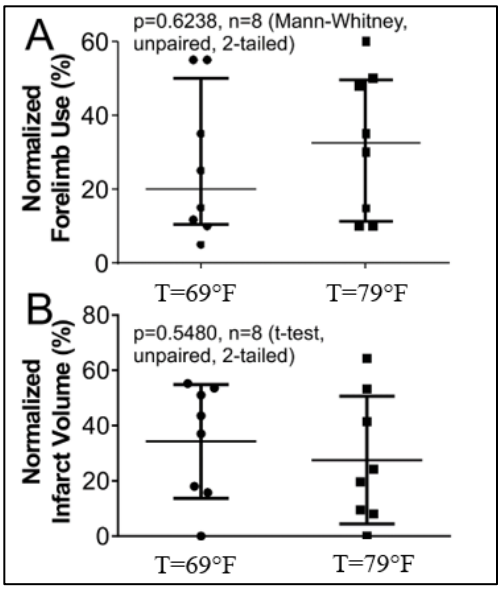

Figure. 2.6. The difference in infarct volume and cylinder test between the two temperature groups is not significant. Animals housed at 69°F do not show significant improvement in neurological deficits when compared to those housed at 79°F. (A) Result of cylinder test in animals from both groups do not show significant difference using Mann-Whitney unpaired, 2-tailed non-parametric test (p=0.6238). (B) Corresponding infarct volume also do not show significant difference (p=0.5480).

2.6 DISCUSSION

Neuroprotective effects of isoflurane have been extensively investigated and reported previously [185,187,189,191,192]. Nevertheless, isoflurane is routinely used for induction and maintenance of surgical anesthesia in preclinical stroke studies. It is common however in stroke studies to not monitor and report anesthesia durations. This practice contradicts the STAIR guidelines aimed at elevation of transparency, rigor and reproducibility of stroke research [208]. In this study, we used a common tMCAO model of ischemic stroke to demonstrate that a typical range of anesthesia durations required for this model and related experimental or surgical procedures (e.g., Laser Doppler flowmetry) acts as a significant source of data variability evidenced by measurements of infarct volume and neurological deficits. The study thus concludes that variability in anesthesia durations across experimental groups should be monitored, minimized, standardized and become a required reportable parameter in studies utilizing tMCAO models of stroke.

Specifically, this study demonstrates that the severity of brain injury and neurological deficits after a 90 min tMCAO in young adult male rats is inversely related to the duration of isoflurane anesthesia in the presence or absence of N2O: e.g., a 90-min isoflurane anesthesia nearly

completely protected brain tissue from tMCAO-induced injury and neurological deficits and thus, would be expected to obscure the effects of stroke treatments in pre-clinical trials. Thus, isoflurane anesthesia may directly interfere with outcomes of novel drug testing acting as an unrestricted, untraceable source of data variability, potentially causing false-positive or false-negative results. This finding is alarming because anesthesia is an important, required step in many animal models of ischemic stroke and other brain injuries. Although anesthetics such as isoflurane and N2O are known to produce neuroprotection [184,185,187,189,191-195], the impact of variable anesthesia duration during a typical 90 min tMCAO on the severity of ischemic brain injury has escaped research attention until now. Both N2O (as a 70% mixture with 30% O2) and air (100%) can be used as vehicles for isoflurane, but N2O exhibits neuroprotective efficacy and thus, may enhance neuroprotective efficacy of isoflurane. For that reason, we tested both vehicles, but did not detect apparent effects of N2O (compare effect seizes and behavioral scores in Figures. 2.3 and 2.4). Since the effects of isoflurane + N2O mixtures were remarkably similar, isoflurane was likely the main player in neuroprotective action of anesthetic mixtures tested in this study. This study reveals the high susceptibility of infarct volume and behavioral deficits to isoflurane + N2O surgical anesthesia. Since the effects of isoflurane + N2O mixtures were remarkably similar (compare effect seizes and behavioral scores in Figures. 2.3 and 2.4), isoflurane was likely the main player in neuroprotective action of anesthetic mixtures tested in this study. Interestingly, our results also demonstrate differential vulnerability of cortical and subcortical brain tissues to tMCAO performed under short (i.e., 20 min) vs. long (i.e., 40 min) isoflurane anesthesia (Figures. 2.3 and 2.4): subcortical tissues appear to be more vulnerable to tMCAO and/or less protected by isoflurane.

Our results may extend to subchronic studies that require multiple rounds of sedation and exposure to isoflurane. Recurring exposures to isoflurane anesthesia as those that may be required in subchronic treatments would be expected to cause cumulative effects over time and add to the variability of data and adverse neuroprotective effects after each round of anesthesia. Therefore, the anesthesia duration, frequency and total number of anesthesia rounds need to be diligently recorded and reported in studies utilizing isoflurane anesthesia.

To minimize undesirable effects of isoflurane anesthesia on the outcome of novel drug testing, we propose to standardize isoflurane anesthesia procedures in pre-clinical experimental surgeries and make the duration of surgical anesthesia a required reportable parameter in pre-clinical studies of ischemic and possibly, other types of brain injury. Specifically, the results of this study (Figures. 2.3 and 2.4) suggest that a 20–30 min duration of isoflurane anesthesia is nearly optimal for a 90-min suture tMCAO model of ischemic stroke in young adult male rats: this duration simultaneously minimizes adverse neuroprotective effects of isoflurane and permits a successful completion of tMCAO. Thus, our data suggest that surgical procedures and experimental paradigms conducted in young adult male rats that require isoflurane anesthesia lasting 90 min should be avoided because in these or similar experimental settings, anesthesia strongly interferes with tMCAO-induced brain injury and neurological deficits in the absence of any treatment (Figures. 2.3 and 2.4). Specifically, anesthesia lasting >90 min would nearly completely eliminate any traces of brain injury and neurological deficits thereby negating any chance of detecting the therapeutic efficacy of a novel stroke treatment. If such prolonged or recurring anesthesia treatments are unavoidable, their limitations and adverse effects on data and data variability should be fully disclosed and the specific details of anesthetic procedures should be reported.

One limitation of this study is that it does not investigate the effect of anesthesia duration on evolution of brain injury because our experimental protocol employed only a 24 hrs delay between tMCAO and behavioral/ histological assays. As ischemic brain injury may not be fully matured 24 h after tMCAO, the long-term (e.g., days, weeks) effects of isoflurane anesthesia on brain injury and neurological deficits cannot be reliably predicted from the presented data. Prolonged or recurrent isoflurane anesthesia may impede stroke maturation and help reduce other post-stroke sequelae not analyzed in this study: e.g., edema, synaptic degeneration and excessive inflammation. Furthermore, isoflurane may alter rCBF and some other physiological parameters during MCAO [217]. However, this factor is not a concern because in our study, all experimental groups were subjected to exactly the same experimental conditions and the same doses of isoflurane while anesthesia duration was the only independent variable across groups. Thus, all observed effects were functions of anesthesia duration, directly or indirectly. Interestingly, we did not observe a significant continuous increase in rCBF during tMCAO as observed in [218]. This discrepancy may have resulted from at least two differences between these two studies: (1) the use of different suture filaments (4-0 monofilament nylon suture from Doccol Corporation (Sharon, MA, USA) in our study vs. a No. 270 guide wire with a smooth surface, blunted tip and constant diameter of 300 μm from VYGON (Aachen, Germany) use in [218]; and (2) the use of smaller (~280 g) and presumably younger Sprague-Dawley rats in our study vs. ~400 g Wistar rats used in [218]. Blood vessels of older rats may lose flexibility and smooth internal surface thus, a filament occlusion may not be as complete as in younger rats. Another limitation of this study is that animals were maintained on spontaneous ventilation and physiological parameters were not measured and controlled. If ventilation and physiological parameters were controlled, the absolute outcome values may have been different. Nevertheless, intubation and controlled stable physiological

parameters during MCAO and anesthesia do not prevent anesthetic neuroprotection as multiple studies have demonstrated [185,187,218,219]. In this study, we extend these previous results by demonstrating that anesthesia duration is a crucial factor modulating the level of neuroprotection by isoflurane during tMCAO. The fact that intra-group data variability is significantly smaller than data variability across groups (leading to statistical significance across groups) indicates that variability in physiological parameters (e.g., blood gases, blood pressure, body temperature etc.) is not a significant source of data variability as long as anesthesia duration is kept constant. Thus, we propose that isoflurane anesthesia duration needs to be monitored, minimized, standardized and reported to ensure research transparency, rigor and reproducibility. Perhaps, a warning in [218] that "isoflurane is not useful for studies on neuroprotection" is somewhat overstated because as long as isoflurane anesthesia during a 90 min tMCAO is short (e.g., ~20 min) and kept constant across groups, brain injury and neurological deficits will remain to be severe and can be treated with experimental drugs in preclinical trials. As the mechanisms of general anesthesia and its neuroprotective, metabolic and immune effects are far from being fully understood, the results presented in this study for isoflurane surgical anesthesia during tMCAO in young adult male rats cannot be blindly generalized to other anesthetics, animal species and experimental models. Additional research efforts may be required to determine anesthetic agents, optimal durations, frequencies and total number of anesthesia rounds (for animals subjected to multiple sedations) appropriate for specific research projects, animal species, surgical procedures and experimental models.

2.7. CONCLUSIONS

Anesthesia duration in pre-clinical stroke studies is usually not monitored and/or reported. In this study, we used a common tMCAO model of ischemic stroke to demonstrate that a typical range of

anesthesia durations prior, during and after tMCAO required for this model and related experimental or surgical procedures (e.g., Laser Doppler flowmetry) acts as a significant source of data variability as evidenced by measurements of infarct volume and neurological deficits. The study further demonstrates that severity of brain injury and neurological deficits after tMCAO is inversely related to isoflurane anesthesia duration and thus, a sufficiently prolonged isoflurane anesthesia (e.g., >40 min) may completely obscure the effects of stroke treatments in pre-clinical trials. Thus, in experiments that utilize variable durations, frequencies and/ or total number of isoflurane anesthesia rounds across groups, the effects of drug treatments cannot be meaningfully compared. The study thus concludes that variability in anesthesia durations across experimental groups should be monitored, minimized and standardized to become a required reportable parameter in studies utilizing tMCAO models of stroke. The results of this study may not be blindly generalized to anesthetics other than isoflurane, animals other than young adult male rats and animal models other than 90 min suture tMCAO. Additional research efforts may be required to determine anesthetic agents, optimal durations, frequencies and total number of anesthesia rounds appropriate for specific research projects, animal species, surgical procedures and experimental models. These efforts are essential to further elevate transparency, rigor and reproducibility of pre-clinical stroke research as recommended by the STAIR guidelines [208].

Chapter 3

Sub-chronic vs. acute efficacy of positive allosteric modulation after focal ischemia in rats.

In preparation, as of 10-26-2017

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3.1. ABSTRACT

Ischemic brain stroke is one of the leading cause of death and disabilities around the world. There is yet to be an effective therapy against stroke despite substantial investment of time and money. In this study we propose a novel and highly effective therapeutic treatment – to augment endogenous protection against stroke-induced brain damage by using positive allosteric modulator (PAMs) of α7 nicotinic acetylcholine receptors (α7nAChRs) using a subchronic treatment paradigm. We compared the therapeutic efficacy of multiple PAM injection against single PAM injection in treating stroke in rats over 72 hours period. Our data suggests that the PAM treatment is effective even when initiated as late as 4 hours post-stroke onset. The efficacy of delayed treatment against stroke-induced damage is remarkable as this provides therapeutic advantage in clinical situations where prompt treatment is not always possible. Another important finding of our study is that early PAM treatment is effective only if it is supplemented by multiple subchronic PAM injections, suggesting that continuous systemic presence of PAM is essential for its beneficial effect against stroke. Our novel approach to managing brain injury and neurological deficits after stroke holds significant promise and presents an exciting therapeutic opportunity in drug discovery/development and may enable health care providers to overcome current limitations associated with the lack of effective treatments after cerebral stroke

3.2. INTRODUCTION

Neuronal α7 nicotinic acetylcholine receptor activation produces robust protection against ischemic stroke which reduces ischemic brain damage and neurological deficits[12,38,51]. This protection is attributed to cytoprotective[38,51] and anti-inflammatory properties[85,114] of α7 nAChRs activation. Positive allosteric modulation of α7 nAChRs is known to reduce ischemia-induced brain injury in *in vivo* and *ex vivo* studies in rats[48,49,74,113,125]. Our previous studies have confirmed the therapeutic efficacy of α7 PAMs in short term treatment (<24 hours) of cerebral ischemia[38,51,122]. In our current study we tested this high therapeutic potential of prototypical (PNU) and one novel α7 PAM (EPGN) under acute and subchronic treatment paradigms in transient middle cerebral artery occlusion model (MCAO) in rats. Neurobehavioral tests such as Bederson and Cylinder (both measure motor function asymmetry) showed that single i.v. injection of PNU at 90 min after MCA occlusion offered significant protection against neurological damage but the beneficial effect gradually diminished (~48 hours) and vanished completely at 72 hours after the MCAO suggesting that systemic presence of PNU is essential for its therapeutic efficacy. This result was in accordance with our previous data where it was shown that only short term (~3 hours and not 24 hours) PNU preconditioning is therapeutically effective against stroke[38]. This led to our hypothesis that delayed sub-chronic treatments with prototypical and novel α7 PAMs will reduce brain injury and neurological deficits after ischemic stroke in a challenging experimental paradigm characterized by delayed (4 hrs after MCAO) multiple (daily) sub-chronic (72 hrs) treatments in the optimized MCAO model. We tested our hypothesis in a fully blinded subchronic (72 hours) study by treating animals with multiple PNU injections at various intervals to maintain continuous systemic PNU level and comparing results with single acute treatment. We compared therapeutic efficacy of PNU in the following three treatment paradigms (Tables 3.1 and 3.2) using

MCAO model of rat: 1) single acute treatment (i.v. injection immediately prior to reperfusion, i.e., 90 min after MCAO onset; Figure 3.2); 2) combined multiple sub-chronic treatment (single i.v. injection immediately prior to reperfusion followed by multiple s.c. injections daily for 72 hrs; Figures 3.3 & 3.5) and 3) delayed multiple sub-chronic treatment (multiple s.c. injections daily for 72 hrs without i.v. injection prior to reperfusion; Figure 3.4). The rationale for using subchronic treatment paradigm was that continuous systemic presence of PNU (half-life ~ 10 hours[258]) is essential to maintain therapeutic effect against stroke damage. Administration of PNU by s.c. route is a form of sustained release drug delivery system that ensures continuous systemic bioavailability of PNU. Within the subchronic treatments (paradigms 2 and 3) we observed that the combined multiple treatment (i.v. + s.c.; i.v immediately prior to reperfusion) and delayed multiple treatments (s.c. only; 1st s.c. at 4 hours post MCAO) were equally therapeutically effective in reducing stroke-induced damage. This was remarkable as it provided evidence that even if prompt treatment after the onset of stroke is not available, the ischemic injury can still be reversed by initiating treatment as late as 4 hours post onset. This result is critical in clinical setting where prompt treatment, although highly desirable, is not always feasible. Another important finding of this study is that single treatment (Paradigm 1) is beneficial for only short term and has to be supplemented by sub-chronic treatment. Our current study did not investigate the length of treatment that is sufficient to fully restore neurological function after the onset of ischemic stroke. Future studies will explore therapeutic effect of long term treatment with PAMs. Our novel therapeutic approach, to augment endogenous α7 nAChRs protection by PAMs against brain stroke, may provide a clinically efficacious therapy.

3.3. MATERIAL AND METHODS

Animals were randomly assigned to groups. Analysis of infarct volumes was blind. Behavioral assays were conducted by the same researcher who conducted MCAO surgeries.

3.3.1 Animals

Young adult male Sprague Dawley (SD) rats (~280 g) were purchased from Charles River (Wilmington, MA, USA) and used in accordance with the Guide for the Care and Use of Laboratory Animals (NIH 865-23, Bethesda, MD, USA). All experimental protocols were approved by the UNTHSC Institutional Animal Care and Use Committee. All animal protocols comply with the ARRIVE guidelines.

3.3.2 Anesthesia

Isoflurane was used to induce and maintain surgical anesthesia for a defined duration during a 90-min suture MCAO. Isoflurane (4% induction; 1.8% maintenance) was purchased from Henry Schein Animal Health (Dublin, OH) and delivered by a mask as a gaseous mixture as 70% N2O +30% O2. To initiate re-perfusion at the end of MCAO, the animals were sedated for 5 min using the same anesthetic mixture and parameters

3.3.3 Transient Middle Cerebral Artery Occlusion (tMCAO).

Our approach was to conduct a tMCAO within a narrow window of experimental parameters to ensure stable ischemic insult and injury.[122] After the effective experimental parameters were established and confirmed by rCBF measurements (See *methods* in Chapter 2), these parameters were kept constant in subsequent experiments supported by intermittent CBF measurements. Specifically, we used only 285±5 g SD rats purchased from Envigo and

accommodated in our animal facility for 5-7 days after arrival. To initiate tMCAO, 4-0 monofilament nylon suture having a coating of silicon rubber on tip (diameter with coating 0.39+/-0.02 mm; coating length 4-5 mm) (Doccol Corporation, Sharon, MA, USA) was used to advance from bifurcation of carotid (i.e., 19-mm). These restrictions ensured stability and reproducibility of experimental parameters across groups as confirmed by rCBF measurements.

Animals were anesthetized with an isoflurane-based mixture (see *Anesthesia*), delivered by a mask. After a midline incision in the neck, the left common carotid artery (CCA) was exposed and permanently dissected. A 4-0 monofilament nylon suture (19-mm) was inserted from the CCA into the left internal carotid artery to occlude the origin of the left MCA. Rats were allowed to regain consciousness in a warmed recovery cage for the rest of MCAO. After 90 min of MCAO, the thread was removed for re-perfusion. The CCA was permanently ligated, and the wound was closed. Rectal temperature was maintained at~37°C using a heating pad. Animals were randomly assigned to groups prior to group labeling. All data were reported.

3.3.4 **Measurements.**

3.3.4. 1 Infarct Volume Measurements

Immediately prior to euthanasia by decapitation 24 hrs after tMCAO, animals were anesthetized for <1 min with the same anesthetic mixture used for surgical anesthesia. The brains were removed. Coronal sections (2-mm thickness) immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline for 20 min at 37°C then, fixed for 2 hrs in 4% paraformaldehyde. The infarct and contralateral hemisphere areas were measured using ImageJ. Infarct volumes were calculated as a percentage of the contralateral slice volume to account for ipsilateral edema.

3.3.4. 2 Neurobehavioral Testing.

Neurobehavioral tests were done 15 min prior to euthanasia. The order of testing (Bederson → cylinder) was always the same for data consistency.

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. A mirror behind the cylinder allowed observing forelimb movements when the rat faced away from the researcher. After an episode of rearing and wall exploration, a landing was scored for the first limb to contact the wall or for both limbs if they made simultaneous contact. The use of the impaired limb was calculated as a percent.

3.3.5 ***Ethics statement***

Young adult male Sprague-Dawley (SD) rats (~280 grams) will be used in the experiments. The animal use is in accordance with the Guide for the Care and Use of Laboratory Animals (NIH 865-23, Bethesda, MD, USA). All experimental protocol is approved by the UNTHSC Institutional Animal Care and Use Committee.

3.3.6 Drug injection schedule

The following injection schedules were used in the experiments and are discussed in detail later in results section.

Table 3.1. PNU injection schedule for sub-chronic treatment. In this fully randomized blinded experiment, each animal is subjected to four PNU injections: 1) i.v. 2.3 mg/kg, immediately prior to reperfusion; 2) s.c. 11.1 mg/kg, 5 hrs after i.v. injection; 3) s.c. 5.5 mg/kg 24 hrs after MCAO; 4) s.c. 5.5 mg/kg 48 hrs after MCAO.

PNU Injection	Injection time (after MCAO onset)	Injection dose (mg/kg)
i.v.	90 min	2.3
s.c.1	6.5 hrs	11.1
s.c.2	24 hrs	5.5
s.c.3	48 hrs	5.5

Table 3.2. EPGN injection schedule for sub-chronic treatment. In this fully randomized blinded experiment, each animal is subjected to four EPGN injections: 1) i.v. 4 mg/kg, 90 min after MCAO onset; 2) s.c.1 20 mg/kg, 6 hrs after MCAO onset; 3) s.c.2 10 mg/kg, 24 hrs after s.c.1; and 4) s.c.3 10 mg/kg, 24 hrs after s.c.2.

EPGN Injection	Injection time (after MCAO onset)	Injection dose (mg/kg)
i.v.	90 hrs	4
s.c.1	6 hrs	20
s.c.2	24 hrs	10
s.c.3	48 hrs	10

3.4. RESULTS

3.4.1 Correlation of behavioral and histological parameters after MCAO. In the absence of functional imaging (e.g., MRI), the state and time course of brain injury and treatment after MCAO cannot be measured in alive animals. We hypothesized that the outcome of behavioral tests may significantly correlate with infarct volume thus, allowing evaluation of the time course of brain injury and treatment after MCAO. To test this hypothesis, we correlated animal performance in a cylinder test with infarct volume 3 days after MCAO. Animals were subjected to a cylinder test 15 min prior to euthanasia. A pool of 56 young adult male rats was used in this retrospective analysis. Both control and treated animals were included in the pool resulting in a large variability in infarct volumes and behavioral measurements (Figure 3.1). The results indicate significant correlation ($r=-07869$; $p<0.0001$) between these important parameters. These results suggest that animal performance in a cylinder test can predict infarct volume and thus, is informative for evaluation of the animal status and time course of brain injury after MCAO during sub-chronic treatments. The 30-40% range of infarct volumes seems to be most critical for performance in the cylinder test because a large drop in the animal performance in the cylinder test occurs within this range. This result justifies the use of cylinder test in evaluation of the state and time course of brain injury after MCAO during sub-chronic treatments (aim 2)

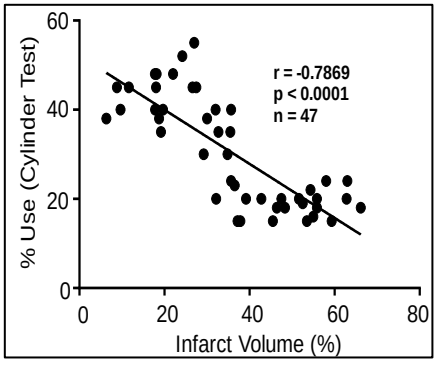

Figure 3.1. Significant correlation between behavioral deficits and infarct volume *(i.e., behavioral deficits predict infarct volume and vice versa).* Significant correlation between % use of the limb contralateral to ischemic injury (cylinder test) and infarct volume (TTC measurement) allows evaluation of both parameters simultaneously without functional imaging and predicts

the state and time course of brain injury and cytoprotective effects of drugs after MCAO during sub-chronic paradigms and treatments.

3.4.2 Time course of cytoprotective effects.

Because animal performance in the cylinder test significantly correlates with infarct volume (Figure 3.1), the outcomes of daily cylinder tests can be used to predict the state and time course of brain injury and cytoprotective effects of drugs after MCAO during sub-chronic paradigms and treatments. Accordingly, we conducted a fully randomized blinded experiment where daily cylinder tests after a single i.v. injection of 2.3 mg/kg PNU 90 min after MCAO onset to determine the time course of therapeutic effects of PNU over time. Treated animals were compared to control animals subjected to matching injections of vehicle (DMSO). The results of this experiment are shown in Figure 3.2. As expected from our previous published reports, [38,51,214] PNU injected i.v. on day 0, produced significant protection 24 hrs after treatment (day 1) vs. controls as evidenced by the cylinder tests conducted 24 hrs after the treatment (Figure 3.2A-C). These protective effects declined in the following 48 hrs and eventually, vanished 72 hrs (day 3) after the treatment (Figure 3.2D). The analysis of infarct volumes on day 3 (Figure 3.E-F) supports 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; Figure 3.2D and $p=0.7768$, n=8; Figure 3.2F), but not day 1 ($p=0.0211$, n=9; Figure 3.2C). One outlier was identified by the ROUT method (Q=1%) in the data pool collected on day 1 (arrowhead; Figure 3.2C). Removing this outlier further strengthened statistical significance ($p=0.0091$, n=8; not shown) and our conclusions. Taken together, these results demonstrated the intrinsic limitations of acute PNU treatment and suggest that multiple sub-chronic PNU treatments may be necessary to maintain therapeutic efficacy of PNU for longer

intervals. This hypothesis was tested in the next series of experiments where s.c injections of PNU were conducted daily for 3 days with or without the first i.v. injection (Figure 3.3).

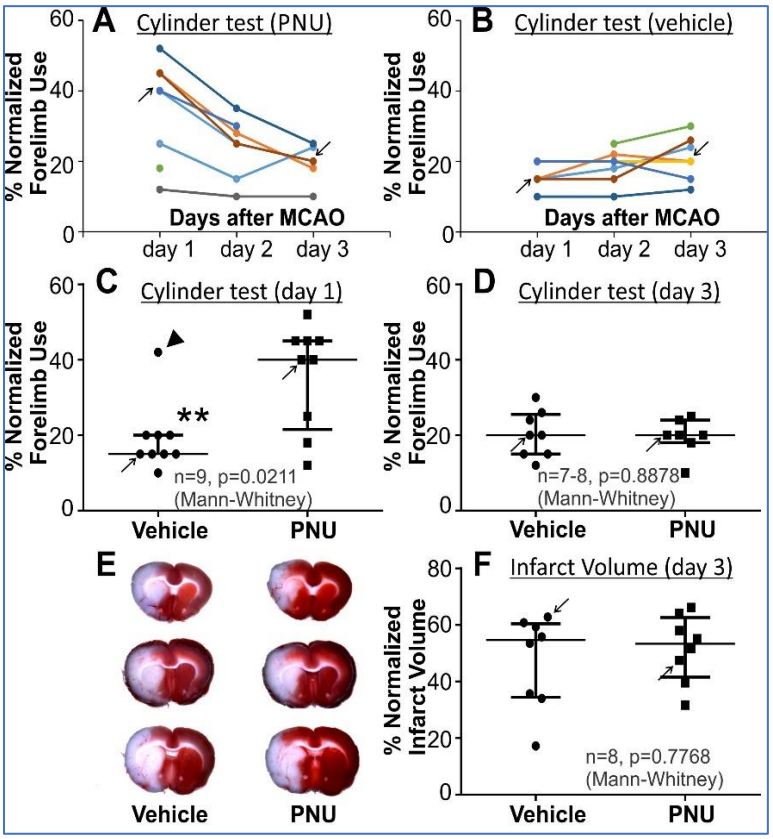

Figure 3.2. Time course of cytoprotective effects of PNU. In this fully randomized blinded experiment, a single i.v. injection of 2.3 mg/kg PNU (test group; A) or DMSO (control group; B) was delivered immediately prior to reperfusion (i.e., 90 min after MCAO onset; day 0) to determine the time course of therapeutic effects of PNU over time. As expected from our previous published reports[12-14], PNU produced significant protection 24 hrs after treatment (day 1) vs. controls as evidenced by the cylinder tests conducted 24 hrs after the treatment (A-C). These protective effects declined in the following 48 hrs and eventually, vanished 72 hrs (day 3) after the treatment (D). The analysis of infarct volumes on day 3 (E-F) supports 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; D and p=0.7768, n=8; F), but not day 1 (p=0.0211, n=9; C). One control and one test animals did not survive day 1. One test animal did not move in behavioral tests and was euthanized 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; C). 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.

3.4.3 Therapeutic efficacy of the combined sub-chronic PNU treatment. In this fully randomized blinded experiment, we determined the therapeutic efficacy of PNU using a combined sub-chronic paradigm. In this paradigm, a single i.v. 2.3 mg/kg PNU injection immediately prior to reperfusion, i.e., at the end of 90 min MCAO (a paradigm which has shown significant efficacy only in the first 24 hrs after treatment; see Figure 3.2) is followed by multiple daily s.c. 5.5-1.1 mg/kg PNU injections (Table 3.1). Behavioral tests and euthanasia were conducted 72 hrs after MCAO. The results of this experiment are shown in Figure 3.3. This combined multiple sub-chronic i.v.+s.c. PNU treatments significantly reduced infarct volume (p=0.0011, n=8; Figure 3.3A-B) and neurological deficits (cylinder test: p=0.0003, n=8; Figure 3.3C; and Bederson: p=0.0014, n=8; Figure 3.3D) 72 hrs after MCAO (Mann-Whitney, two-tailed). These data are consistent with our previous reports where s.c. administration of PNU 24 hrs prior to MCAO failed to reduce MCAO-induced brain injury and neurological deficits[38] suggesting that PNU ($\tau_{1/2}$~10 h) should be present in the system to produce therapeutic effects.

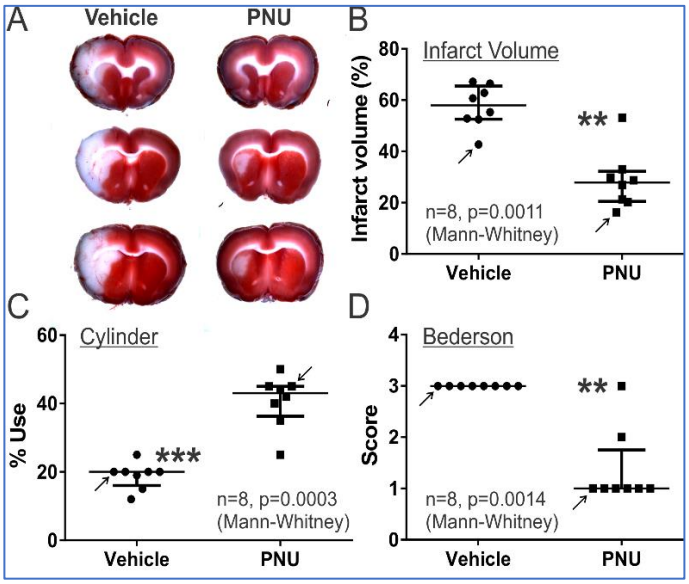

Figure 3.3. Therapeutic efficacy of the combined sub-chronic PNU treatment. In this fully randomized blinded experiment, a single i.v. injection of 2.3 mg/kg PNU (test group) or DMSO (control group) was delivered immediately prior to reperfusion (i.e., 90 min after MCAO onset) and was followed by multiple daily s.c. PNU injections (see administration schedule in Table 3.1) to determine therapeutic effects of sub-chronic PNU. The animals' performance in Bederson and cylinder tests was tested 72 hrs after MCAO immediately prior to euthanasia and sample brain tissue collection for

infarct volume analysis. Combined sub-chronic i.v.+s.c. PNU significantly reduced infarct volume (p=0.0011, n=8; A-B) and neurological deficits (cylinder test: p=0.0003, n=8; C; and Bederson: p=0.0014, n=8; D) 72 hrs after MCAO (Mann-Whitney, two-tailed). Arrows in B-D indicate data points corresponding to data illustrated in A

3.4.4 Therapeutic efficacy of the delayed sub-chronic PNU treatment. In this fully randomized blinded experiment, we determined therapeutic efficacy of PNU in a *delayed* sub-chronic paradigm where the initial i.v. injection was omitted, while the remaining injections were identical to those shown in Table 3.1. Accordingly, the first s.c. PNU injection was done 6.5 hrs after MCAO onset (i.e., 5 hrs after reperfusion) followed by daily s.c. injections 24 hrs and 48 hrs later. Behavioral tests and brain tissues for infarct volume analysis were collected 72 hrs after MCAO. The results of this experiment are shown in Figure 3.4. Delayed sub-chronic s.c. PNU significantly reduced infarct volume (p=0.0043, n=5-6; Figure 3.4A-B) and neurological deficits (cylinder test: p=0.0043, n=5-6; Figure 3.4C; and Bederson: p=0.0122, n=5-6; Figure 3.4D) 72 hrs after MCAO (Mann-Whitney, two-tailed). These data demonstrate significant therapeutic efficacy of delayed sub-chronic s.c. PNU treatment after MCAO providing proof-of-concept for the use of α7 PAMs after ischemic stroke.

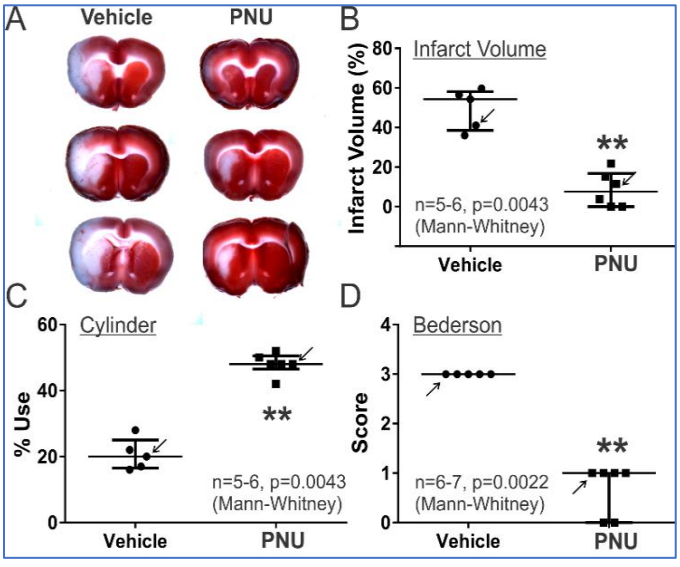

Figure 3.4. Therapeutic efficacy of the delayed sub-chronic PNU treatment. In this fully randomized blinded experiment, we determine therapeutic efficacy of PNU in a *delayed* sub-chronic paradigm where the initial i.v. injection was omitted. The remaining injections were identical to those shown in Table 3.1. Accordingly, the first s.c. PNU

injection was done 6.5 hrs after MCAO onset (i.e., 5 hrs after reperfusion) followed by daily s.c. injections 24 hrs and 48 hrs later. Behavioral tests and brain tissues for infarct volume analysis were collected 72 hrs after MCAO. Delayed sub-chronic s.c. PNU significantly reduced infarct volume (p=0.0043, n=5-6; A-B) and neurological deficits (cylinder test: p=0.0043, n=5-6; **C**; and Bederson: p=0.0122, n=6-7; D) 72 hrs after MCAO (Mann-Whitney, two-tailed). Arrows in B-D indicate data points corresponding to data illustrated in A

3.4.5 Therapeutic efficacy of a novel sub-chronic α7 agent. In this fully randomized blinded experiment, we determined therapeutic efficacy of EPGN (a novel agent synthesized by Epigen Bioscience Inc.) using a combined sub-chronic paradigm. In this paradigm, a single i.v. 4 mg/kg EPGN injection immediately prior to reperfusion, i.e., at the end of 90 min MCAO (a paradigm which has shown significant efficacy only in the first 24 hrs after treatment; see Figure 3.2) is followed by multiple daily s.c. 10-20 mg/kg EPGN injections (Table 3.2). Behavioral tests and euthanasia were conducted 72 hrs after MCAO. The results of this experiment are shown in Figure 3.5. This combined multiple sub-chronic i.v.+s.c. EPGN treatments significantly reduced infarct volume (p=0.002, n=8; Figure 3.5A-B) and neurological deficits (cylinder test: p=0.0009, n=8; Figure 3.5C; and Bederson: p=0.0014, n=8; Figure 3.5D) 72 hrs after MCAO (Mann-Whitney, two-tailed). These data support therapeutic efficacy of this novel EPGN compound as a potential treatment after ischemic stroke

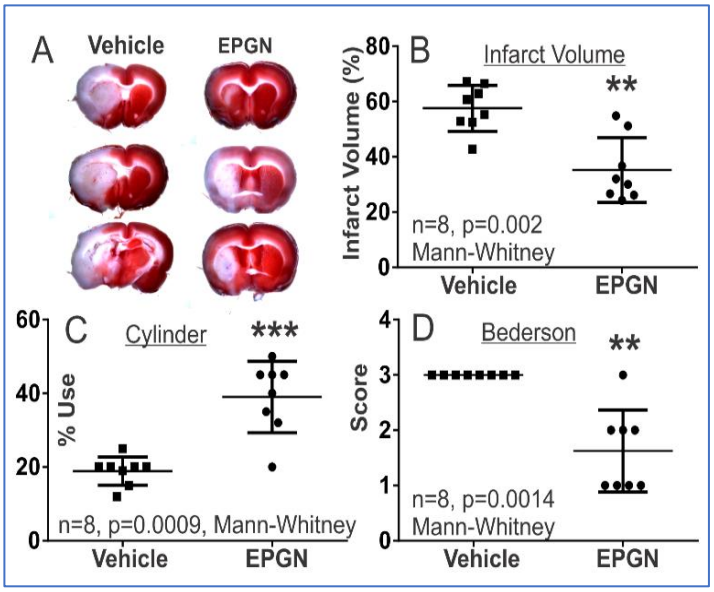

Figure 3.5. Therapeutic efficacy of the combined sub-chronic EPGN treatment. In this fully randomized blinded experiment, a single i.v. injection of 4 mg/kg EPGN (test group) or DMSO (control group) was delivered immediately prior to reperfusion (i.e., 90 min after MCAO onset) and was followed by multiple daily s.c. EPGN injections (see administration schedule in Table 3.2) to determine therapeutic effects of sub-chronic EPGN. The animals' performance in Bederson and cylinder tests was tested 72 hrs after MCAO immediately prior to euthanasia and sample brain tissue collection for infarct volume analysis. Combined sub-chronic i.v.+s.c. EPGN injections significantly reduced infarct volume (p=0.002, n=8; A-B) and neurological deficits (cylinder test: p=0.0009, n=8; C; and Bederson: p=0.0014, n=8; D) 72 hrs after MCAO (Mann-Whitney, two-tailed).

3.5. DISCUSSION

The key finding pertinent to aim 2 is that multiple sub-chronic treatments with Type II α7 PAMs (i.e., PAMs that inhibit α7 nAChR desensitization [11] significantly reduce brain injury (evaluated by infarct volume measurements) and neurological deficits (evaluated from Bederson's and cylinder tests) after MCAO. While the efficacy of acute treatments with α7 PAMs (i.e., <24 hrs) has been extensively explored and confirmed in previous studies [38,214], our early tests indicated that therapeutic benefits of the treatment vanish within 48 hrs after the treatment (Figure 3.2) and thus, sub-chronic paradigms are required to maintain the therapeutic efficacy of α7 PAMs for a prolonged time. We have previously reported that only a short-term PNU pre-conditioning (e.g., 3 hrs, but not 24 hrs prior to MCAO) generates therapeutic efficacy [38] suggesting that PNU (half-life

time ~10 hrs) [220] has to be present in the system to be effective. These observations highlight the importance of sub-chronic treatment paradigms (e.g., daily s.c. injections; see Tables 3.1-2) for PAM-based interventions.

In this proof-of-concept study, we used fully randomized blinded experiments to test sub-chronic regiments of one prototypical (i.e., PNU) and one novel (i.e., EPGN) α7 PAMs and confirmed their efficacy after MCAO in young adult male rats. Future studies will extend these findings to female and aged animals and will test other biological variables according to the STAIR guidelines. Experiments with α7 agents employed the following three treatment paradigms (see Tables 3.1-2): 1) single acute treatment (i.v. injection immediately prior to reperfusion, i.e., 90 min after MCAO onset; Figure 3.2); 2) combined multiple sub-chronic treatment (single i.v. injection immediately prior to reperfusion followed by multiple s.c. injections daily for 72 hrs; Figures 3.3 & 3.5) and 3) delayed multiple sub-chronic treatment (multiple s.c. injections daily for 72 hrs without i.v. injection prior to reperfusion; Figure 3.4). Remarkably, delayed multiple treatments (Figure 3.4) were as effective as combined multiple treatments (Figure 3.3). The latter included early i.v. injections immediately prior to reperfusion. Although the efficacy of any stroke treatment is time sensitive and the treatment should be applied as promptly as possible, these observations suggest that brain tissues and functions can be still successfully rescued even if the presentation to medical attention is substantially delayed (up to 4-6 hrs) as long as α7 PAM treatments are maintained sub-chronically (Figures 3.3-4). In fact, our data indicate that the face value of the early acute PAM treatment (<90 min after MCAO onset) is limited if it is not supported by the following multiple sub-chronic treatments because therapeutic efficacy of a single acute PAM treatment vanishes within 48 h (Figure 3.2).

3.6. CONCLUSIONS

The results pertinent to aim 2 are expected to develop, confirm and justify sub-chronic treatment paradigms and regiments using PNU, a prototypical PAM and EPGN, a novel α7 agent. These paradigms and novel compounds are ready for immediate in vivo testing for compatibility with various biological variables, tPA and large animal species (e.g., rabbits). Our contribution is multifold and includes the development of proof-of-concept for sub-chronic efficacy of α7 agents as well as the identification of novel agents with in vivo efficacy/potency suitable for post-stroke treatment. This contribution is expected to generate novel clinically-suitable α7 agents that convert endogenous choline and ACh into potent therapeutic agents not currently available to health care providers. Because α7 nAChRs and phosphatidylcholine-based cell membranes are common in the mammalian brain, including humans [221,222], the endogenous α7-dependent cytoprotective anti-inflammatory mechanisms may be an evolutionarily-shaped common mammalian motif that can be significantly amplified by PAMs. Thus, there is a rational basis to expect that the therapeutic utility of PAMs revealed in our studies in rats will naturally extend to humans [79,214,223]. This novel approach to managing brain injury and neurological deficits after stroke holds significant promise and presents an exciting therapeutic opportunity in drug discovery/development and may enable health care providers to overcome current limitations associated with the lack of effective treatments after cerebral stroke.

IV OVERALL CONCLUSIONS

This research project has achieved two important goals: 1) First, it established high standards for research rigor and reproducibility by identifying and mitigating sources of data variability (specific aim 1); and 2) It generated an important proof-of-concept for the use of PAMs as cytoprotective anti-inflammatory agents with significant therapeutic benefits after focal ischemia in rodent stroke models (specific aim 2). It is common in stroke studies to not monitor and report anesthesia durations. This practice contradicts the STAIR guidelines aimed at elevation of transparency, rigor and reproducibility of stroke research. The main finding of aim 1 is that a typical range of anesthesia durations required for MCAO surgeries acts as a significant source of data variability evidenced by measurements of infarct volume and neurological deficits. Thus, an indiscriminate use of isoflurane surgical anesthesia may potentially cause false-positive or false-negative results. We thus concluded that variability in anesthesia durations across experimental groups should be monitored, minimized, standardized and become a required reportable parameter in studies utilizing tMCAO models of stroke. These concepts were fully implemented in our proof-of-concept studies pertaining to specific aim 2 where prototypical and novel PAMs were tested as potential cytoprotective anti-inflammatory agents in an MCAO stroke model in young adult male rats. The results of these studies demonstrated that selective PAMs offer the opportunity for a multi-faceted therapy that affords persistent direct cytoprotection and management of central and peripheral anti-inflammatory pathways. This concept is novel, unconventional and substantively different from previous strategies with α7 agonists (e.g., 4OH-GTS-21, EVP-6124, ABT-126). PAMs are not in clinical trials (www.clinicaltrials.gov) for stroke and may provide health care providers with a new option to overcome limitations of available therapies. These scientific, clinical and commercial opportunities will be explored in our future endeavors.

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