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Boosting Endogenous Resistance of Brain to Ischemia

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Abstract Most survivors of ischemic stroke remain physically disabled and require prolonged rehabilitation. However, some stroke victims achieve a full neurological recovery suggesting that the human brain can defend itself against ischemic injury, but the protective mechanisms are unknown. This study used selective pharmacological agents and a rat model of cerebral ischemic stroke to detect endogenous brain protective mechanisms that require activation of α7 nicotinic acetylcholine receptors (nAChRs). This endogenous protection was found to be (1) limited to less severe injuries; (2) significantly augmented by intranasal administration of a positive allosteric modulator of α7 nAChRs, significantly reducing brain injury and neurological deficits after more severe ischemic injuries; and (3) reduced by inhibition of calcium/calmodulin-dependent kinase-II. The physiological role of α7 nAChRs remains largely unknown. The therapeutic activation of α7 nAChRs after cerebral ischemia may serve as an important physiological responsibility of these ubiquitous receptors and holds a significant translational potential.

Keywords PNU120596 · PNU-120596 · Nicotinic receptors · Choline · Allosteric modulator · Stroke · Neuroprotection

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Introduction

Choline and choline derivatives such as cytidine-5′-diphosphocholine (i.e., CDP-choline, also known as citicoline) stimulate brain phosphatidylcholine synthesis, tissue repair, and neurological function and have been viewed as highly bio-available and safe treatments after ischemic stroke, traumatic brain injury, and other neurological disorders. However, the recent failure of the International Citicoline Trial on Acute Stroke (ICTUS) [1] has dampened the enthusiasm for choline-based therapies in neurological disorders. Although choline and choline derivatives are important building blocks of cellular membranes in the brain, a different important property of endogenous choline may hold significant therapeutic potential [2–4]: i.e., the ability of choline to selectively activate α7 nicotinic acetylcholine receptors (nAChRs).

It is an intriguing concept that the mammalian brain can defend itself against ischemic injury and that the brain's inner capacity to resist ischemic injury can be elevated by the injury itself. In the absence of clinically effective post-stroke therapies, endogenous brain protection may serve as a compelling therapeutic guidance from nature. While post-stroke rehabilitation programs (e.g., targeted neural plasticity) can be highly effective [5], the potential positive impact of a successful early post-stroke therapy on the patient progress cannot be overestimated because the majority of cell damage and death occur within the first two hours after the onset of stroke. Among early post-stroke pharmacological interventions, the strategies that enhance the brain's innate capacity to resist ischemic injury are especially attractive because they could allow clinicians to team up with endogenous protective mechanisms that are already in place to selectively target cerebral ischemic injury with a high spatiotemporal precision [4, 6, 7].

The α7 nAChRs are commonly expressed in neuronal and non-neuronal tissues throughout the brain including brain

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regions that are highly susceptible to ischemic injury [8–13]. Despite their broad distribution in the brain, the physiological role of α7 nAChRs is not known, but the predominant absence of classical α7 nAChR synapses in the central nervous system suggests extensive pre- and/or extra-synaptic functions [14]. The existing literature indicates that activation of α7 nAChRs by endogenous (i.e., choline and ACh) or exogenous (e.g., nicotine) agonists benefits survival and function of individual neurons and neuronal networks in various in vivo, ex vivo, and in vitro models of cerebral ischemic stroke, traumatic brain injury, and other neurological disorders [2, 3, 12, 15–23]. However, the normal physiological levels of extracellular choline (<10 μM) [24, 25] and ACh (<10 nM) [26] are sub-threshold for α7 activation [27–29] due to the low potency of choline (EC_{50} ~0.5 mM) [30] and ACh (EC_{50} ~0.12 mM) [31], as well as their tendency to induce α7 desensitization [28, 32]: IC_{50} (choline) ~40 μM [28] and IC_{50} (ACh) ~1.7 μM [31]. As a result, the therapeutic effects of α7 agonists may develop tolerance [33, 34]. Therefore, endogenous nicotinic agonists have been dismissed as therapeutic agents even though the extracellular concentration of choline can be considerably elevated by energy deprivation, cellular dysfunction, injury, and death due to the cell membrane phosphatidylcholine breakdown [29, 35–40] providing a large source of this selective α7 agonist. Significant elevations in the extracellular level of choline have been recently demonstrated by direct measurements in the peri-infarct areas after a middle cerebral artery occlusion (MCAO) model of cerebral ischemic stroke in rats [37].

The positive allosteric modulation of α7 nAChRs has been proposed as a powerful alternative to the desensitizing and somewhat indiscriminate action of nicotinic agonists as an approach to counteracting neurocognitive deficits [41, 42], acute and chronic nociception [23, 33], and cerebral ischemia [2, 3, 6]. There are two types of positive allosteric modulators (PAMs) of α7 nAChR. Both types potentiate α7 responses, but only the type-II PAMs (abbreviated here as α7-PAMs) reverse α7 desensitization. The type-II PAMs, such as PNU120596 (abbreviated hereafter as PNU), are extremely selective for α7 nAChRs [6], but do not directly activate α7 nAChRs. Instead, PNU-like α7 PAMs enhance and prolong activation of α7 nAChRs by nicotinic agonists [41] including choline [27, 43].

We have previously reported that intravenous (i.v.) or subcutaneous (s.c.) PNU treatment up to 6 h after a transient 90-min suture MCAO significantly reduced brain injury and neurological deficits in young adult rats [2, 3]. We then have proposed that the therapeutic action of PNU most likely arises from augmenting the protective action of endogenous α7 agonists [2, 3] after MCAO in a spatiotemporally restricted manner exactly where and when it is most needed: near the site and time of focal cerebral ischemia [4, 6]. However, the direct evidence of endogenous α7-dependent protective action and its enhancement by α7-PAMs after MCAO have not been produced. In this study, we use selective pharmacological agents and a transient suture MCAO (tMCAO) in young adult rats to identify endogenous α7-dependent protection that can be significantly augmented by PNU.

While the i.v. treatment provides an unrestricted access to blood circulation and, thus, precise dose and time regimens can be ensured, it requires a fully functional cerebral circulation near the peri-infarct area to be fully effective. This may not be readily available after ischemic stroke even after a successful re-perfusion initiated by the recombinant tissue plasminogen activator (rtPA). By contrast, the intranasal (i.n.) treatment allows drug delivery to the brain via both a dense i.n. vasculature and the olfactory/trigeminal nerve pathway [44, 45]. Thus, the i.n. treatment may not be as dependent on the quality of cerebral circulation after ischemic injury. In this study, we explore the therapeutic potential of i.n. PNU treatment using a tMCAO in young adult rats and show that i.n. treatment significantly reduces both infarct volume and neurological deficits.

The mechanisms responsible for α7-mediated neuroprotection involve Ca^{2+}-dependent ERK1/2- [16, 46] and PI3K/Akt-dependent [47, 48] intracellular signaling pathways. Both pathways can be activated downstream of Ca^{2+}/calmodulin-dependent kinase-II (CaMKII) [16, 49, 50]. Because PNU only amplifies activation of α7 nAChRs, PNU is expected to amplify activation of these same intracellular pathways in neurons of the penumbra after focal ischemia. In this study, we use KN93, a potent inhibitor of CaMKII, to test the involvement of CaMKII in the effects of PNU after a MCAO.

Therefore, this study employs a tMCAO model of cerebral ischemic stroke in young adult rats to test the hypothesis that endogenous α7-dependent brain protection is available after focal cerebral ischemia and can be augmented by i.n. PNU via a CaMKII-dependent mechanism to significantly reduce brain injury and neurological deficits.

Materials and Methods

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. The UNTHSC animal facility is AAALAC accredited.

Transient Middle Cerebral Artery Occlusion

Our approach was to conduct tMCAO within a very narrow and confirmed window of experimental parameters to ensure stable ischemic insult and injury in each experiment. Once these parameters have been established and confirmed by cerebral blood flow (CBF) measurements (see Fig. 1), the parameters were then kept constant in all subsequent experiments supported by intermittent CBF measurements. Specifically, we used only 275–290-g Sprague Dawley rats purchased from Charles River and accommodated in our animal facility for 7 days after arrival. To initiate tMCAO, only 19-mm 4-0 monofilament nylon suture size was used. These restrictions ensured stability of experimental parameters across groups and reproducibility of outcomes during and after tMCAO.

A transient MCAO was induced using a suture technique as previously described [51]. The animals were anesthetized with 4 % isoflurane mixed with 67 % N_2O + 29 % O_2, delivered by a mask. After a midline incision in the neck, the left common carotid artery (CCA) was exposed and dissected. A 19-mm, 4-0 monofilament nylon suture was inserted from the CCA into the left internal carotid artery to occlude the origin of the left MCA. After occlusion, 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 136 animals were used in MCAO experiments. Of these, nine rats (i.e., 6.6 %) did not survive the surgery. This mortality factor is reflected by the differences in sample sizes of experimental groups. All the animals subjected to MCAO have shown ischemic infarcts 24 h after MCAO. In all experiments, the animals were randomly assigned to groups prior to group labeling and all data were reported. However, the allocation concealment was not used because only one researcher conducted all in vivo experiments and data analysis (the statistical analysis was conducted by a different researcher); thus, the study cannot be viewed as blinded.

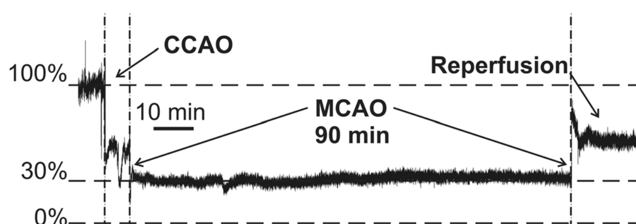

Fig. 1 Cerebral blood flow measurements. A typical example of regional cerebral blood flow (rCBF) measurements before, during, and after tMCAO. In these experiments, the rCBF was continuously recorded and evaluated using a laser-Doppler flowmeter (see the section "Materials and Methods") before and during occlusion of the common carotid artery (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 90-min MCAO was defined as an abrupt sustained reduction in rCBF by at least 70 % followed by a recovery to the flow level corresponding to CCAO

Cerebral Blood Flow Measurements

The rats were anesthetized with 4 % isoflurane mixed with 67 % N_2O + 29 % O_2, 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 probe (Periflux system 5000; Perimed, Stockholm, Sweden) was attached to the skull (−1 mm bregma; 5 mm medial-lateral) in the left hemisphere ipsilateral to the MCAO using superglue. Regional cerebral blood flow (rCBF) was recorded before and during CCA occlusion (CCAO) and then during and after MCAO 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 (Fig. 1).

Drugs

PNU-120596 was purchased from Selleck Chemicals (Houston, TX, USA). Methyllycaconitine (MLA) was purchased from Abcam Biochemicals (Cambridge, MA, USA). KN92/KN93 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Other chemicals were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Solutions and Drug Administration

Intravenous (i.v.) PNU (1 mg/kg) significantly reduces brain injury and neurological deficits after tMCAO [2, 3]. Because the proportion of injected drug volume absorbed in the intranasal (i.n.) cavity is uncertain as some of the drug may leak out of the nostrils or enter the gastrointestinal tract, the i.n. treatment may generate errors in dosing. To account for that potential loss of treatment, in this study, we used a higher predicted dose (10 mg/kg) for i.n. PNU administration as compared to i.v. 1 mg/kg PNU used previously [3].

To make a 0.2–1-M stock solution, PNU was dissolved in dimethyl sulfoxide (DMSO). Then, a 10-μl micropipette with a small tip was used to inject up to 60 μl of the stock solution (i.e., PNU + DMSO) or DMSO alone (i.e., vehicle) in one or both nostrils of the animal over a 2-min period to achieve the final dose of 10 mg/kg PNU. A water-based MLA stock solution (20 mM) or water alone (i.e., vehicle) was injected subcutaneously 10 min after the onset of MCAO. The amount of DMSO injected in each animal did not exceed 0.5 ml/kg.

Intracerebroventricular Administration of KN92/KN93

The anesthetized animals were implanted with an osmotic minipump (Alzet 1003D; Alza Corporation, CA, USA). The cannula was placed into the left lateral ventricle, 2.0 mm

lateral to the midline, 1.0 mm posterior to the bregma, and 4.0 mm ventral to the pial surface. KN92 or KN93 was dissolved in DMSO to 10 mM and diluted in artificial cerebrospinal fluid (ACSF) to achieve 20 μM. The animals were infused intracerebroventricularly for 24 h with 1 μl/h of KN93, KN92, or vehicle starting at 20 min prior to MCAO onset.

Focal Pressure Puffs of KN93 in Acute Brain Slices KN93 was dissolved in DMSO at 10 mM and then diluted in ACSF to achieve 20 μM. To apply KN93 focally, the tip of the application pipette similar to that used for patch-clamp recordings was positioned within 10 μm from the recorded neuron. Prolonged pressure puffs (12-s duration at 10-psi pressure) delivered KN93 focally to the recorded neurons, and the effects of KN93 on the persistent levels of α7 nAChR activity could be determined. Due to diffusion, the apparent concentration of KN93 that the recorded neuron is exposed to during a pressure puff may be somewhat lower than the original concentration (i.e., 20 μM) present in the application pipette. Thus, the application parameters used in this study (i.e., 12-s puff duration, 10-psi pressure, 10-μm pipette distance from the recorded neuron) have been optimized in our earlier study [52] to eliminate the drug concentration drop during each pressure puff.

Infarct Volume Measurements

The anesthetized animals were euthanized by decapitation 24 h after MCAO. The brains were removed and coronal sections (2-mm thickness) immersed in 2 % 2,3,5-triphenyltetrazolium chloride in saline for 20 min at 37 °C, then fixed for 2 h in 4 % paraformaldehyde. The infarct and contralateral hemisphere areas were measured using the ImageJ software. Section areas were multiplied by the distance between sections to obtain the respective volumes. The infarct volume was calculated as a percentage of the contralateral slice volume to account for a possible edema in the ischemic hemisphere.

Neurobehavioral Testing

The order of testing (Bederson➔cylinder➔ladder rung walking) was always the same to keep the testing conditions identical for all animals.

Bederson Test The Bederson score was used to assess the neurological deficit using a four-level scale: 0, normal; 1, forelimb flexion; 2, decreased resistance to lateral push; and 3, circling.

Cylinder Test Forelimb use was analyzed by observing the rat's movements over 3-min intervals in a transparent, 18-cm-wide, 30-cm-high poly-methyl-methacrylate cylinder. A mirror behind the cylinder made it possible to observe and record forelimb movements when the rat was facing away from the examiner. 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. Percentage use of the impaired limb was calculated.

Ladder Rung Walking Test The ladder rung walking test is sensitive for quantifying skilled locomotion. The degree of motor dysfunction after MCAO was measured by counting the number of foot faults of the impaired limbs per round [3]. Baseline and post-operative testing sessions consisted of three traverses across the ladder. An error was scored for any foot slip or misstep. The number of errors of the affected forelimb and hindlimb in each trial was counted. The mean number of errors in three traverses was calculated.

Measurements of PNU in Blood and Brain Samples Using Mass Spectrometry

The young adult male Sprague Dawley rats (~280 g) were subjected to i.n. PNU (10 mg/kg). The animals were then anesthetized with 4 % isoflurane mixed with 67 % N_2O + 29 % O_2, delivered by a mask 3 h after PNU injection, and blood samples were collected intracardially. The animals were then euthanized by decapitation; brain tissues from the motor-somatosensory cortical areas and the corresponding striatal regions were rapidly collected. All the samples were immediately flash frozen in liquid nitrogen and stored at −80 °C at UNTHSC (Fort Worth, TX, USA). The samples were then shipped on dry ice overnight to Carbon Dynamics Institute, LLC (Sherman, IL, USA) for mass spectrometry (MS) determinations. Subsamples were macerated with methanol for 3 min, and the resultant slurry was centrifuged at 4 °C for 15 min at 15,000×*g*. The blood samples were centrifuged at 4 °C for 15 min at 19,000×*g*. The resultant supernatant for both preparations was further purified using a mixed-mode polymeric exchange solid-phase extraction (SPE). The eluate was lyophilized and reconstituted in the initial mobile phase. PNU was analyzed by a 1-μl injection of each sample aliquot into a Waters 2795 Alliance equipped with an XBridge BEH130 C_{18} 2.1 mm × 150 mm, 3.5-μm particulate column (110 Å) at a flow rate of 0.150 ml/min; a binary mobile phase gradient at 50:50 (Δ2.5 %) A:B in 20 min (mobile phase A = 0.01 % formic acid; mobile phase B = acetonitrile) until proper peak shape, separation, and reduction of interferences were obtained (Fig. 2a, b). A Waters Quattro Ultima triple stage quadrupole mass spectrometer equipped with an electrospray ionization source was calibrated with methionine-arginine-phenylalanine-alanine peptide for both

the single and double charge states (*m*/*z* 524.2 and 262.6, respectively) to provide a 0.1-amu mass accuracy for each $[M+H]^+$ parent ion (m_p^+) (Fig. 2a–c). The m_p^+ ion 312 from Q1 was passed through a collision chamber (Q2), operating in radio-frequency-only mode (25 V), and focused for subsequent ionization as a consequence of the collision of the rapidly moving m_p^+ with an inert gas (Ar) at ~1.8 mTorr to produce the unique product fragmentation spectrum that was subsequently scanned through a third mass filter (Q3) where selected daughter ions (m_d^+) were collected in multiple resonance monitoring (transitions 312 > 173, 188, and 158) (Fig. 2d). A total of six animals were used for evaluation of PNU levels in the blood and brain tissue samples.

Acute Hypothalamic Slices: Tissue Preparation and Whole-Cell Patch-Clamp Recordings

Three to four coronal whole-brain slices (260-μm thickness) containing the hypothalamic tuberomammillary (TM) nuclei were cut in a sucrose-rich solution at 3 °C using a 7000smz-2 vibrating microtome (Campden Instruments Ltd., Lafayette, IN, USA). The sucrose-rich solution was of the following composition (in mM): 250 sucrose, 3 KCl, 1.23 NaH_2PO_4, 5 $MgCl_2$, 0.5 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose (pH 7.4, when bubbled with carbogen, i.e., 95 % O_2 and 5 % CO_2). The brain slices were then transferred to a temporary storage chamber where they were maintained for ~1 h at 30 °C and then up to 8 h at room temperature in an oxygenated ACSF of the following composition (in mM): 125 NaCl, 3 KCl, 1.23 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose (pH 7.4, when bubbled with carbogen).

For patch-clamp recordings, slices were transferred to a recording chamber perfused with ACSF at room temperature. Recordings were made using a MultiClamp-700B amplifier and Digidata-1440A A/D converter (Molecular Devices, Sunnyvale, CA, USA). Data were sampled at 10 kHz and filtered at 2 kHz. Recording pipettes were pulled using a Sutter P-97 puller (Sutter Instruments, Novato, CA, USA). The pipette resistance was 4–6 MΩ. After formation of a giga-seal (>2 GΩ), the whole-cell configuration was established. Choline and PNU-120596 were added to ACSF, and KN93 was pressure applied (at 5–8 psi) via a picospritzer (Parker Hannifin, Cleveland, OH, USA) using pipettes identical to those used for recordings. The application pipette tips were positioned ~10 μm away from the recorded neuron. Recordings were conducted at room temperature. The membrane voltage was clamped at −60 mV in all experiments. The extracellular solution was identical to ACSF that was used for

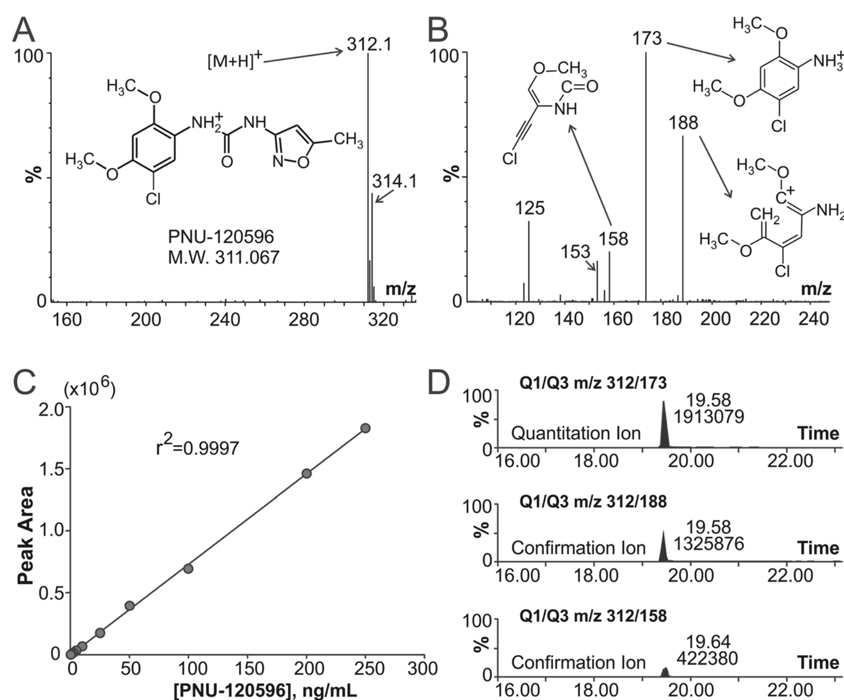

Fig. 2 Evaluation of PNU levels in the brain and blood samples using mass spectrometry. The samples of blood as well as cortical and striatal tissues were collected 3 h after i.n. injection of 10 mg/kg PNU (see "Materials and Methods"). **a** Full mass spectrum of PNU showing a $[M+H]^+$ at *m*/*z* 312.1 and the 2-Da shift due to chloride at 314.1. The structure shown is one possible protonated molecule. **b** Production mass spectrum of PNU showing unique fragment ions from a collision-induced dissociation at 25 V. The product ion structures are theoretical fragments using distonic ion formations and the following common reactions: resonance reactions, ring formations, and hydride shifts. **c** A typical calibration curve for the determination of PNU in tissue and plasma samples. The standards were run at the following concentrations: 0.3, 1.0, 2.5, 5.0, 10, 25, 50, 100, 200, and 250 ng/ml. **d** Typical chromatograms for the determination of PNU in tissue. The Q1/Q3 *m*/*z* 312/173 was used as the quantitation ion while the Q1/Q3 *m*/*z* 312/188 and Q1/Q3 *m*/*z* 312/158 were used for further confirmation

preparation and storage of slices. The recording electrodes were filled with an intracellular solution of the following composition (in mM): 140 K-gluconate, 1 NaCl, 2 $MgCl_2$, 2 Mg-ATP, 0.3 Na-GTP, and 10 HEPES (pH 7.4 adjusted with KOH). The membrane voltage was not corrected for the liquid junction potential: V_{LJ} ~16 mV. A custom-made perfusion pump was used to perfuse slices in the recording chamber at a rate of 1.5 ml/min.

Statistical Analysis

Data obtained from behavioral assays (i.e., based on a scoring system) were analyzed using two-tailed non-parametric Kruskal-Wallis or Mann-Whitney tests. Statistical significance of continuous data (i.e., infarct volume) was defined by a one-way ANOVA followed by individual *F*-test comparisons among groups or a two-tailed Student's test. The alpha level was set at 0.05. The results are presented as mean ±S.E.M.

Results

Evaluation of PNU Levels in Blood and Brain Tissue Samples

The samples of blood and brain tissues were collected 3 h after i.n. 10 mg/kg PNU injection and analyzed for PNU content using mass spectrometry (see "Materials and Methods"). The analysis demonstrated the presence of PNU in all the samples at the following concentrations ($n=6$): 2.2 ± 0.6 ng/ml (blood), 10.8 ± 2.8 ng/g (cortex), and 17.4 ± 5.5 ng/g (striatum). The statistical significance of differences in the levels of PNU in blood, cortex, and striatum were evaluated using a one-way ANOVA with individual *F*-test comparisons. This analysis indicated that the concentration of PNU in the samples significantly depends on the sample type: $F(2, 15)=4.551$, $p=0.0285$, $n=6$. The corresponding post-test comparisons detected the following levels of significance among PNU concentrations in the sample groups ($n=6$ per group): cortex vs. striatum ($t=1.304$, df = 15, $p=0.2120$), blood vs. cortex ($t=1.705$, df = 15, $p=0.1089$), and blood vs. striatum ($t=3.008$, df = 15, $p=0.0088$). Thus, cortical and striatal brain tissues appear to accumulate PNU equally. However, the concentration of PNU in striatal brain tissues was significantly greater than that found in blood which is consistent with our previous results [2].

Endogenous α7-Dependent Protection After tMCAO

I.V. PNU treatments after a 90-min tMCAO have been demonstrated to significantly reduce infarct volume and neurological deficits [2, 3], and these effects of PNU have been proposed to arise from the augmented protective action of α7 nAChRs activated by extracellular choline elevated focally by ischemic injury [2, 6]. However, the presence of endogenous protection in untreated animals and its limitations have not been directly detected. To identify this endogenous protective action in the absence of PNU, we used s.c. 10 mg/kg MLA, a selective α7 antagonist. The animals were randomly selected, subjected to a 90-min tMCAO, and treated either with s.c. saline or s.c. MLA 10 min after tMCAO onset. Infarct volume and neurological deficits were determined 24 h after tMCAO.

MLA failed to significantly increase infarct volume after a 90-min tMCAO (unpaired, two-tailed Student's *t* test; $t=0.3572$; df = 12; $p=0.7271$; 95 % CI −10.75, 7.72; $n=6$–8; Fig. 3a, b), suggesting that α7 activation does not produce significant protective action in the absence of PNU in this experimental paradigm. These results also suggested that ischemic brain injury resulting from a 90-min tMCAO is too extensive (~45 %; Fig. 3a, b) for endogenous protection, and thus, the use of PNU or other PNU-like α7-PAMs is absolutely required to achieve significant α7-dependent protection in these settings. To test whether a less severe injury is more receptive to endogenous protection, the animals were subjected to a 60-min tMCAO and treated with s.c. saline or s.c. MLA 10 min after tMCAO onset. Infarct volume and neurological deficits were determined 24 h after tMCAO. Reducing the duration of tMCAO from 90 to 60 min resulted in a ~50 % reduction of infarct volume (~22 vs. ~45 %; open columns in Fig. 3c, d vs. a, b). MLA significantly increased infarct volume after a 60-min tMCAO from 20 to 35 % (unpaired, two-tailed Student's *t* test; $t=2.272$; df = 14; $p=0.0393$; 95 % CI 0.7724, 26.72; $n=7$–9; Fig. 3c, d), an increase of ~75 %, supporting our hypothesis that endogenous α7-dependent protection is more effective after a less severe ischemic injury. The results of behavioral tests were consistent with these data: MLA failed to produce significant neurological deficits after a 90-min tMCAO (unpaired, two-tailed Mann-Whitney, $n=11$–12): Bederson ($p=0.3556$; 95 % CI 0.0, 1.0), cylinder ($p=0.6127$; 95 % CI −10.0, 2.5), and limb placing ($p=0.4478$; 95 % CI −1.0, 0.0) (Fig. 4a–c). By contrast, significant neurological deficits after MLA were detected in two tests after a 60-min tMCAO (unpaired, two-tailed Mann-Whitney, $n=11$–12): Bederson ($p=0.0427$; 95 % CI 0.0, 2.0) and cylinder ($p=0.0227$; 95 % CI −45.0, 0.0; Fig. 4d, e). The remaining test (limb placing) conducted after a 60-min tMCAO failed to detect significant differences between MLA-treated and control groups: $p=0.5940$; 95 % CI −7.0, 2.0 (Fig. 4f). Therefore, these results revealed significant endogenous α7-dependent brain protection after a 60-min tMCAO and suggested that this protective action of α7 nAChRs does not extend to more severe injuries modeled here by a 90-min tMCAO.

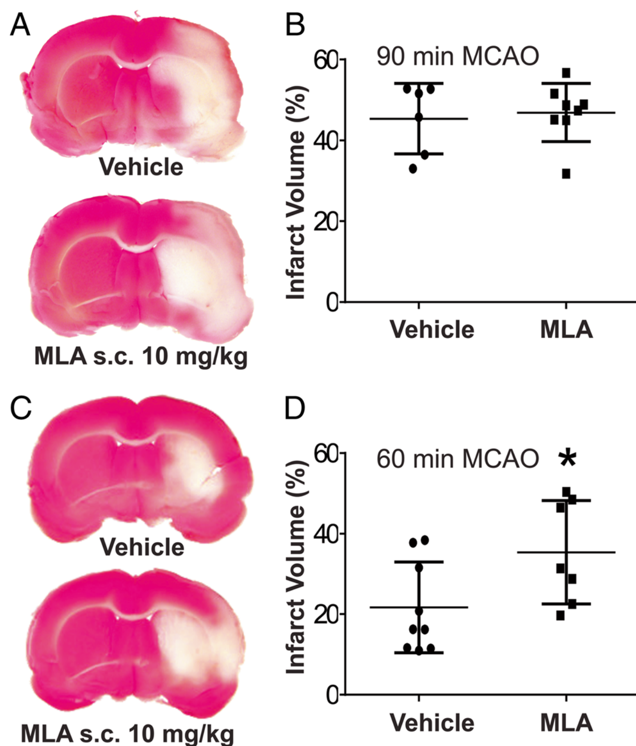

Fig. 3 Endogenous α7-dependent protection after tMCAO: ischemic infarct volume. Methyllycaconitine (MLA; s.c., 10 mg/kg), a selective α7 nAChR antagonist, failed to significantly increase infarct volume after a 90-min tMCAO (unpaired, two-tailed Student's t test; $t=0.3572$; df = 12; $p=0.7271$; 95 % CI −10.75, 7.72; $n=6$–8; **a**–**b**) suggesting that α7 activation does not significantly save neurons in the absence of PNU in this experimental paradigm (*circles* vs. *squares*; **b**). These results also suggested that ischemic brain injury resulting from a 90-min tMCAO is too extensive (~45 %; **a**–**b**) and the use of PNU-like α7-PAMs is absolutely required to achieve significant α7-dependent protection in these settings. To test whether a less severe injury is more receptive to endogenous protective mechanisms, we used a 60-min tMCAO. Reducing the duration of tMCAO from 90 to 60 min resulted in a ~50 % reduction of infarct volume (~22 vs. ~45 %; *circles*; **b** and **d**). MLA significantly increased infarct volume after a 60-min tMCAO from 20 to 35 % (unpaired, two-tailed Student's t test; $t=2.272$; df = 14; $p=0.0393$; 95 % CI 0.7724, 26.72; $n=7$–9; **c**–**d**), an increase of ~75 % (*circles* vs. *squares*; **d**), indicating that endogenous α7-dependent protection is greater after a less severe ischemic injury. Statistical significance was defined by the p value (*<0.05)

Protective Action of i.n. PNU

I.N. drug delivery utilizes both the vast i.n. vasculature and the olfactory/trigeminal nerves [44, 45]. Thus, we expected to detect a high therapeutic utility of i.n. PNU treatment after tMCAO. To test this hypothesis, the animals were subjected to a 90-min tMCAO and treated with either i.n. vehicle (i.e., DMSO) or i.n. PNU (10 mg/kg) 30 min after tMCAO onset with or without MLA pre-treatment (i.e., PNU ± MLA groups). To account for a potential loss of therapeutic action due to a leak of PNU out of the animals' nostrils or entering the gastrointestinal tract, the i.n. PNU dose was increased tenfold as compared to the dose used in our previous study utilizing intravenous injections [3]. Infarct volume and neurological deficits were determined 24 h after tMCAO. I.N. PNU significantly reduced infarct volume (one-way ANOVA with individual F-test comparisons; $F(2, 12)=9.730$, $p=0.0031$, $n=5$; squares vs. circles, Fig. 5a, b) and neurological deficits (Kruskal-Wallis with Dunn's multiple comparisons test; Fig. 5c–e) in control ($n=10$) vs. PNU-treated ($n=13$) groups in the following behavioral tests: Bederson ($H=23.00$, $p<0.0001$; Fig. 5c), cylinder ($H=20.94$, $p<0.0001$; Fig. 5d), and limb placing ($H=25.85$, $p<0.0001$; Fig. 5e). The corresponding post-test multiple comparisons tests detected the following levels of significance among vehicle- and PNU-treated groups: infarct volume ($t=3.889$, df = 12, $p=0.0022$), Bederson ($p=0.0002$), cylinder ($p=0.0036$), and limb placing ($p<0.0001$). These effects of PNU were reversed by MLA (s.c. 10 mg/kg; $n=12$) injected 10 min after tMCAO onset (triangles vs. squares) as evidenced from significant effects produced by MLA (i.e., PNU vs. PNU + MLA groups) in all tests: i.e., infarct volume ($t=3.748$, df = 12, $p=0.0028$), Bederson ($p=0.0001$), cylinder ($p<0.0001$), and limb placing ($p<0.0001$). Significant differences were not detected between control and MLA-treated groups in all tests ($p>0.05$, circles vs. triangles; Fig. 5b–e). These results support the high therapeutic utility of i.n. PNU after a 90-min tMCAO and confirm the involvement of α7 nAChRs.

KN93 Inhibits Protective Effects of i.n. PNU

Because PNU only amplifies α7 activation by agonists [41], we hypothesize that protective action by PNU after tMCAO arises from amplified CaMKII-dependent signaling pathways downstream of α7 activation [16, 49, 50]. To test this hypothesis, we used KN93, a potent inhibitor of CaMKII and its phosphorylating activity, that prevents association of calmodulin with CaMK without any effects on PKA, PKC, and Ca^{2+}-phosphodiesterase [53]. Intracerebroventricular (i.c.v.) infusion of KN93 completely eliminated the effects of i.n. PNU after a 90-min tMCAO, as evidenced by statistically significant changes in infarct volume (one-way ANOVA with individual F-test comparisons; $F(2,14)=5.522$, $p=0.0171$, $n=5$–7; Fig. 6a, b) and neurological deficits (Kruskal-Wallis with Dunn's multiple comparisons test; Fig. 6c–e) among PNU-, PNU + KN92-, and PNU + KN93-treated groups ($n=8$–9) in the following behavioral tests: Bederson ($H=8.798$, $p=0.0123$; Fig. 6c), cylinder ($H=13.27$, $p=0.0013$; Fig. 6d), and limb placing ($H=11.81$, $p=0.0027$; Fig. 6e). The corresponding post-test multiple comparisons tests detected the following levels of significance among PNU- and PNU + KN93-treated groups (circles vs. squares): infarct volume ($t=2.943$, df = 14, $p=0.0107$), Bederson ($p<0.05$), cylinder ($p<0.05$), and limb placing ($p<0.05$). A similar i.c.v. treatment with KN92, an inactive analogue of KN93, failed to significantly alter the effects of PNU in all tests ($p>0.05$;

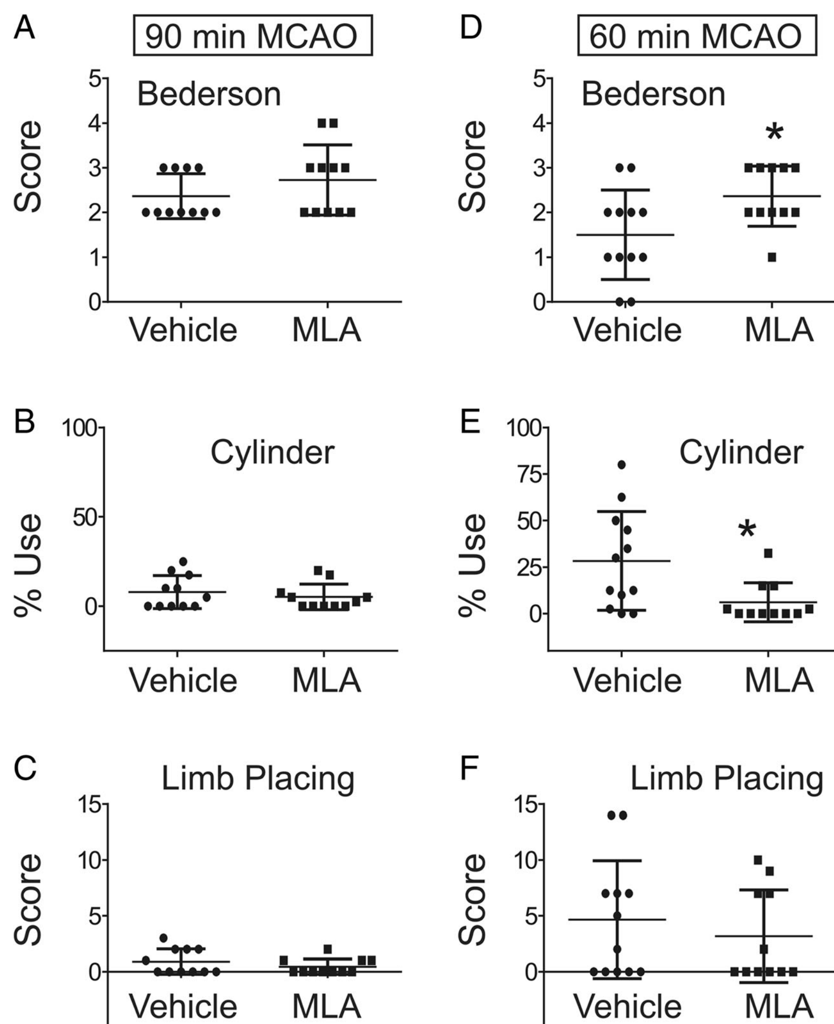

Fig. 4 Endogenous α7-dependent protection after tMCAO: behavioral tests. The results of behavioral tests were consistent with infarct volume data: MLA (*squares* as compared to *circles*; i.e., vehicle groups) failed to produce significant neurological deficits after a 90-min tMCAO (unpaired, two-tailed Mann-Whitney; $n = 11–12$; **a**–**c**): Bederson ($p = 0.3556$; 95 % CI 0.0, 1.0), cylinder ($p = 0.6127$; 95 % CI −10.0, 2.5), and limb placing ($p = 0.4478$; 95 % CI −1.0, 0.0). By contrast, significant neurological deficits after MLA treatment were detected in two tests after a 60-min tMCAO (unpaired, two-tailed Mann-Whitney; $n = 11–12$; **d**–**e**): Bederson ($p = 0.0427$; 95 % CI 0.0, 2.0) and cylinder ($p = 0.0227$; 95 % CI −45.0, 0.0). The remaining test (limb placing) conducted after a 60-min tMCAO failed to detect significant differences between MLA-treated and control groups: $p = 0.5940$; 95 % CI −7.0, 2.0 (**f**). Statistical significance was defined by the *p* value (*<0.05)

triangles vs. circles, Fig. 6b–e). Accordingly, significant differences were detected between PNU + KN92 and PNU + KN93 groups in all tests ($p < 0.05$; triangles vs. squares, Fig. 6b–e).

A statistically significant increase in infarct volume was detected after i.c.v. KN93 treatment alone (i.e., without PNU) (unpaired, two-tailed Student's *t* test; $t = 2.373$; df = 9; $p = 0.0417$; 95 % CI 0.4539, 18.97; $n = 5–6$; Fig. 6f–h), supporting the presence of endogenous α7-dependent protective action after tMCAO. These data support the requirement for CaMKII activity downstream of α7 activation in generating the therapeutic efficacy of both endogenous α7-dependent brain protection and PNU after a 90-min tMCAO.

KN93 Does Not Directly Block α7 nAChRs

To eliminate a possibility that KN93 directly blocks α7 nAChRs, we conducted electrophysiological patch-clamp recordings of α7 nAChR-mediated responses using hypothalamic tuberomammillary (TM) neurons in acute brain slices. TM neurons act as a unique reliable source of native functional homomeric α7 nAChRs [27, 31, 32, 54]. Acute brain slices containing the TM nuclei were perfused with 1 μM PNU and 20 μM choline to model conditions and persistent activation of α7 nAChRs that occurs in the ischemic peri-infarct areas in our in vivo experiments after tMCAO and i.n. PNU treatment [2, 37]. Prior to patch-clamp recordings, the acute brain slices were pre-incubated in 1 μM PNU for at least 50 min to equilibrate the concentration of PNU within the slice as we have justified previously [27, 43]. Choline (20 μM) added to the artificial cerebrospinal fluid (ACSF) produced a clearly detectable persistent whole-cell current upon its entrance in the recording chamber (Fig. 7a). This choline-mediated current stabilized shortly after initiation, as we discussed previously [27, 43]. KN93 (20 μM) was dissolved in ACSF containing 1 μM PNU + 20 μM choline and administered focally to the recorded neuron using a pressure application pipette identical to that used for patch-clamp recordings. The puff pipette was positioned within

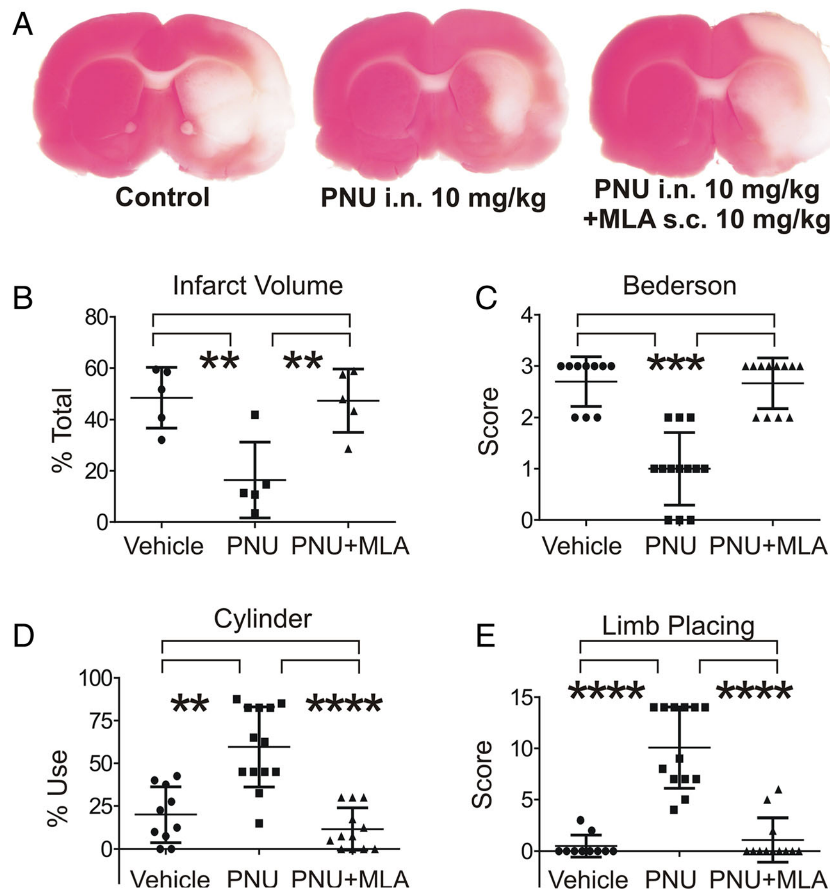

Fig. 5 Significant brain protection by i.n. PNU. Animals were subjected to a 90-min tMCAO and randomly assigned to three groups treated with i.n. vehicle or i.n. PNU (10 mg/kg) 30 min after the tMCAO onset with or without MLA pre-treatment (i.e., PNU ± MLA groups). Infarct volume and neurological deficits were determined 24 h after tMCAO. I.N. PNU significantly reduced infarct volume (one-way ANOVA with individual *F*-test comparisons; $F(2,12) = 9.730$, $p = 0.0031$, $n = 5$; *squares* vs. *circles*; **a**–**b**) and neurological deficits (Kruskal-Wallis with Dunn's multiple comparisons test; **c**–**e**) in control ($n = 10$) vs. PNU-treated ($n = 13$) groups in the following behavioral tests: Bederson ($H = 23.00$, $p < 0.0001$; **c**), cylinder ($H = 20.94$, $p < 0.0001$; **d**), and limb placing ($H = 25.85$, $p < 0.0001$; **e**). The corresponding post-test multiple comparisons tests detected the following levels of significance among vehicle- and PNU-treated groups: infarct volume ($t = 3.889$, df = 12, $p = 0.0022$), Bederson ($p = 0.0002$), cylinder ($p = 0.0036$), and limb placing ($p < 0.0001$). These effects of PNU were reversed by MLA (s.c. 10 mg/kg; $n = 12$) injected 10 min after tMCAO onset (*triangles* vs. *squares*) as evidenced from significant effects produced by MLA (i.e., PNU vs. PNU + MLA groups) in all tests: i.e., infarct volume ($t = 3.748$, df = 12, $p = 0.0028$), Bederson ($p = 0.0001$), cylinder ($p < 0.0001$), and limb placing ($p < 0.0001$). Significant differences were not detected between control and MLA-treated groups in all tests ($p > 0.05$, *circles* vs. *triangles*; **b**–**e**). Statistical significance was defined by the *p* values (**<0.01; ***<0.001; ****<0.0001)

10 μm from the recorded neuron and connected to a picospritzer. Prolonged pressure puffs (12-s duration at 10-psi pressure) of KN93 (gray box above current traces) did not inhibit the level of persistent α7 nAChR activation (Fig. 7b). On the contrary, a small but significant increase in the current levels was detected (Fig. 7c). Data were recorded from $n = 6$ TM neurons obtained from $m = 2$ preparations/rats. The level of activation was quantified by measuring the mean current over two time windows of equal durations: i.e., T1 (12 s prior to KN93) and T2 (12 s during KN93) (Fig. 7b). These experiments determined that 20 μM KN93 does not directly inhibit α7 nAChR activity mediated by 20 μM choline in the presence of 1 μM PNU. The concentration of KN93 selected for these experiments (i.e., 20 μM) was similar to that used in our i.c.v. injections.

Discussion

This study used selective pharmacological agents and a tMCAO model of ischemic stroke in young adult rats to detect endogenous brain protection that requires activation of α7 nAChRs during the acute post-tMCAO phase, i.e., in the first 24 h after tMCAO. The therapeutic utility of this protection was found to be limited to less severe ischemic injuries modeled in this study by a 60-min tMCAO, and did not appear to extend to more severe injuries modeled by a 90-min tMCAO (Figs. 3 and 4). An immediate implication of these

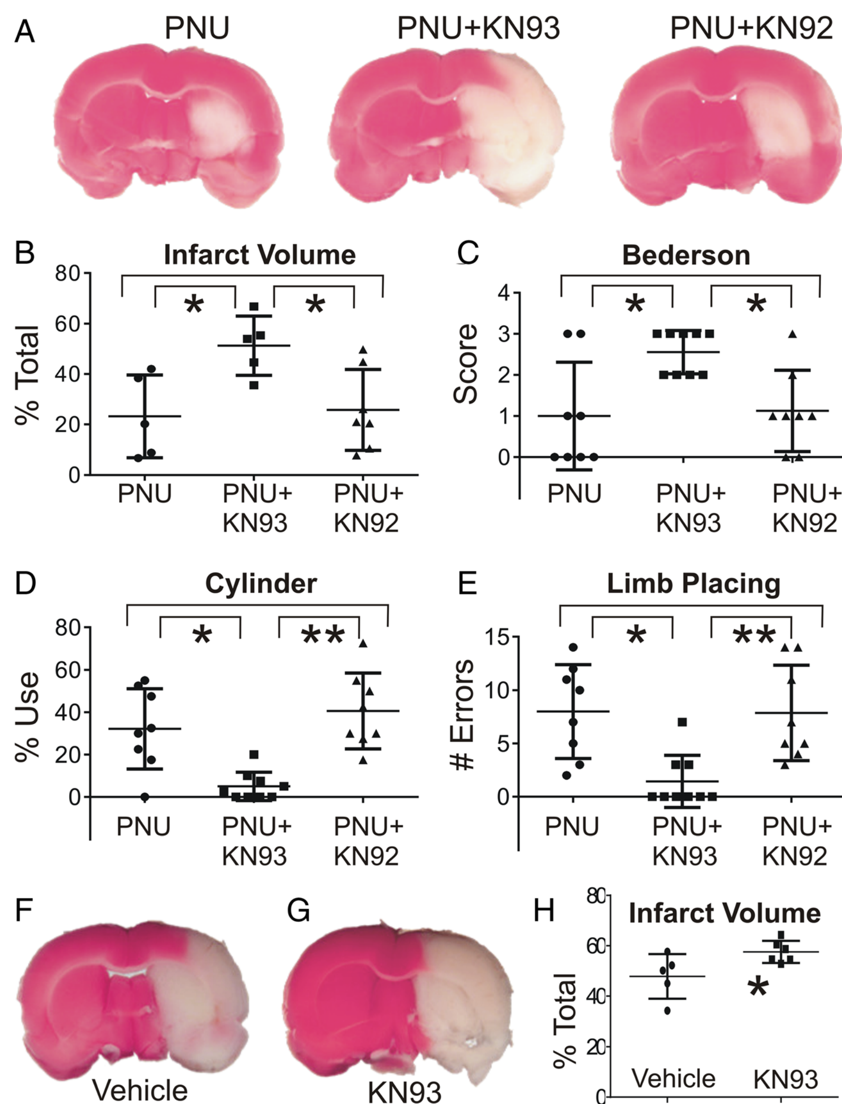

Fig. 6 KN93 inhibits protective effects of i.n. PNU. I.C.V. infusion of KN93 completely eliminated protective effects of i.n. PNU after a 90-min tMCAO, as evidenced by statistically significant changes in infarct volume (one-way ANOVA with individual F-test comparisons; $F(2,14)=5.522$, $p=0.0171$, $n=5$–7; **a**–**b**) and neurological deficits (Kruskal-Wallis with Dunn's multiple comparisons test; **c**–**e**) among PNU-, PNU + KN92-, and PNU + KN93-treated groups ($n=8$–9) in the following behavioral tests: Bederson ($H=8.798$, $p=0.0123$; **c**), cylinder ($H=13.27$, $p=0.0013$; **d**), and limb placing ($H=11.81$, $p=0.0027$; **e**). The corresponding post-test multiple comparisons tests detected the following levels of significance among PNU- and PNU + KN93-treated groups (*circles* vs. *squares*): infarct volume ($t=2.943$, df = 14, $p=0.0107$), Bederson ($p<0.05$), cylinder ($p<0.05$), and limb placing ($p<0.05$). A similar i.c.v. treatment with KN92, an inactive analogue of KN93, failed to significantly alter the effects of PNU in all tests ($p>0.05$; *triangles* vs. *circles*, **b**–**e**). Accordingly, significant differences were detected between PNU + KN92 and PNU + KN93 groups in all tests ($p<0.05$; *triangles* vs. *squares*, **b**–**e**). A statistically significant increase in infarct volume (**f**, **g**) was detected after i.c.v. KN93 treatment alone (i.e., without PNU) (unpaired, two-tailed Student's t test; $t=2.373$; df = 9; $p=0.0417$; 95 % CI 0.4539, 18.97; $n=5$–6; *squares* vs. *circles*, **h**), supporting the presence of endogenous α7-dependent protection after tMCAO. Statistical significance was defined by the p values (*<0.05; **<0.01)

results is that truly unprotected animals may not exist, at least in the first 24 h after tMCAO, because endogenous α7-dependent mechanisms protect both brain tissues and neurological functions even in untreated animals subjected to tMCAO. These results also suggest that ischemic brain injury resulting from a 90-min tMCAO is too extensive (~45 %; Fig. 3) and resistant to endogenous α7-dependent protection. Thus, the use of α7-PAMs is absolutely required to achieve significant α7-dependent protection in these or similar settings (Fig. 5).

Our experimental protocol employed a 24-h delay between tMCAO and behavioral/histological assays. A potential limitation of this approach is that ischemic brain injury may not be fully matured 24 h after tMCAO, and thus, the long-term (e.g., days, weeks) efficacy of PNU treatment cannot be reliably predicted. However, we view the initial 24 h after stroke to be especially critical because a timely administered PNU

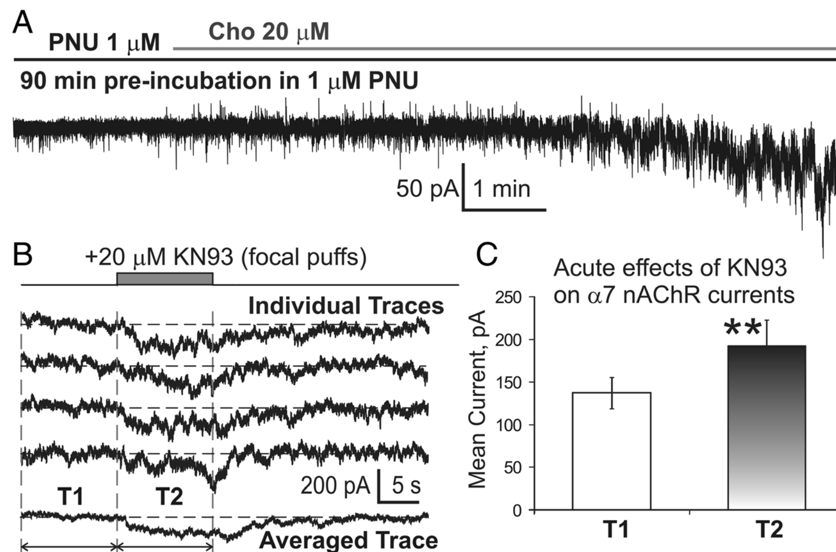

Fig. 7 KN93 does not directly block α7 nAChRs. To test whether KN93 directly blocks α7 nAChRs, electrophysiological patch-clamp recordings of α7 nAChR-mediated responses were conducted using hypothalamic TM neurons that express high densities of native functional α7 nAChRs [31, 54]. Acute hypothalamic slices were pre-incubated in 1 μM PNU for 50 min to equilibrate the concentration of PNU within the slice. An addition of 20 μM choline to the ACSF produced a clearly detectable persistent whole-cell current (**a**) that stabilized shortly after initiation. KN93 (20 μM) was dissolved in ACSF containing 1 μM PNU + 20 μM choline and focally applied by pressure. Prolonged pressure puffs (12 s duration at 10 psi) of KN93 (*gray box above current traces*) did not inhibit the level of persistent α7 nAChR activation (**b**). On the contrary, a small but significant increase in the current levels was detected (**c**). Data were recorded from $n = 6$ TM neurons obtained from $m = 2$ preparations/rats. The level of activation was quantified by measuring the mean current over the time windows with equal duration: i.e., T1 (12 s prior to KN93) and T2 (12 s during KN93) (**b**). Statistical significance was defined by the p value (**<0.01)

treatment that reduces brain injury in the first few hours after stroke may impede stroke maturation and help reduce long-term post-stroke sequelae including edema, synaptic degeneration, and excessive inflammation and, thus, may improve clinical outcomes. The long-term therapeutic efficacy of PNU-like α7-PAMs is being investigated in our ongoing research.

The rational basis for the therapeutic use of PNU or PNU-like α7-PAMs as a post-stroke treatment in mammals is tripartite [4, 6, 55] and arises from (1) the potent neuroprotective effects of α7 nAChR activation [2, 3, 12, 15–23, 56]; (2) the presence of endogenous extracellular choline, elevated by ischemic injury [6, 36, 37, 39, 40]; and (3) the ubiquitous expression of α7 nAChRs in the mammalian brain, including regions highly vulnerable to ischemia [8–10]. Because PNU does not directly activate α7 nAChRs but only amplifies α7 activation [27] by injury-elevated choline near the site and time of the injury [4, 6], the challenge of a timely focal delivery of PNU treatment to the ischemic penumbra is naturally resolved: while systemically applied PNU is somewhat homogenously distributed throughout the body by circulation, its therapeutic effects would take place mostly, or even exclusively, in the brain areas with elevated extracellular choline [37, 40], i.e., exactly where and when it is most needed: in the ischemic penumbra, post-injury. Therefore, the high spatio-temporal precision of α7-PAM therapies after ischemic stroke is an important benefit of this approach.

Because α7 nAChRs and phosphatidylcholine-based cell membranes are common in the mammalian brain, including rodents and humans [30, 57], the endogenous α7-dependent brain protection may act as an evolutionarily shaped common mammalian mechanism that can be significantly amplified by PNU-like α7-PAMs. Thus, there is a rational basis to expect that the therapeutic utility of PNU revealed in our studies in young adult rats will extend to the human brain. Furthermore, in addition to anti-ischemic action, PNU-like α7-PAMs exhibit pro-cognitive [34] and anti-nociceptive [33] properties by the same mechanism: augmenting the endogenous α7-dependent cholinergic tone.

While in our previous studies PNU was administered either i.v. or s.c [2, 3, 55], in this study, we explored the i.n. route. One potential advantage of i.n. treatment is that it allows drugs be delivered to the brain via both circulation (because of a dense i.n. vasculature) and direct transport (because of a direct olfactory/trigeminal nerve pathway). In ischemic stroke, a fully functional cerebral circulation in the peri-infarct areas is unlikely even after rtPA treatment and, thus, the dual delivery pathway provided by i.n. treatment may yield additional benefits and enhance treatment efficacy. One disadvantage of i.n. treatments is potential errors in dosing and, thus, a potential need to use higher drug concentrations because the proportion of injected drug volume absorbed in the i.n. cavity is uncertain as some of the drug may leak out of the nostrils or enter the gastrointestinal tract. To account for that potential loss of

treatment, we used a higher dose (10 mg/kg) for i.n. PNU administration in this study as compared to 1 mg/kg i.v. PNU used previously [3]. Nevertheless, our data suggest a high therapeutic utility for i.n. PNU treatment that is worth further consideration.

The levels of PNU found in the blood (~2 ng/ml), cortical (~11 ng/g), and striatal (~17 ng/g) samples collected 3 h after i.n. injection at 10 mg/kg were lower than those found in the blood (~63 ng/ml), cortical (~158 ng/g), and striatal (~150 ng/g) samples collected 3 h after s.c. injection at 30 mg/kg and reported previously [2]. These differences may reflect lower doses and/or lower bioavailability of i.n. PNU as well as delays in PNU delivery to blood and brain tissues after i.n. injection as compared to s.c. injection. Nevertheless, the therapeutic efficacy of i.n. 10 mg/kg PNU (Fig. 5a, b; this study) was found to be very similar to that of i.v. 1 mg/kg PNU (Fig. 5d–f [2]), suggesting that i.n. PNU administration presents a compelling strategy.

The results of this study support our hypothesis that the mechanism underlying therapeutic effects of PNU after focal cerebral ischemia involves a desensitization reversal and enhanced activation of α7 nAChRs [27, 41, 43] in the ischemic penumbra and peri-infarct areas leading to activation of CaMKII-dependent intracellular signaling pathways and therapeutic efficacy (Fig. 6). These mechanisms are directly linked to α7 nAChR activation as proposed previously for the action of α7 agonists [16, 46–48]. Because PNU, as a typical α7-PAM, is highly selective and does not directly activate any receptors but simply enhances activation of α7 nAChRs by nicotinic agonists [6, 27, 41, 43], these results are consistent with the notion that the effects of PNU are directly derived from enhanced activation of α7 nAChRs and α7-dependent intracellular pathways. The finding that KN93 significantly increases infarct volume without PNU treatment (Fig. 6f–h) supports the presence of endogenous CaMKII-dependent protection after MCAO that may include an α7-dependent component (Fig. 5). The possibility that KN93 reversed the effects of PNU by a direct inhibition of α7 nAChRs was eliminated by electrophysiological experiments using α7-expressing TM neurons in acute slices [54] where direct inhibitory effects of KN93 on persistent α7-mediated currents were not detected (Fig. 7). These results are consistent with the predicted inhibition of CaMKII by KN93 and a reduced neuroprotective efficacy of PNU in the presence of KN93.

By re-activating desensitized α7 nAChRs [41], PNU augments and prolongs endogenous α7-dependent cholinergic tone in the ischemic penumbra [6], significantly reducing brain injury and neurological deficits in the first 24 h after tMCAO (Fig. 5). This ability of PNU to re-activate desensitized α7 nAChRs [41] is critical because the extracellular concentration of choline in the ischemic core/penumbra is elevated manifold within the first few hours after MCAO onset [37], and as a result, a certain portion of α7 nAChRs in the ischemic penumbra is expected to be desensitized (IC_{50} ~40 μM) [28] and unable to fully participate in protective action [37].

Because the normal physiological level of cerebral extracellular choline is low (i.e., <10 μM) [24, 25, 29] and subthreshold for α7 activation [28], it is unlikely that extracellular choline is protective in the absence of injury or α7-PAMs. Thus, the finding that MLA significantly increases infarct volume and neurological deficits after tMCAO suggests that endogenous α7-dependent brain protection is indeed produced by extracellular choline elevated by tMCAO [37] and, thus, is a focal, not global, phenomenon. However, the same low physiological levels of choline in the absence of injury could pre-condition brain tissues in the presence of PNU and, thus, enhance neuronal resistance to injury, as we reported previously in oxygen-glucose deprivation (OGD) experiments in acute hippocampal slices [2]. In those experiments, preincubation of slices in 20–200 μM choline plus 1 μM PNU significantly delayed terminal anoxic depolarization of CA1 pyramidal neurons and thus enhanced their resistance to OGD [2]. Therefore, in addition to amplifying the effects of injury-elevated extracellular choline focally in the ischemic penumbra post-MCAO, α7-PAMs may also produce a global, α7-dependent (but injury-independent) protective preconditioning among α7-expressing neurons resulting from synergistic action of α7-PAMs and normal (i.e., low; <10 μM) physiological levels of choline. This global preconditioning would remain undetected in the absence of injury (e.g., tMCAO) or insult (e.g., OGD), but could be revealed by experimental insults that do not elevate extracellular choline: e.g., OGD in acute slices continuously perfused with ACSF [2]. The benefits from PNU-induced pre-conditioning appear to be short lived with the rate matching the rate of PNU clearance (~8 h [42]) because s.c. PNU (which is effective when injected 3 h prior to tMCAO) becomes ineffective when injected 24 h prior to tMCAO [2]. Therefore, PNU appears to produce both a *focal* protective action in the ischemic penumbra where extracellular choline is elevated by ischemic injury and a *global* protective pre-conditioning in brain areas unaffected by MCAO where extracellular choline remains sub-threshold for α7 activation in the absence of α7-PAMs [29, 39].

While PNU treatment is effective after a 90-min tMCAO, the contributing protective pathways are not fully understood. In fact, because α7 nAChRs are ubiquitously expressed in neuronal and non-neuronal tissues, the therapeutic efficacy of α7-PAMs may result from multiple and relatively independent components. For example, we have shown previously that physiologically relevant concentrations of choline (20–200 μM) in the presence of 1 μM PNU produced

neuroprotection in an OGD model of cerebral ischemia in acute hippocampal slices [2]. These data supported the neuroprotective component of α7-PAM action. α7-PAMs may also enhance activation of α7 nAChRs expressed in the autonomic neuronal circuitry which provides neurogenic control over vascular tone (e.g., adrenergic, nitrergic) [58, 59] and, thus, may cause elevation of the collateral blood supply within the ischemic penumbra. Because α7 nAChRs are expressed in glial and immune cells [4, 7, 55, 60, 61], α7-PAMs may contribute to brain protection by augmenting endogenous α7-dependent cholinergic anti-inflammatory mechanisms activated by injury [4, 7, 11–13, 23, 55, 60–64]. On the other hand, excessive prolonged activation of α7 nAChRs by high concentrations of α7 agents can be toxic for biological cells as demonstrated and discussed previously [65–70]. Thus, it is essential for future ex vivo and in vitro studies to employ physiologically and clinically relevant drug concentrations and treatment conditions available in the literature [2, 24, 25, 37, 42, 68]. The use of clinically relevant experimental conditions in ex vivo and in vitro studies should not be a matter of preference because in the absence of clinically effective therapies, the continuing suffering of stroke victims and the associated financial burden demand prompt effective solutions.

In conclusion, although the physiological role of α7 nAChRs in the central nervous system is largely unknown, the results of this study suggest that the therapeutic activation of α7 nAChRs in the penumbra after focal cerebral ischemia serves as an important physiological responsibility of these ubiquitous receptors and can be significantly augmented by α7-PAMs and, thus, may hold an attractive translational potential.

Clinical Perspectives

This study used a transient focal cerebral ischemia model of ischemic stroke to detect the early (i.e., the first 24 h post-tMCAO) endogenous choline-/α7-/CaMKII-dependent mechanisms of brain self-protection that can be augmented by positive allosteric modulators of α7 nAChRs, such as PNU120596. Because PNU120596 only amplifies α7 activation by injury-elevated extracellular choline in the ischemic peri-infarct region, the challenge of a timely focal delivery of PNU120596 treatment to the ischemic penumbra is naturally resolved: while systemically applied PNU120596 is distributed throughout the body by circulation, its therapeutic effects take place mostly, or even exclusively, in the brain areas with elevated extracellular choline, i.e., exactly where and when it is most needed: in the ischemic penumbra, post-injury. Therefore, the high spatiotemporal precision of α7-PAM therapies at the early stage after ischemic stroke is an important benefit of this approach. Because α7 nAChRs and phosphatidylcholine-based cell membranes are common in the mammalian brain, including humans, the endogenous choline-/α7-dependent protection may serve as an evolutionarily shaped common mammalian brain protective mechanism that can be significantly amplified by α7-PAMs. Thus, there is a rational basis to expect that the therapeutic utility of acute α7-PAM treatment will naturally extend to humans. Here, we discuss an unconventional approach to managing acute brain injury and neurological deficits at the early stage (i.e., the first 24 h) after cerebral ischemia. This approach can become a starting point for developing clinically efficacious α7-PAM-based therapies that may enable health-care providers to overcome current limitations associated with the lack of effective treatments after stroke.

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Compliance with Ethical Standards The use of animals was 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. The UNTHSC animal facility is AAALAC accredited.

Conflict of Interest The authors declare that they have no conflict of interest.

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