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Pharmacological Phosphodiesterase Inhibition as a Therapeutic for Cortical and Striatal Stroke

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Pharmacological Phosphodiesterase Inhibition as a Therapeutic for Cortical and Striatal Stroke

A thesis submitted in partial satisfaction
of the requirements for the degree of
Master of Science in Physiological Science

by

Nora Abduljawad

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ABSTRACT OF THE THESIS

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as a Therapeutic for

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by

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Master of Science in Physiological Science

University of California, Los Angeles, 2019

Professor Rachelle Hope Watson, Co-Chair

Professor Stanley Thomas Carmichael, Co-Chair

Stroke is the leading cause of adult disability, but there is no drug regimen available that promotes recovery after stroke. Recovery of function after stroke involves molecular signaling events mediated by cAMP and cGMP signaling, such as CREB activation and axonal sprouting. Cyclic nucleotide signaling is regulated by a variety of phosphodiesterase (PDE) isoforms, which degrade cAMP and cGMP and exhibit region-specific distribution in the brain. PDE10A is highly enriched in the basal ganglia/striatum. A novel PDE10A inhibitor, TAK-063, was tested for its effect on functional recovery and neuroplasticity after both striatal and cortical stroke. Inhibition of PDE10A improves recovery of function after striatal but not cortical stroke, consistent with its brain distribution, in young adult mice. Recovery of motor function correlated with increases in striatal BDNF, downstream of the cAMP signaling cascade, as well as axonal sprouting and angiogenesis. However, PDE10A inhibition did not improve functional recovery in aged mice after striatal stroke. PDE2A is the most prevalent phosphodiesterase expressed in cortex. A second novel PDE inhibitor specific to PDE2A, PDE2A-T1, was tested for its effect on tissue repair and functional gains after cortical stroke. PDE2A-T1 treatment improved behavioral

outcomes in both young adult and aged mice after stroke and increased the density of cortical axonal connections in aged animals after stroke. This is the first demonstration of brain region-specific enhanced functional recovery after stroke, and indicates that differential molecular signaling between brain regions can be exploited to improve recovery based on stroke subtype, with an important effect of age on therapeutic efficacy.

The thesis of Nora Abduljawad is approved.

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Acknowledgments

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Introduction

Globally, 15 million people suffer a stroke each year [1]. In the United States, stroke burdens 800,000 new patients annually, with 80% suffering ischemic lesions caused by a blockage of blood vessels and disruption of blood flow to the brain [2]. Improvements in acute care, such as the development of thrombectomy procedures and intravenous thrombolytic treatments, have led to reductions in ischemic stroke-related mortality [1,3]. However, damage to the brain occurs rapidly, leading surviving patients to suffer serious, long-term losses in cognitive, motor, and linguistic functionalities [4-6, 63]. As a result, stroke has become a leading cause of adult disability worldwide [1,6]. Moreover, this stroke disability can be progressive, since initial functional gains made with neurorehabilitation are often lost, as patients gradually minimize their use of affected functions [3]. Despite this great burden, there is currently no drug treatment that promotes neural repair or functional recovery after stroke. Developing pharmacological treatments for this chronically disabling disease is thus a strong research priority.

Disruption of blood flow to brain tissue results in initial cell death and damage. However, the brain also has a limited ability for subsequent repair after stroke. The progression of cell death in the stroke core, or infarct, results in the release of cytokines and diffusible free radicals that alert neighboring cells to initiate a neural repair response [7-10]. Spatially, stroke thus comprises a focal site of permanent, irreparable cell death, surrounded by areas of reorganization and plasticity in adjacent, peri-infarct tissue. The neuroplasticity or repair program induced in this peri-infarct tissue includes an integrative, coordinated response across multiple cell types and scales, spanning synapse to circuit. Dendritic spine morphogenesis, physiological alterations in neuronal excitability, generation of new neurons and their migration to areas of injury, formation of new blood vessels, recruitment and differentiation of glial cells, activation of neuronal growth programs leading to formation of new axonal connections, and functional re-mapping of cortical circuits are all processes that participate in the brain's

response to stroke in animal models or humans [7, 11-17]. However, the extent to which these repair processes occur endogenously remains limited in time and scope after stroke [3, 13, 16]. Consequently, this endogenous repair program is insufficient to produce full repair and recovery. The goal of ongoing research programs is to identify mechanisms that extend or increase the amount of early post-stroke plasticity, or re-activate plasticity programs at later stages after stroke. Boosting the brain's repair potential in this way would produce more sustained neural repair and potentially greater, lasting functional improvement.

Developing effective therapeutics for stroke might begin with recognizing the similarities between the processes participating in post-stroke neural repair and the systems governing plasticity during regular learning and memory formation [3, 18-21]. On a molecular level, both learning and post-stroke recovery are associated with gene expression changes in signaling pathways involving Nogo/Nogo receptors, GAP-43, c-Jun, MARCKS, stathmin, and RB3 [4, 13, 22-23]. As described above, recovery also involves cellular events such as dendritic spine plasticity and neurogenesis, as well as long-term potentiation-dependent changes in synaptic activity, events with well-characterized roles in memory formation [23-25]. At the circuit level, both recovery after stroke and learning involve honing down diffuse networks of connected brain regions to sets of core areas directly involved in specific tasks [3]. Even cognitively, functional recovery requires "re-learning" tasks lost due to stroke, a process that relies on the same neuropsychiatric principles that mediate learning for the first time [26]. These parallels have led to the hypothesis that repair and recovery after stroke might depend on the brain's ability to harness systems that mediate structural and functional plasticity during normal learning and memory formation.

This hypothesis was initially tested using non-stroke specific drugs that enhance learning potential broadly, such as serotonin reuptake inhibitors, dopamine agonists, and various central nervous system stimulants [12, 27]. Clinical trials employing these compounds failed, likely due to the action of these drugs on neurotransmitter systems not primarily implicated in stroke-

induced damage or recovery [12, 27]. As a result, ongoing work has instead shifted towards characterizing the molecular learning and memory systems specifically altered or recruited after stroke, as well as developing therapeutics to target these context-relevant systems.

Two neurotransmitter systems that impact synaptic plasticity in learning and memory have been implicated in recovery after stroke. The first of these is tonic, or extrasynaptic, inhibitory GABA signaling [20, 28]. During traditional learning, blocking tonic GABA signaling promotes neuronal excitability in the hippocampus and enhances learning and memory in many animal models [30, 31]. After stroke, tonic GABA inhibition is reported to increase and persist, likely due to downregulation of GABA transporters in astrocytes [32]. Under physiological conditions, astrocytes help maintain neurons in their excitable state, partially by uptake of excess GABA from the extracellular environment. This reduced GABA uptake and resulting dampened neuronal excitability is protective in the acute setting after stroke, since stroke causes a positive feedback cycle of excitotoxicity-mediated neuronal death [7, 12, 29]. However, following this initial period of cell death, reduced neuronal excitability hinders the brain's capacity for plasticity and repair [12, 20, 21]. One context-relevant system that has been targeted as a post-stroke therapeutic is tonic GABA inhibition; both genetic deletion and pharmacological antagonism of tonic GABA receptors improves functional recovery and promotes cortical circuit reorganization in animal models of stroke [20].

A second neurotransmitter system with dual roles in learning and stroke recovery is excitatory glutamatergic signaling through AMPA receptors (AMPARs). Trafficking of AMPARs to synapses is a key step in the induction and maintenance of long-term potentiation (LTP), the cellular correlate of learning and memory formation in the brain [33, 34]. Drugs that enhance glutamatergic transmission through AMPAR signaling increase neuronal excitability and improve learning in humans and animal models [35, 36]. After stroke, dampened neuronal excitability resulting from increased tonic GABA inhibition might be offset by potentiation of excitatory AMPAR signaling. Preclinical studies have demonstrated that treatment with positive allosteric

modulators of AMPARs after stroke does in fact promote functional recovery, as well as peri-infarct induction of BDNF, a neurotrophin that causally regulates multiple aspects of cortical plasticity such as neurogenesis, neuronal circuit reorganization, and dendritic spine morphogenesis [37-39]. Together, studies of tonic GABA and AMPAR signaling in stroke demonstrate that learning and memory systems that enhance peri-infarct excitability are effective, context-appropriate therapeutic targets for stroke recovery and repair.

Within neurons, a central molecular node that regulates excitability and firing potential is the transcription factor CREB (CRE response element binding protein) [40-42]. A wide range of cellular inputs lead to intracellular signaling cascades that converge upon downstream CREB activation. The resulting CRE-dependent transcription increases neuronal excitability and synaptic restructuring [40, 44]. In this way, CREB couples experience-dependent neuronal stimulation with gene transcription and long-term cellular and molecular changes associated with plasticity, learning, and memory [45, 46]. This role for CREB has been well-appreciated in the learning and memory field. CREB has been shown to be necessary for LTP and long-term memory maintenance; CREB potentiation also enhances performance in a wide range of learning and memory paradigms, indicating a role that is non-specific to particular types of learning [47-50]. Thus, like tonic GABA inhibition and AMPAR transmission, CREB is a molecular target that increases neuronal excitability, with learning and memory functions that could be co-opted for stroke repair.

Importantly, CRE-dependent transcription has additional roles in the allocation of neurons into a memory's trace or engram, defined as the cluster of neurons that co-activate to encode or store a particular memory [51-56]. In other words, CREB expression during learning preferentially commits neurons to an active neuronal ensemble that comprises the circuit-level representation of memory. Selective ablation of these neurons disrupts this particular memory [57]. CREB might allocate neurons into other types of task-specific engrams or circuits, such as the motor circuit for a particular limb movement, or the sensory circuit for a particular type of

visual stimulus [18, 58-60]. Critically, CREB mediates reorganization of these task-specific neuronal circuits in response to altered input or experience [45, 61-62]. For example, rodent monocular or whisker deprivation paradigms promote CREB-dependent reorganization of the neuronal networks that represent visual or somatosensory information, respectively [62-64]. In human studies of blind individuals, brain areas exhibiting reorganization of functional connectivity, such as traditional visual processing areas, show increased CREB expression, suggesting a role for CREB in mediating the network reorganization observed in these areas [65]. This re-organization role is particularly relevant to the context of repair after stroke. Stroke causes task-specific neuronal engrams to be disrupted, either directly due to death of participating neurons or indirectly via functional disconnection or lack of coincident firing of surviving neurons [15, 66-68]. Activation of CREB in surviving neurons after stroke might be one molecular way to encourage neuronal excitability and circuit reorganization, which is correlated in both preclinical and human studies with greater post-stroke functional recovery.

Recent preclinical studies have focused on testing this idea, to establish whether CREB can enhance the limited endogenous plasticity observed after stroke and promote functional recovery [18]. In these studies, CREB levels were manipulated virally in a subset of peri-infarct neurons after stroke to the motor cortex. Upregulation of CREB after stroke improved motor function on a variety of motor assessments. Further, selective inactivation of CREB-transfected neurons after stroke resulted in the loss of initial CREB-induced functional gains, to the point of worsened performance even beyond untreated stroke controls. Releasing this selective inactivation reinstated the initial motor improvement. This functional recovery was correlated with both structural and functional plasticity of cortical networks. In addition to increased axonal sprouting and connectivity within spared cortical motor regions, viral up-regulation of CREB resulted in accelerated remapping of lost motor or sensory functions into adjacent, surviving pre-motor and somatosensory regions. Further, neuronal CREB induction activated sets of genes within cellular pathways that include nervous system development and tissue

development, highlighting the critical status of CREB as a transcription factor able to regulate cellular processes relevant to stroke repair as well as learning and memory. Together, these results show that CREB is a molecular switch that can turn recovery on or off, by altering neuronal transcriptional profiles in ways that promote cortical circuit plasticity after stroke. These studies thus identify CREB as a specific learning and memory molecule that can accelerate or enhance endogenous plasticity mechanisms after stroke.

Currently, there is no drug regimen available that promotes neural repair or functional recovery after stroke. A compound able to target CREB pharmacologically could have strong clinical potential as a post-stroke therapeutic. One intracellular signaling cascade that activates CREB and involves druggable molecular targets is the cyclic nucleotide/protein kinase pathway [69, 70]. In this pathway, activation of G protein-coupled receptors on a cell's surface prompts adenylyl and/or guanylyl cyclase to synthesize cyclic nucleotides via conversion of AMP/GMP to their cyclic forms cAMP/cGMP, respectively. In turn, cyclic nucleotides act as intracellular second messengers and activate protein kinases, which translocate to the cell's nucleus to phosphorylate CREB and other transcription factors. Phosphorylation of CREB at the serine-133 site results in CREB-dependent transcription of plasticity-related genes, such as BDNF [70]. Within this and other cyclic nucleotide signaling cascades, phosphodiesterases (PDEs) are the sole enzymes that hydrolyze the second messengers cAMP and cGMP, thus regulating the duration and amplitude of cyclic nucleotide signaling [71]. Inhibiting PDEs prevents the degradation of cyclic nucleotides and is one way of increasing cyclic nucleotide availability for intracellular signaling. This signaling includes participation in pathways that potentiate CREB phosphorylation and CREB-mediated transcription. A variety of pharmacological PDE inhibitors have been developed and can increase cyclic nucleotide signaling in multiple tissue types, including the brain [72, 73]. Currently, these PDE inhibitors are clinically approved for the treatment of acute heart failure, pulmonary hypertension, and erectile dysfunction [74-76]. Increasing preclinical evidence indicates that PDE inhibitors can also improve performance on

learning and memory tests, as well as attenuate disease progression in a variety of neurological diseases, such as schizophrenia and Alzheimer's disease [77, 78].

Phosphodiesterases comprise a superfamily of enzymes encoded by 21 genes [79]. This family is further divided into 11 sub-families (PDE1 through PDE11) based on sequence homology, structural features, catalytic properties, and substrate affinity for cAMP, cGMP, or both. Further, each PDE family includes multiple mRNA splice variants, giving rise to over 100 different PDE isoforms in total. Importantly, PDE families and their isoforms are expressed in tissue- and cell-type specific manners [80]. For example, PDE1 and PDE3 are the most prevalent PDEs expressed in the heart, while PDE8 is the dominant PDE in thyroid tissue [80]. Within the brain, PDE10 is maximally expressed in the striatum/basal ganglia, and PDE2 is the most abundant PDE isoform expressed in the hippocampus as well as cortical regions [71, 80]. Both striatum and cortex are common sites of human stroke [81]. This regional distribution of PDEs and opportunity for region-specific targeting make PDEs an especially popular pharmacological target broadly; in the context of stroke, this allows for selective manipulation of cyclic nucleotide signaling, as well as potential downstream CREB activation and repair-associated plasticity events, in affected stroke regions and circuits. Due to the diversity of human stroke subtypes, treatments tailored to specific stroke pathologies could have increased clinical potential through reduced side effects and potentially improved, focused efficacy in affected brain regions.

The current study first aimed to test the efficacy of pharmacological phosphodiesterase inhibition in enhancing functional recovery after stroke. This was performed in two preclinical models of ischemic stroke, to investigate whether selective targeting using different PDE inhibitors could promote region-specific recovery based on stroke subtype. In previous work, a novel, striatum-specific PDE10A inhibitor, TAK-063, was tested in a model of striatal stroke as well as a separate model of cortical stroke. Treatment with TAK-063 ameliorated motor deficits following striatal but not cortical stroke. The current study demonstrated that, unlike with

striatum-specific PDE10A inhibition, cortex-specific PDE2A inhibition using the novel inhibitor PDE2A-T1 improved motor recovery following cortical stroke in both young adult and aged mice.

This study next aimed to identify potential cellular and molecular mechanisms underlying the functional recovery induced by targeted PDE inhibition. Following striatal stroke, striatum-specific PDE10A inhibition increased peri-infarct protein levels of BDNF, a neurotrophin induced downstream of CREB activation and correlated with neural repair and recovery, as well as angiogenesis in contralesional striatum and axonal connectivity of motor cortex with contralesional striatum. Following cortical stroke, cortical PDE2A inhibition increased axonal connections within peri-infarct motor systems, consistent with previous studies demonstrating the role of CREB in enhancing axonal sprouting and cortical circuit reorganization after stroke. No changes in infarct size, post-stroke neurogenesis, gliogenesis, or microglial proliferation were observed with either treatment. Together, these studies comprise the first demonstration of region-specific functional recovery after stroke, and demonstrate that differential molecular signaling between brain regions can be exploited to improve recovery based on stroke subtype. These studies also demonstrate that PDE inhibitors can be administered systemically to promote plasticity processes that act downstream of CREB activation, a molecular learning and memory target that enhances neuronal excitability, neural repair, and functional recovery after stroke.

Materials and Methods

Animals

All procedures were performed in accordance with National Institutes of Health Animal Protection Guidelines. 10-week old male C57BL/6 mice from Jackson Laboratories (Bar Harbor, MA) were used for young adult studies. 18-month old male C57BL/6 mice obtained from the National Institute of Aging (NIA) were used for aged studies. Animals were housed under pathogen-free conditions in a light-controlled environment with a reverse light cycle, with free access to irradiated pellets (LabDiet PicoLab, Rodent Diet 20) and sterilized, acidified water. Housing temperatures were maintained between 69 to 70˚ F and standard humidity between 30 to 70%.

TAK-063 Preparation

Lyophilized TAK-063, a PDE10-specific inhibitor, was obtained from Takeda Pharmaceuticals. TAK-063 was solubilized in a solution of 0.5% methyl cellulose (MC) in sterile saline. A stock solution of 5 mg/ml TAK-063 was made fresh twice weekly. The solution was vortexed and sonicated for 20 min in an ultrasonic bath, with intermittent vortexing during sonication, and kept at 4C. Further dilutions were made using MC saline as needed. Diluted solutions were sonicated for 10 minutes prior to drug injection.

PDE2A-T1 preparation

Lyophilized PDE2A-T1, a PDE2-specific inhibitor, was obtained from Takeda Pharmaceuticals. PDE2A-T1 was solubilized step-wise in 10% DMSO, 10% Cremophor EL, 30% PEG 400, 10% propylene glycol, and 40% H2O. At each step, the PDEA-T1 solution was vortexed. After addition of H2O, the solution was vortexed until completely translucent. Fresh PDE2A-T1 solution was prepared twice weekly. Once daily, 50 uL of either vehicle or 3.0 mg/kg PDE2A-T1 solution was injected intraperitoneally into test subjects. Daily injections were initiated five days after stroke.

Drug delivery

Daily intraperitoneal injections of drug or vehicle were initiated 5 days after stroke induction. TAK-063 was injected at escalating doses of either 0.3 mg/kg, 3.0 mg/kg, or 10.0 mg/kg. PDE2A-T1 was injected at 3.0 mg/kg. No adverse health effects were observed over the course of drug treatment.

Stroke production: L-NIO Injection

Mice were anesthetized with 2.5% isoflurane and placed in a stereotaxic apparatus. The left common carotid artery was exposed through a ventral midline incision in the neck, which was shaved and aseptically prepared. After separation of the artery from the vagus nerve, the artery was permanently occluded via cauterization. Mice were subsequently placed on a stereotaxic apparatus where their heads were also shaved and aseptically prepared. Skulls were exposed using a midline incision. A single burr hole was made in the skull (0.95 AP, 3.0 ML). A 33-gauge needle fitted to a 25 uL Hamilton syringe was angled at 10° over the burr hole. 3uL of the vasoconstrictor L-NIO (27mg/mL) was infused at a rate of 0.3uL/min into the brain (0.95 AP, 3.0 ML, 2.6 DV). Five minutes after injection, the needle was retracted and the incision was closed. Body temperature was monitored and maintained at 37°C (±3°C) during the procedure.

Cortical stroke production: Photothrombosis

Mice were anesthetized with 2.5% isoflurane and placed in a stereotaxic apparatus. Heads were shaved and aseptically prepared. Skulls were exposed using a midline incision. Each mouse was injected with 200 uL of a sterile solution of 10 mg/mL Rose Bengal in PBS. After 5 minutes, a 200 mV light source was shone focally onto forelimb motor cortex (AP 0.0, ML 1.5, DV 0.0) for 15-18 minutes. The skull was rinsed using sterile solution, and the incision was closed. Body temperature was monitored and maintained at 37°C (±3°C) during the procedure.

5-Ethynyl-2'deoxyuridine preparation

A stock solution of 5 mg/ml of 5-ethynyl-2'deoxyuridine (EdU) was prepared in potable water. EdU, along with the antibiotic trimethoprim–sulfamethoxazole (TMS), was added to mouse drinking water days 3-10 after stroke, for a final EdU concentration of 200 ug/mL. Water was refreshed every 48 hours.

BDA Injections

10 weeks after stroke, the neuroanatomical tracer biotinylated dextran amine (BDA, 10k MW, Invitrogen) was injected into motor cortex ipsilateral to the cortical stroke site. Following anesthesia with 2.5% isoflurane and placement in a stereotaxic apparatus, a burr hole was made in the skull 0.0 mm anterior and 1.5 mm lateral to bregma. Using a Hamilton syringe and automated injection apparatus, 0.3 uL of BDA was infused at a rate of 0.03 uL/min, 0.75mm ventral to the surface of the brain. Body temperature was monitored and maintained at 37°C (±3°C) during the procedure. Animals were euthanized 1 week after BDA injections.

Behavioral Testing

Motor performance on three different behavioral tests was assessed at regular intervals following stroke, beginning 1 week after injury. The grid walking and cylinder tests, administered monthly, assessed gait and spontaneous forelimb use, respectively. The pasta matrix test, administered bimonthly in the aged PDE2A-T1 studies, measured skilled reach ability. Mice were trained on the pasta matrix for 5 weeks prior to injury. Motor performance on each task was normalized to pre-stroke baseline performance. Mice were tested up to 9 weeks post-stroke, a time point that precedes spontaneous motor recovery. Sample size was 10-12 animals for young adult studies and 8-10 animals for aged studies.

Grid-walking Task

Mice were placed on a suspended wire grid and video recorded while walking freely for 5 minutes each. The total grid area was 32 cm x 20 cm, with 1 cm x 1 cm squares. Videos were subsequently analyzed offline by evaluators blinded to the treatment groups. Total number of steps taken and the total number of foot-faults by the stroke-affected forelimb, defined as total slips through the grid, were recorded. Number of foot-faults was reported as percentages of total steps taken and normalized to pre-stroke baseline performance.

Cylinder Task

Mice were placed in a transparent, plastic cylinder (15 cm x 10 cm) and video recorded for 5 minutes to capture exploratory vertical rearing behavior onto the cylinder. Two mirrors were placed at angles vertically behind the cylinder to allow the video camera to capture rears in the opposite plane of view. Videos were subsequently analyzed offline by evaluators blinded to the treatment groups. The number of vertical rears made using the right forelimb, left forelimb, or both forelimbs was determined to determine forelimb preference during spontaneous, exploratory behavior. Preference for use of the stroke-affected limb was calculated as: (right rears - left rears/total rears) and normalized to pre-stroke baseline performance.

Pasta Matrix Task

Mice were placed in a plastic chamber and were trained to reach through a narrow slit in the chamber to break and retrieve pieces of dry pasta. Dry capellini pasta was cut into 3.2-cm pieces and oriented vertically on a heavy-duty plastic block with holes drilled in that fit individual pieces of pasta. Mice were trained daily for 5 weeks prior to baseline testing. After stroke, mice were tested bimonthly for 9 weeks. The number of pasta breaks per 20-minute session was averaged across two consecutive days of testing and normalized to pre-stroke baseline performance.

Immunohistochemistry

Brains were perfused with 4% paraformaldehyde, cryoprotected, and frozen. Tissue sections were incubated in primary antibodies overnight at 4C, following permeabilization and blocking. The following primary antibodies were used: rat anti-GFAP (Invitrogen), goat anti-IBA-1 (Abcam), and rat anti-Glut-1 (Abcam), rabbit anti-Olig2, (Millipore), rabbit anti-NeuN (Abcam), and rat anti-CD31 (BD Biosciences). Secondary antibodies were as follows: Alexa Fluor 488 donkey anti-rabbit, Alexa Fluor 488 donkey anti-rat, and Alexa Fluor 647 donkey anti-rabbit, and Alexa Fluor 647 donkey anti-rat, and Alexa Fluor 647 streptavidin (Jackson ImmunoResearch). Confocal z-stack images from peri-infarct and contralesional striatum, as well as confocal z-stack images of ipsilesional cortex, were obtained at 40x magnification. Number of cells and co-localization of nuclear cell markers through the z-stack was quantified using Imaris Bitplane Software, n = 4-5 animals per group.

Axonal sprouting analysis

Following BDA injection and tissue harvesting, brain sections were stained for BDA using a streptavidin-conjugated fluorophore. Confocal z-stack images of each full section were obtained at 20x magnification, and maximum intensity projection images were analyzed. Using ImageJ, integrated densities were recorded from various brain regions, including white matter, cortex, and striatum of both hemispheres. After background subtraction, each section's intensity data were normalized to the integrated density of the section's BDA injection site. Mean integrated density across six sections was calculated and plotted for each animal, n = 4 animals per group.

Nissl Staining and infarct analysis

Coronal sections were run through ascending alcohol solutions (50%, 75%, 95% and 100%), placed in a 1:1 alcohol/chloroform solution for 45 minutes, rehydrated in descending alcohols, rinsed in distilled water, and finally stained in a cresyl violet solution. Using ImageJ and MBF StereoInvestigator softwares, the volumes of both ipsilesional and contralesional hemispheres

or cortices were calculated for a full series of coronal sections through the striatal strokes or cortical strokes, respectively. Values were plotted as ratios of the ipsilesional to contralesional volumes.

ELISA

Tissue was collected from peri-infarct striatum 3 weeks after striatal stroke. Striatal tissue was dissected in a 1 mm radius around the stroke infarct core, including the core itself, and flash frozen in liquid nitrogen. Equal volumes of tissue were homogenized in 100 ml of RIPA buffer (Sigma) supplemented with Protease and Phosphatase Inhibitor Tablet (Invitrogen). Tissue was incubated in homogenization buffer for 30 minutes on ice, followed by a 5 minute spin at 14,000 rpm. The supernatant was collected, and total protein concentrations were determined using the Pierce™ BCA Protein Assay kit (ThermoFisher Scientific). Amount of BDNF protein was measured using the BDNF (mouse) ELISA kit (Abnova), per the manufacturer's instructions.

Statistics

All reported values represent means and standard errors of the means (SEMs). For PDE10A young adult studies, behavioral data were analyzed with general linear model and Tukey's HSD post-hoc test. For all other data, differences between two means were assessed by unpaired two-tailed Student's t-test, and differences among multiple means were assessed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer's post-hoc tests.

Results

Part I: Striatum-specific PDE inhibition as a pharmacological therapeutic for striatal stroke

PDE10A Inhibition Improves Motor Recovery After Striatal Stroke With No Improvement in Motor Recovery After Cortical Stroke

Mice were given a stroke in the striatum or in the motor cortex in separate cohorts. Behavioral testing of forelimb motor function was performed up to 9 weeks post stroke to measure exploratory rearing and gait control in forelimb function.

A competitive inhibitor of PDE10A, TAK-06316, was administered intraperitoneally at three escalating doses beginning 5 days post stroke, a time point at which brain sensitivity to damage-enhancing effects of plasticity drugs is lost. One week after surgery, the cylinder task, a measure of forelimb use in exploratory rearing, indicated that both striatal and cortical stroke mice were impaired on the task relative to sham controls (Figure 1A, C). The stroke + 3.0 mg/kg dose of TAK-063 produced a tendency toward recovery of function in the affected forelimb in the striatal stroke over the 9-week treatment compared with stroke + vehicle controls (Figure 1A, n = 8-10 for each treatment group, p = 0.04258). Furthermore, no differences between stroke + 0.3 mg/kg and 10 mg/kg treatment groups when compared to stroke + vehicle were observed in the striatal stroke model. There was no effect of TAK-063 on motor recovery in cortical stroke at individual time points and over the 9-week treatment in stroke + 3.0 mg/kg compared with stroke + vehicle controls (Figure 1C, n = 8-11, P = 0.6367).

The grid-walking task, a measure of limb use in gait, showed contralateral forelimb deficits in stroke + vehicle, stroke + 0.3 mg/kg, stroke + 3.0 mg/kg, and stroke +10 mg/kg groups compared with sham + vehicle and sham + 3.0 mg/kg treatment controls (Figure 1B, D). In striatal stroke, a significant difference was observed at the individual 6-week (P = 0.04317) and 9-week (P = 0.03387) time points in stroke + 3.0 mg/kg versus stroke + vehicle controls.

TAK-063 showed a trend toward improvement in overall recovery, with a difference in stroke + 3.0 mg/kg over the 9-week treatment compared with stroke + vehicle controls (Figure 1B, n = 8-10 for each treatment group, P = 0.05160). TAK-063 did not enhance recovery of motor function in cortical stroke (Figure 1D, n = 8-11, P = 0.9934). Overall, the combined results obtained from both behavioral tests indicate that a daily dose of TAK-063 leads to improvement in gait and forelimb motor control in striatal stroke but not cortical stroke.

PDE10A Inhibition Leads to Increases in BDNF

PDE10A is predominantly expressed in medium spiny neurons of the striatum. We next investigated if treatment with TAK-063 leads to increases in BDNF levels at 3 weeks following striatal stroke, a time point where heightened levels of BDNF post- stroke have been reported and just before significant behavioral recovery with this drug is observed (Figure 1A, B; Supplemental Figure 2). There is a significant loss of BDNF levels in the ipsilateral striatum with stroke (Figure 2). Striatal stroke + 3.0 mg/kg of TAK-063 significantly induced BDNF in ipsilateral, but not contralateral striatum (Figure 2). This low level of striatal BDNF does not change significantly with TAK-063 delivered at a dose that is ineffective in promoting behavioral recovery (i.e., at 1.0 mg/kg) (Figure 2A).

PDE10A Inhibition Alters Motor System Connections after Stroke

Neural repair after cortical stroke involves axonal sprouting within the ipsilateral peri-infarct cortex and tissue reorganization in other rodent models of ischemic brain lesions also includes axonal sprouting in cortical projections to the contralateral striatum from the stroke site. Increases in BDNF promote axonal sprouting after motor cortex and striatal lesions. To determine if the improvement in forelimb motor recovery and induction of BDNF in the striatum following TAK-063 were associated with axonal sprouting after striatal stroke, the neuroanatomical tracer BDA was used to quantitatively map axonal projections from the

forelimb motor cortex 6 weeks after stroke, a time of improved motor recovery (Figure 1A, B). The pattern of axonal sprouting in stroke and stroke + TAK-063 at the recovery-inducing dose of 3.0 mg/kg was compared with sham + vehicle and sham + 3.0 mg/kg TAK-063. The density of BDA-positive axons was assessed in the striatum for all animals. The mean integrated density of labeled axons linearly correlates with axon number. Data was normalized to the integrated density of the BDA injection site to account for differences in injection size (Figure 3A-D). Striatal stroke causes a loss in the projection from the motor cortex to the contralateral striatum (Figure 3C). Stroke + 3.0 mg/kg TAK-063 compared with stroke + vehicle showed significant increases (Figure 3C, n = 4-5 for each treatment group, P = 0.0009) in density of BDA positive fibers in the contralateral striatum (Figure 3C; data representative of two independent experiments). Axonal projections to ipsilateral striatum were mapped; no differences were observed in ipsilateral striatum (Figure 3D). These data suggest that TAK-063 either preserves the normal pattern of corticostriatal connections or that TAK-063 induces axonal sprouting in the partially damaged corticostriatal system and restores the absolute level of this system through compensatory axonal sprouting responses.

Effects of PDE10A Inhibition Post-Striatal Stroke on Angiogenesis

Angiogenesis plays a role in tissue reorganization in stroke and has been linked to recovery in associational studies in humans. There may be an induction of proliferative angiogenesis (endothelial cell division) in the absence of changes in the overall morphology of the vascular bed. To determine endothelial proliferation in the tissue adjacent to the infarct, mice were given a striatal stroke and were treated with a marker of cell proliferation, EdU, for 6 weeks. Stroke + 3.0 mg/kg TAK-063 increased the total number of proliferative cells in the contralateral striatum compared with stroke + vehicle and stroke + 0.3 mg/kg; there was no significant difference between stroke + vehicle and stroke 0.3 mg/kg (Figure 4A, upper panel). Co-localization of the EdU nuclear signal with the endothelial marker CD31, showed a

significant increase in proliferating endothelial cells with stroke + 3.0 mg/kg TAK-063 compared with stroke + vehicle (Figure 4A, lower and right panels). Interestingly, in the non-stroke (control) condition, TAK-063 induced angiogenesis in the striatum (Figure 4A, lower and right panels) ($n = 3\text{-}5$ per treatment group, $P < 0.0001$). No differences were observed in ipsilateral striatum.

Effects of PDE10A Inhibition on Post-Striatal Stroke Neurogenesis and Gliogenesis

Angiogenesis is associated with neurogenesis after stroke, and during brain development, angiogenesis regulates oligodendrocyte precursor cell (OPC) differentiation and myelination. With the angiogenesis induced by TAK-063 after stroke, we examined neurogenesis and OPC proliferation in the region of significant angiogenesis (the contralateral striatum). There were no differences in a maker of mature neurons, NeuN+ EdU+ cells, in TAK-063-treated mice compared with controls in either striatal hemisphere (Figure 4B, C). Olig2 is a broad marker of cells throughout all but the last stages of OPC differentiation. Quantification of Olig2+ EdU+ cells indicates that there is no significant effect of stroke or of TAK-063 treatment on OPC proliferation (Figure 4D).

Delayed PDE10A Inhibition Does Not Affect Infarct Volume

Brain volume was assessed between TAK-063 treatment groups as a determinant of stroke size for both the striatal and cortical stroke models at 9 weeks post-stroke. At this late stage after stroke, the size of the stroke and of any secondary tissue loss can be determined by comparing the ipsilateral cerebral striatum to the contralateral cerebral striatum. No differences were found in the area of infarct between the striatal stroke groups irrespective of treatment (Figure 5).

PDE10A Inhibition Does Not Promote Functional Recovery in Aged Mice after Striatal Stroke

Stroke risk increases with age, and the molecular and cellular responses to stroke differ between young and aged brain. To assess the role of selective PDE10A inhibition in promoting motor recovery after stroke in aged mice, 3.0 mg/kg of TAK-063 was administered to 18-month old mice after striatal stroke. This dose was chosen as it promoted functional improvement, BDNF induction, and axonal connectivity in young adult mice. Daily intraperitoneal injection of either TAK-063 or vehicle was initiated 5 days after striatal stroke induction and continued for 2 months. Mice were tested on the grid-walking task, which assesses gait by quantifying the number of steps errors mice make with their stroke-affected forepaw. After stroke, aged mice receiving either vehicle or TAK-063 display a statistically significant deficit in performance on the gridwalking task compared to sham operated animals (Figure 6, $p = 0.0026$, $p < 0.0001$ for stroke + vehicle and stroke + 3.0 mg/kg groups, respectively). Over the course of 2 months of treatment, there was no difference in motor performance between aged mice receiving 3.0 mg/kg of TAK-063 daily and aged vehicle controls after stroke (Figure 6, $p = 0.4121$ and $p = 0.2359$ 1 month and 2 months after stroke, respectively). This suggests that unlike in young adult mice, 3.0mg/kg of TAK-063 is not effective at promoting functional recovery after striatal stroke in aged mice.

Part II: Cortical PDE inhibition as a therapeutic for cortical stroke

PDE2 inhibition promotes motor recovery after cortical stroke in young adult mice

To assess the role of PDE2 inhibition in promoting recovery of motor function following cortical stroke, young adult mice (2-3 months old) receiving stroke to forelimb motor cortex were treated with daily intraperitoneal injection of either vehicle or 3.0 mg/kg of PDE2A-T1, a novel PDE2-specific inhibitor. Two behavioral measurements of motor function were tested 1 week, 5 weeks, and 9 weeks following stroke: the grid walking task, a measure of gait control, and the

cylinder task, a measure of spontaneous forelimb ability. Treatment with 3.0 mg/kg of PDE2A-T1 improved performance on the grid walking task 1 week and 9 weeks after stroke (Figure 7A, $p = 0.0478$ and $p = 0.0452$, respectively; n = 12-15 animals per group). Improvement was quantified as reduced number of step errors taken with injured forepaw relative to stroke and vehicle control. Nine weeks after stroke, mice treated with stroke and PDE2A-T1 recovered to sham performance levels, whereas mice treated with stroke and vehicle maintained a statistically significant deficit relative to sham animals ($p = 0.0143$; n = 12-15 animals per group). Moreover, a statistically significant main effect of PDE2A-T1 treatment was observed on performance in the cylinder task (Figure 7A, $p = 0.0429$; n = 12-15 animals per group). Improvement in the cylinder task was quantified as increased spontaneous use of injured forepaw during exploratory rearing. These results suggest a novel role for PDE2-specific inhibition in promoting motor recovery after cortical stroke.

PDE2A inhibition promotes motor recovery after cortical stroke in aged mice

Stroke risk increases with age, and the molecular and cellular responses to stroke differ between young and aged brain. To assess the efficacy of selective PDE2 inhibition at promoting motor recovery after stroke in aged mice, 19-month old mice receiving stroke to forelimb motor cortex were treated daily with intraperitoneal injection of either vehicle or 3.0 mg/kg of PDE2A-T1. Three behavioral assessments of motor function were tested beginning 1 week after stroke: the grid walking and cylinder tasks, tested monthly, and the pasta matrix reaching task, tested bimonthly to assess skilled reach ability. Similar to the young adult animals receiving stroke and PDE2A-T1, treatment with 3.0 mg/kg of PDE2A-T1 promoted improved performance in the grid walking task 1 week and 9 weeks after stroke (Figure 7B, $p = 0.0031$ and $p = 0.0255$, respectively; n = 8-9 animals per group). PDE2 inhibition also promoted improvement in the pasta matrix reaching task 9 weeks after stroke, quantified as an increased number of successful reaches through the matrix relative to stroke and vehicle control (Figure 7B, $p <$

0.0001; n = 8-9 animals per group). Unlike the young adult animals receiving PDE2A-T1, no statistically significant improvement was observed in the performance of aged mice treated with PDE2A-T1 compared to vehicle controls after stroke. However, a non-significant trend towards improvement 5 weeks after stroke was observed, and a limited number of aged animals was used (Figure 6B, 8-9 aged animals per group vs. 12-15 young adult animals).

PDE2A inhibition increases motor system connections after cortical stroke in aged mice

To measure the pattern and extent of motor system connections after stroke and vehicle or drug treatment, aged mice receiving a unilateral stroke to forelimb motor cortex were injected with the neuroanatomical tracer biotinylated dextran amine (BDA) in motor cortex ipsilateral to the stroke site. BDA was injected nine weeks after stroke, the time point at which behavioral recovery following daily treatment with PDE2 inhibitor was observed. The density of BDA-positive axons was assessed in ipsilesional cortex for all animals. The mean integrated density of labeled axons linearly correlates with axon number. Data was normalized to the integrated density of the BDA injection site to account for differences in injection size. Animals treated with 3.0 mg/kg of PDE2A-T1 showed increased density of axonal labeling in cortex ipsilateral to stroke (Figure 8, $p = 0.0299$; n = 4-5 animals per group), indicating that PDE2 inhibition either preserves existing axonal connections or promotes sprouting of new connections following stroke. Preservation or remapping of motor system connections might be one potential process by which PDE2A-T1 enhances functional recovery after stroke.

PDE2A inhibition does not enhance neuronal, glial, microglial, or vascular cell proliferation after cortical stroke in aged mice

To assess the effect of PDE2 inhibition on cell progenitor pools after cortical stroke, aged mice receiving motor cortex stroke were treated with the thymidine analog EdU in drinking water. Mice were treated with EdU for days 3-10 after stroke, to assess potential early cell

proliferation in response to stroke. PDE2A-T1 treatment began 5 days after stroke and continued for 8 weeks. Immunohistochemical labeling of neurons (NeuN), oligodendrocyte lineage cells (Olig2), vascular cells (CD31), and microglia (IBA1) was co-located with chemical EdU staining. Treatment with PDE2A-T1 was not correlated with increased proliferation of any quantified cell type in peri-lesional cortex (Figure 9, $p = 0.3946$, $p = 0.2052$, $p = 0.9035$, and $p = 0.2553$, respectively; n = 4-5 animals per group).

PDE2A inhibition does not affect cortical loss or infarct size after cortical stroke in aged mice

Tissue was collected from aged mice receiving photothrombotic stroke to motor cortex, along with daily injections of vehicle or 3.0 mg/kg PDE2A-T1, 9 weeks after stroke. At this late stage, the size of the stroke and of any secondary tissue loss can be determined by comparing the ipsilateral cerebral striatum to the contralateral cerebral striatum. Tissue was Nissl stained and imaged, and cortical volumes were quantified using ImageJ. No change in cortical volume was observed with PDE2A-T1 treatment after stroke in aged mice (Figure 10, $p = 0.0937$; n = 5-6 animals per group).

Discussion

Brain region-specific treatments for stroke subtypes

The above data demonstrate that pharmacological PDE inhibition is a viable therapeutic strategy for promoting functional recovery after stroke. Importantly, PDE inhibition is site-specific in its role of improving recovery after stroke, with recovery patterns matching the spatial localization of each targeted PDE enzyme. The PDE inhibitor TAK-063, which is specific to the striatal PDE10A enzyme, promotes recovery of motor function after striatal stroke but not cortical stroke in young adult mice. Motor performance after cortical stroke is instead improved by treatment with PDE2A-T1, which targets the most highly expressed PDE in cortex. Together, these studies consist the first report of a pharmacological therapy for stroke recovery that is brain region-specific.

The adoption of brain region-specific treatments overcomes a key limitation in the development of pharmacological therapeutics for neurological disease: unwanted side effects. Most notably, early clinical trials attempting to utilize PDE inhibition as a cognitive enhancer and/or therapeutic for stroke were hampered by the nausea and emetic responses evoked by the PDE4 inhibitors used in these studies [82]. As the maximally expressed PDE isoform in the brain, PDE4 remains an attractive target for drug development; however, its high midbrain expression resulted in patient nausea and emesis, leading to poor drug tolerability [82]. This illustrates the necessity of targeting brain regions that are directly implicated in the diseases or neurological contexts under study, though this has proven challenging with systemically delivered pharmacological therapeutics. The current study attempts to address this challenge via the use of PDE inhibitors spatially restricted to the brain regions affected by each stroke subtype. This was achieved by the use of novel, isoform-specific PDE inhibitors with minimum activity against other, non-specific PDE isoforms expressed in non-targeted brain regions. Even

more precise localization of PDE isoforms and splice variants would aid in the development of increasingly specific PDE inhibitors that continue to minimize side effects.

Stroke in the striatum and in cortical regions governing motor function is very common [81], and each of these stroke subtypes involves distinct neuronal circuits and cellular architectures. The striatum is home to medium spiny neurons and cholinergic interneurons organized in striosomal and matrix compartments, whereas cortical neurons exhibit a much greater diversity of subtypes as well as laminar cellular organization [83-84]. These different environments could contribute to potential differences in the progression or recovery mechanisms induced after stroke. In this study, PDE inhibition promoted an increase in the density of motor system axonal connections, possibly via axonal sprouting, in both cortical and striatal models of stroke. However, the spatial localization of these connections varied across stroke models. Following TAK-063 treatment in striatal stroke, axonal connections were observed between ipsilesional motor cortex and contralateral striatum. The pattern observed after PDE2A-T1 treatment in cortical stroke was an increase in peri-infarct cortical motor system projections, a pattern consistently associated with functional recovery in studies of cortical strokes spanning motor regions [13-15, 18-19]. Additionally, while neither TAK-063 nor PDE2A-T1 promoted neurogenesis or gliogenesis, TAK-063 after striatal stroke increased angiogenesis in the contralesional striatum. The differences in recovery mechanisms across these different brain regions point to a need for a deeper understanding of the molecular, cellular, and circuit-level mechanisms governing pathogenesis and recovery following different stroke subtypes. The present findings support the possibility of region-specific neural repair, recovery, and treatment in stroke that is distinct based on stroke subtype, with subcortical-basal ganglionic stroke displaying distinct repair mechanisms and benefiting from a separate drug than cortical stroke. This subtype-specific tailoring of treatment might be extended to other common stroke types, such as white matter stroke [85].

Critical time window for PDE inhibition after stroke

Ischemic cell death occurs rapidly after stroke onset. Within hours, all cell types within the ischemic core die, producing irreversible damage [3, 63]. In this study, treatment with PDE inhibitors was initiated at a 5-day delay after stroke, a point that is considered part of the sub-acute phase of stroke that follows the initial period of post-stroke cell death in rodent models [12, 20, 86]. Consistent with this timeline, PDE treatment did not improve functional performance through amelioration of cell death or neuroprotection, evidenced by a lack of difference in stroke size between vehicle- treated mice and mice receiving TAK-063 or PDE2A-T1 treatment after stroke. The action of this drug as an enhancer of post-stroke repair and plasticity following damage, rather than as a neuroprotective agent, is a key distinction. Despite the historical focus of the stroke field on neuroprotection, of the >1000 experimental reports of neuroprotection in preclinical studies and the >100 targets tested in clinical trials, no treatments or drug regimens have shown enough efficacy to approve their use as protective agents in stroke [87-88]. With the failure of neuroprotection as a treatment strategy to date, a focus on enhancing and/or reactivating plasticity programs in adjacent, surviving tissue could prove more effective clinically compared to attempts at preventing inevitable cell death [89]. Especially with the recent, welcome reduction in stroke mortality rates due to improvements in acute care, and the consequent growing number of stroke survivors living with chronic disabilities, there is a dire need for treatment options that can effectively promote repair and functional recovery despite cell death-induced damage [1-4].

With this delay in mind, the specific time point to begin PDE inhibition following ischemic cell death was carefully chosen. Preclinical studies suggest that enhancing plasticity at times when acute injury and death are still occurring destabilizes the brain's ability to deal with stress and thus hinders endogenous neuroprotection, resulting in larger strokes [12, 20, 86]. Studies that enhance plasticity and excitability after stroke using tonic GABA inhibition or potentiation of glutamatergic AMPAR signaling demonstrate that initiation of treatment sooner than 5 days

post-stroke worsens stroke outcome by increasing cell death and infarct size. However, initiation of treatment after this sensitive window improves functional recovery with no impact on infarct size [20]. In both animal and human studies, early and increased initiation of rehabilitation therapies, such as paradigms for forced use of stroke-affected limbs, may worsen long-term functional outcomes when compared to treatments initiated later in time after stroke onset [90]. What is common to these clinical and preclinical studies is their attempts to promote recovery via enhancement of neuronal excitability or activity, whether physiologically by manipulating neurotransmitter systems or behaviorally by forced use [91, 153]. The detrimental effects of early treatment are thus possibly the result of increasing excitability and promoting energy-dependent plasticity programs during a time at which excitotoxic cell death and rapid depletion of cellular ATP stores are taking place [12]. Together, these observations suggest an inflection point in the timeline of stroke, where an initial sensitive period of cell death and damage transitions to a period of endogenous repair and plasticity in the more subacute time window after stroke. As potentiators of cyclic nucleotide signaling, downstream CREB activation, and resulting excitability and plasticity, PDE inhibitors were administered in this study at a 5-day delay after stroke. Future work will need to identify an optimal delivery timeline for human patients to receive maximum benefit from PDE inhibitors after stroke.

Cellular and molecular mechanisms of PDE inhibition after stroke

There are several possible mechanisms of action for PDE inhibition in ischemic stroke. First, PDE10A inhibition via daily TAK-063 treatment increases protein levels of BDNF in ipsilesional striatum when compared to vehicle treatment after striatal stroke. BDNF is a neurotrophic growth factor correlated with functional recovery in several preclinical models of stroke [37-39]. For example, potentiation of glutamatergic AMPAR signaling promotes behavioral recovery after stroke via induction of BDNF in peri-infarct tissue [37]. Direct delivery of BDNF to peri-infarct cortical tissue via sustained hydrogel release improves motor

performance and promotes axonal sprouting in animal models of cortical stroke [38]. Conversely, mutations in the BDNF gene prevent recovery in both animal models and clinical studies of stroke [92]. In brain injury models, including stroke, BDNF is acutely induced in affected brain regions; however, this induction only lasts for a limited time window after stroke, and BDNF does not cross the blood brain barrier [38-39, 93-94]. Since BDNF is transcribed downstream of CREB phosphorylation and activation, PDE inhibition might be one pharmacological means for increasing BDNF levels in a sustained, region-specific manner after its initial period of induction after stroke [95-98]. Future studies should quantify BDNF levels in peri-infarct cortex after cortical stroke and cortical PDE inhibition using PDE2A-T1, particularly given BDNF's role in promoting functional recovery and axonal sprouting in this region.

Another mechanism of action for PDE inhibition after stroke is axonal sprouting. Axonal sprouting within motor or somatosensory systems has been repeatedly demonstrated in many rodent and in primate stroke models to correlate with functional recovery [13-15, 18-19]. In this model of striatal stroke, PDE10A inhibition increased the density of axonal connections of ipsilesional motor cortex with contralateral striatum. This could potentially represent the sprouting and rerouting of ipsilesional corticostriatal projections, which would have been damaged or lost due to striatal stroke, to the contralateral, uninjured striatum after PDE10A inhibition. Similarly, PDE2A inhibition following stroke to the motor cortex increased the density of motor system connections in peri-infarct cortex. This pattern could represent the sprouting of new axonal connections into adjacent cortical regions spared after stroke, which suggests a possible takeover by these surviving regions of motor functions lost due to stroke [18, 51-53]. This explanation is consistent with recent studies demonstrating the causal role of CREB activation and CREB-mediated transcription on improving behavioral recovery, increasing axonal sprouting in peri-infarct cortical regions, and promoting functional reorganization of damaged cortical maps in the same rodent model of cortical stroke [18].

Alternatively, the increased density of axonal connections observed in both the striatal and cortical stroke models may represent a selective sparing of these contralateral connections in response to PDE inhibition after stroke. The methodology used for axonal labeling and axonal density quantification in this study cannot definitively eliminate this second possible interpretation. Future studies might use multiple axonal tracers conjugated to different fluorophores to distinguish between pre-stroke axonal connections and those that sprout after stroke, as well as more quantitative methods of mapping axonal projections, which could also determine potential sprouting directionality [13-14]. These follow-up experiments could more definitively determine whether PDE inhibition selectively protects axonal tracts or stimulates plasticity and regenerative axonal sprouting. That said, an axonal sprouting effect is consistent with a growing body of literature supporting roles for CREB- and BDNF-induced reparative plasticity after stroke [18-19, 37-39]. Additionally, the timing of treatment suggests mechanisms other than protection [12].

The potential enhancement of post-stroke axonal sprouting after PDE inhibition suggests a possible induction of growth programs within peri-infarct neurons in response to drug treatment [13, 99-100]. An initial induction of neuronal growth programs within surviving neurons is part of the endogenous cellular response of the brain to stroke [99-100]. This growth state comprises transcriptional changes in genes with known functions relevant to axonal sprouting and cortical circuit reorganization [13-15, 99-100]. However, this pro-growth state is limited and does not persist long after stroke; 21 days after ischemic injury, the transcriptional profile of peri-infarct sprouting neurons shows distinct changes compared to their 7-day post-stroke counterparts, with much of the early growth programs turned off and replaced with up-regulation of genes that maintain earlier axonal growth [13]. The axonal sprouting observed in response to PDE inhibition suggests a potential action of these PDE inhibitors in enhancing or re-opening this axonal pro-growth state. This is likely to occur through the action of CREB, a molecular target with known roles in governing plasticity during development, learning and memory, and

following multiple models of neurological disease [18, 43-52, 96, 101-106]. Consistent with this hypothesis, recent studies have demonstrated that viral up-regulation of CREB in peri-infarct excitatory neurons after cortical stroke induces transcriptional changes within these surviving cells, including activation of genes with roles in nervous system development and tissue development [18]. This suggests a role for CREB in mediating a pro-growth state similar to but distinct from developmental growth.

In striatal stroke, this “growth state” might extend to non-neural cell types—specifically, brain endothelial cells. This is supported by data demonstrating enhanced angiogenesis observed in contralesional striatum following PDE10A inhibition. This angiogenesis was quantified as an increase in the number of cells expressing an endothelial-specific marker that had incorporated EdU, which labels proliferating cells, during the first 3-10 days after stroke. Angiogenesis, or the growth of new blood vessels from existing vessel branches, has also long been correlated with functional recovery after stroke, as well as with axonal sprouting after neurological injury [108-111]. After striatal stroke, angiogenesis was observed in the same region as axonal sprouting; however, the same two processes were not observed in ipsilesional striatum. Recent studies have begun to elucidate the role of the contralateral, uninjured hemisphere in recovery after stroke. In larger models of stroke, axonal sprouting is in fact observed involving contralateral cortex, striatum, and spinal cord [112]. Additionally, in rodent models of ischemic cortical stroke, mice that display spontaneous functional recovery early after stroke demonstrate gene expression changes in contralateral cortex that suggest the involvement of cAMP signaling pathways in this endogenous recovery [112]. Among these differentially regulated genes, PDE10A is down-regulated in the contralateral cortex of these spontaneously recovering mice [112]. While the contribution of contralateral tissue to stroke recovery is not fully understood, it is possible that pharmacological inhibition of PDEs such as PDE10A might have specific roles in promoting recovery mechanisms that involve contralesional brain regions, particularly due to the bilateral targeting of these drugs in the brain.

CREB: a molecular regulator of recovery downstream of PDE inhibition

PDE inhibition prevents the hydrolysis of cAMP and cGMP, increasing the availability of these intracellular second messengers to participate in signaling pathways that culminate in CREB activation [72]. Since BDNF is transcribed downstream of CREB activation, the increase in BDNF observed in this study suggests enhanced CREB activation with PDE10A inhibition in contralesional striatum. Additionally, the cellular processes promoted by PDE inhibition in both striatum and cortex, namely axonal sprouting and striatal angiogenesis, have both been demonstrated in multiple models of stroke and other neurological diseases to correlate with increased CREB activation and CRE-dependent transcription. In other disease models and in learning and memory studies, accumulating evidence has similarly demonstrated the ability of PDE inhibitors to increase CREB phosphorylation in the brain. In neuronal culture assays utilizing transgenic CRE-β lactamase cells lines, a wide range of PDE inhibitors leads to potent enhancement of CRE-mediated transcription in these cells [113]. PDE4 inhibition via Rolipram, the subject of various clinical trials, increases hippocampal expression and phosphorylation of CREB and the cAMP-dependent, learning and memory-related protein Arc, as well as CREB expression following brain injury [114-115]. In models of fetal alcohol syndrome, PDE1 inhibition restores phosphorylated CREB levels in visual cortex, in addition to proper visual tuning of cortical maps [116]. Together, these data demonstrate that systemic delivery of PDE inhibitors can effectively enhance CREB phosphorylation in the brain, and that CREB activation is an important mechanism of action of these drugs.

The key role for CREB activation in stroke recovery has been highlighted in several recent studies [18-19, 21, 117]. CREB was shown to play a causal role in regulating behavioral recovery after stroke, acting as a molecular switch that can be turned on or off to mediate functional improvement [18]. In these studies, selective inactivation of neurons in which CREB was induced results in a loss of the motor gains observed after CREB activation, while

subsequent release of this inactivation reinstates the improvement. Functional recovery in this previous study also correlated with increases in axonal sprouting, which is in line with the current results described following PDE inhibition, as well as functional reorganization of cortical maps. In other studies, the molecular learning and memory target CCR5, the chemokine receptor that binds HIV, was shown to promote functional recovery after stroke when genetically knocked-down in neurons or pharmacologically antagonized with the FDA-approved drug maraviroc [19]. Importantly, this functional improvement accompanied genetic up-regulation of CREB in neurons, as well as enhancements in axonal sprouting and dendritic spine plasticity. As discussed previously, potentiation of glutamatergic AMPAR signaling also improves functional recovery after stroke in a BDNF-dependent manner, suggesting a role for increased CREB activation in this process [21]. Together, these studies demonstrate a convergence of mechanism onto CREB activation by the action of many molecular learning and memory systems targeted as therapeutics for stroke. The current study demonstrates a pharmacological method for targeting CREB in the brain after stroke using a systemically injected drug and thus could have important clinical potential.

Age-specific effects of PDE inhibition after stroke

Most preclinical stroke studies are conducted using young adult animals [118]. However, stroke predominantly affects the aging population, with stroke incidence markedly increasing beyond the age of 65 [119]. This suggests that aged models of stroke better represent clinical, human stroke. Indeed, aged mouse models are more likely to incorporate common, age-related stroke comorbidities, such as hyperlipidemia, obesity, and hypertension, which contribute to the severity of stroke pathogenesis [118]. Aged mouse models also more closely represent the aging brain's reduced capacity for plasticity, a consequence of the structural and functional changes demonstrated to affect brain cell types with age [120-122]. These factors make aged mice a stronger model system to test stroke therapeutics preclinically. In this study, 18 to 19-

month old C57BL mice were used in addition to young adult mice to more accurately model human stroke. This represents a mouse age range at which senescent biomarkers are routinely observed, and roughly correlates to 60 years of age in humans [123-124]. (In contrast, the young adult studies were performed in 3-month old mice, which correlates to 20 years of age in humans.)

An important result of this work is that TAK-063 treatment, which inhibits striatal PDE10A, did not promote recovery of function in aged mice after striatal stroke despite its efficacy in young adult mice. This discrepancy in therapeutic efficacy might be attributed to a variety of factors. First, since the severity of stroke increases with age [99, 120, 125-128], greater initial damage in the aged brain might have created a larger barrier to recovery not overcome with TAK-063 treatment. In aged models of stroke, accelerated lesion and glial scar formation, reduced blood brain barrier integrity, greater white matter and blood vessel degeneration, increased oxidative damage, and elevated, more widespread inflammatory responses contribute to greater degrees of injury and more sustained deficits after stroke [126-134]. At least some of these aging mechanisms might act with region specificity in the brain. For example, occlusion of the middle cerebral artery, which damages both cortical and striatal tissue, produces accelerated striatal but not cortical infarcts in aged relative to young adult mice [130]. In this model, the aged striatum but not cortex also displays higher levels of mitochondrial dysfunction, oxidative stress, and pro-oxidant/antioxidant imbalances in response to stroke, contributing to a potential subcortical ischemic vulnerability with age [130]. Thus, the treatment dose and duration used for young adults might not have promoted recovery in aged mice experiencing accelerated or more severe post-stroke damage. This possibility should be further explored.

Second, as noted, the capacity of the aged brain for repair and regeneration diminishes with age [120-122, 134]. It is thus possible that the mechanisms governing the observed recovery in young adult animals were not induced to the same extent in aged animals, limiting recovery. In this study, TAK-063 treatment promoted angiogenesis and axonal sprouting after

striatal stroke in young adult animals. However, both these repair processes exhibit age-dependent decline [13, 15, 19, 136-142]. For example, lower angiogenic responses have been consistently reported in preclinical and clinical studies of aged stroke, hypoxia, and peripheral artery occlusion [136-141]. This reduced angiogenesis can in part be explained by impaired endothelial cell function (namely, lower nitric oxide production and acetylcholine-dependent vessel dilation), as well as decreased endothelial cell VEGF receptor expression, resulting in attenuated responses to pro-angiogenic factors [136-139]. In fact, this decline in VEGF receptor expression might exhibit region-dependency within the brain [136]. On the other hand, axonal regeneration and sprouting in response to CNS injury also diminishes with age [13, 15, 19, 142]. For example, genetic PTEN deletion, which typically promotes axonal regeneration after nerve injury in young adults, does not promote long-range growth or protect against degeneration after spinal cord injury in aged mice [142]. After ischemic stroke, axonal growth-promoting genes are induced at later time points and growth-inhibiting genes/proteins are induced earlier and to a greater extent in aged mice relative to young adult mice [13, 15, 19]. It is thus possible that the lack of recovery observed in aged mice could in part be due to diminished axonal sprouting, as well as angiogenesis, processes with demonstrated impact on functional recovery after stroke. Further studies are necessary to compare the extent of axonal sprouting and angiogenesis induced in the striatum of aged versus young adult mice after stroke and TAK-063 treatment.

Lastly, age-dependent alterations in the signaling cascade underlying TAK-063's mechanism of action might have rendered this drug treatment less effective at promoting recovery in the aging brain. Accumulating evidence in both human and animal models have identified age-related, brain region-specific changes in the cyclic nucleotide to CREB signaling cascade at each level of the pathway, including alterations in GPCR, adenylyl cyclase, cAMP/cGMP, PDE, PKA/PKC, and CREB expression and/or activity [143]. Of note, PDE enzymes are reported to exhibit more resistance to pharmacological modulation during aging, with rolipram binding activity reduced by 22-25% in the aging striatum and cortex (but not

cerebellum) of nonhuman primates, and sildenafil activity lowered by half in aged rodent models of stroke [144, 145]. In aging D1 neurons, dopamine agonists are less effective at inhibiting the binding of PDE inhibitors to their PDE substrates, suggesting a depression of cyclic nucleotide-dependent signaling cascades with age [144]. Downstream of this, the DNA binding activity of CRE has been reported to decrease with age, particularly in the basal forebrain and striatum [146]. Age-dependent changes in cyclic nucleotide-protein kinase-CREB signaling are more pronounced when the aged brain is challenged with stress or injury. After traumatic brain injury, aged mice show larger reductions in cAMP levels compared to young mice, and this correlates with reduced LTP and cognitive performance [147]. Aged mice also experience lower injury thresholds for TBI-induced cAMP reductions, as well as less complete recovery of cognitive abilities following PDE inhibitor treatment [147]. In models of neurotoxic injury to the striatum, compensatory BDNF production in the lesioned striatum is not observed in aged animals despite an observed BDNF increase in young adult mice [148]. This lack of BDNF induction is region-specific, as the same neurotoxic injury to the midbrain leads to BDNF increases in both young adult and aged mice [148]. Further, regional declines in BDNF signaling with age have been reported to depend on brain region-specific changes in regulators of BDNF transcription and post-transcriptional processing, as well as regional changes in phosphorylation of BDNF's high-affinity receptor TrkB [135]. Additional studies are needed to identify potential age-dependent changes in cyclic nucleotide, CREB, and/or BDNF regulation after striatal stroke. However, it remains possible that the lack of recovery promoted by TAK-063 might involve an age-related reduction in cyclic nucleotide pathways, CREB activity, and/or BDNF signaling, some of the key molecular targets affected by PDE inhibition.

Together, these age-dependent differences highlight the need for a revised PDE inhibition treatment strategy for striatal stroke. This might include increasing the dose administered to aged animals beyond that of young adults, or perhaps combining TAK-063 treatment with other drugs or rehabilitative therapies to effect maximum functional recovery.

Previous studies using the PDE5 inhibitor sildenafil as a treatment for cortical stroke demonstrated that aged mice required 5 times the dose of young adults to achieve functional recovery with treatment (10 mg/kg versus 2 mg/kg; 3 mg/kg was administered in the current study) [145]. In these earlier studies, aged mice demonstrated only half the increase in cyclic nucleotide levels as young adult mice did with the same dose of PDE inhibitor [145]. In other studies of cortical stroke, aged mice required a combined treatment of BDNF and a BDNF-inducing AMPAkine drug to promote similar functional recovery and induce BDNF levels to those observed in young adult animals receiving BDNF treatment alone [149]. This suggests that a certain threshold for BDNF potentiation and/or upstream CREB activation exists to observe functional recovery in aged mice, which might not have been reached in this study with the particular dose of drug used. Further studies are required to determine the optimal TAK-063 dosage to use for aged mice, as well as how well tolerated these higher doses of drugs are. Alternative strategies might include combining a more effective TAK-063 dose with a rehabilitation paradigm such as forced paretic limb use, a behavioral therapy demonstrated to increase measures of brain plasticity after stroke including peri-infarct neuronal excitability, neurogenesis, and axonal sprouting [91, 150-154].

Despite the challenges associated with repairing the aged brain after stroke, the second drug tested in this study, PDE2A-T1, was able to promote functional recovery and tissue-level repair in aged mice after cortical stroke. Several key distinctions can be made between the PDE2A-T1 and TAK-063 studies which might account for this difference. First, the brain regions injured in each study varied considerably, with the PDE2A-T1 study conducted in mice receiving cortical strokes, and the TAK-063 study conducted in mice receiving subcortical, striatal strokes. As discussed above, there is evidence to support that a number of age-related mechanisms associated with increased stroke severity, lower plasticity capacity, and potentially reduced drug efficacy are heightened in subcortical brain regions relative to cortical areas. It is thus possible that a subset of cellular and molecular processes involved in stroke damage or recovery might

be differentially regulated in striatum versus cortex and/or differentially targeted by regional PDE inhibition. Further studies comparing the pathophysiology of stroke in different brain regions are needed to explore this hypothesis and to develop more effective treatments for stroke subtypes. Second, the specific targets of the two PDE inhibitors in this study differ, which might have implications for the molecular mechanisms induced by these drugs. PDE2A-T1 targets the cGMP-stimulated PDE2 enzyme, which exhibits bias towards cGMP hydrolysis in the brain, while TAK-063 targets PDE10, a dual-substrate enzyme with higher cAMP specificity [72, 73]. While both cyclic nucleotides act in signaling cascades that activate CRE-mediated transcription, it is possible that additional mechanisms induced by cGMP potentiation underlie some of the drug-induced recovery observed after cortical stroke and PDE2A-T1 treatment [69-70, 155]. In the brain, the NO/cGMP pathway is associated with neurogenesis, angiogenesis, and axonal sprouting [145, 155-158]. Notably, cGMP levels and activity of the nitric oxide/cGMP pathway are reduced in the aging brain, and after stroke, aged mice exhibit decreased cortical levels of cGMP relative to young adult mice [143, 145, 159-161]. Additional studies might investigate differences in cGMP- versus cAMP-mediated mechanisms of repair after stroke, and the extent to which each cyclic nucleotide signaling system is potentiated after TAK-063 and PDE2A-T1 treatment. Lastly, it should be noted that the technical method of stroke production varied across studies, with cortical strokes produced via photothrombosis and striatal strokes produced via localized vasoconstrictor injection. Any variations in the pathophysiology induced by each respective stroke model, or the potential differing sensitivity of the behavioral assessments towards detecting deficits from each stroke model, should be taken into account for a more comprehensive comparison of the two PDE inhibitors.

Together, these aged mouse studies highlight the importance of validating preclinical findings in the appropriate disease models. This would aid in the development of stroke therapeutics that are met with more clinical success. It is possible that the use of young adult

animals in preclinical stroke research is one reason for the failure of many stroke drugs in clinical trials, despite demonstrated preclinical promise [118, 161-162].

Future directions

The current studies identified axonal sprouting and angiogenesis as neural plasticity measures potentially induced by CREB activation with PDE inhibition after stroke. In addition to these mechanisms, CREB is a known enhancer of neuronal excitability [41, 42], and enhancing neuronal excitability specifically has been shown to promote functional recovery after stroke [3, 23, 18, 20-21, 149, 163-166]. Future studies could aim to understand the effects of PDE inhibition specifically on peri-infarct and contralateral excitability after stroke. This can be investigated at multiple scales, such as the study of dendritic spine plasticity and turnover, the electrophysiological properties of various neuronal cell types in the PDE inhibition territory, or circuit-level excitability via calcium imaging. These excitability changes can also be investigated in the context of combined neurorehabilitative and drug treatment studies, to probe for a potential synergistic effect of behavioral and pharmacological enhancements in excitability and recovery after stroke.

However, CREB activation is just one potential mechanism of PDE inhibition action. PDEs and cyclic nucleotides are involved in a wide variety of signal transduction pathways outside of those that activate CREB and CRE-mediated transcription, some of which also regulate cellular excitability [70-73, 79, 95]. Alternative molecular effectors known to act downstream of PDE inhibition include: cyclic nucleotide-gated ion channels (CNGs), the exchange protein activated by cAMP (Epac), mitogen activated protein kinases (MAPK), Ras/Raf and ERK kinases, and cAMP-GEF proteins, which activate the RAP-1 pathway [167-171]. Due to the complex nature of intracellular signaling, these alternative and/or complementary mechanisms of action for PDE inhibition in stroke should also be considered. While the examination of these separate signaling pathways and mechanisms is outside the

scope of this current study, investigating these other systems are potential targets for future work and might uncover additional cellular and molecular mechanisms for neural repair after stroke.

Additional work might also focus on characterizing the cellular and subcellular compartmentalization of PDEs, as well as the action of increasingly specific PDE inhibitors, in the brain. For example, the novel PDE10A inhibitor used in this study, TAK-063, is known to preferentially increase cyclic nucleotide expression in the striatal D2 medium spiny neurons (MSNs) and only partially in D1 MSNs [172]. In other words, beyond its region-specific properties, TAK-063 is also a neuronal subtype-biased drug. In models of neuropsychiatric and Huntington's diseases, this property allows TAK-063 to restore the pathological imbalance in neurotransmission observed between striatal D1 and D2 in preclinical models of these diseases, leading to TAK-063 induced behavioral improvement [172]. Imbalances in the D1/D2 system are implicated in various hypokinetic and hyperkinetic movement disorders resulting from lesions to the basal ganglia, including the striatum [174]. These disorders can present following ischemic lesions to these regions, sometimes at a delay after stroke [175]. It is possible that an additional mechanism of action of PDE10A inhibition via TAK-063 treatment might be to restore a potential imbalance in this striatal system. As noted, aging D1 neurons exhibit depressions in cAMP signalling [144]; identification of PDE10A inhibitors that function in D1 rather than or in addition to D2 MSNs might be a more effective therapeutic for aged striatal stroke. In addition to investigating the effect of TAK-063 on D1/D2 MSN balance after stroke, future studies could also identify whether the cortical PDE inhibitor used in this study, PDE2A-T1, also exhibits any cell type selectivity. These cortical studies might aim to identify potential cell type- and neuronal subtype-specific expression of PDEs in cortex, whether PDE expression patterns change with stroke, and the potential role of PDE inhibitors in regulating the excitation-inhibition balance after stroke. Characterizing the distribution of PDEs and the effects of PDE inhibitors in this way would lead to a better understanding of the mechanisms underlying PDE inhibition and

potentially aid in the development of increasingly specific PDE inhibitors that target these differentially localized PDE isoforms.

Currently, selective PDE inhibitors are being investigated as potential therapeutics for a wide range of neurological diseases, including schizophrenia, Alzheimer's disease, Huntington's disease, and even malignant brain tumors [71-73]. Outside of the brain, PDE inhibitors have found clinical success in ameliorating symptoms of pulmonary hypertension, acute cardiac failure, and erectile dysfunction [73]. With an increased understanding of the mechanisms of PDE inhibitor action, the physiological roles and compartmentalization properties of different PDE isoforms [176-177], and the dynamic changes in cyclic nucleotide signaling and localization during health and disease [178-179], more targeted PDE inhibitors can be developed and applied strategically towards the treatment of peripheral and brain disease, including stroke [71-73, 77-80].

Figures

Figure 1: Functional effect of TAK-063 on forelimb use and gait post-striatal and cortical stroke in young adult mice

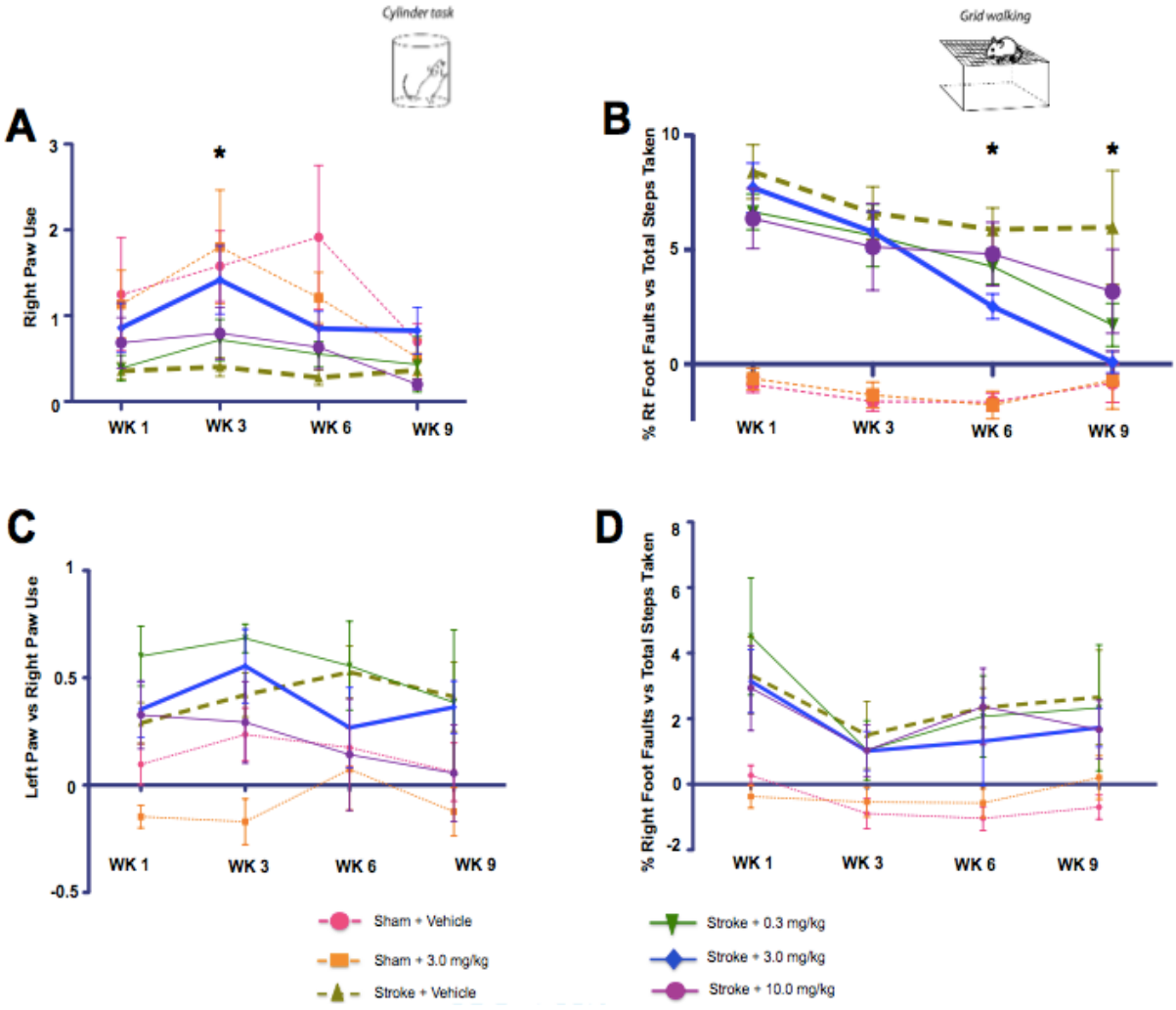

Figure 1: (A) In post-striatal stroke of the left hemisphere, the Cylinder task revealed that Stroke + 3.0 mg/kg versus Stroke + Vehicle had an overall increased right paw use over the 9-week treatment; *P = 0.04258. (B) Differences in gait observed for Stroke + Vehicle versus Stroke + 3.0 mg/kg at 6 weeks; *P = 0.04317 and at 9 weeks; *P = 0.03387 in post-striatal stroke. For overall differences between Stroke + Vehicle versus Stroke + 3.0 mg/kg, P = 0.05160. (C) In post-cortical stroke of the left hemisphere, the forelimb task revealed no differences in Stroke + Vehicle versus treatment groups. (D) Differences in gait were also not observed in Stroke + Vehicle versus treatment groups in post-cortical stroke. Error bars represent mean ± SEM for n = 8-10 per group in striatal stroke model and n = 8-11 in cortical stroke model. Data are reported as difference from baseline. Data were analyzed by GLMs with Tukey's HSD.

Figure 2: TAK-063 treatment-mediated alterations in BDNF expression in striatal tissue

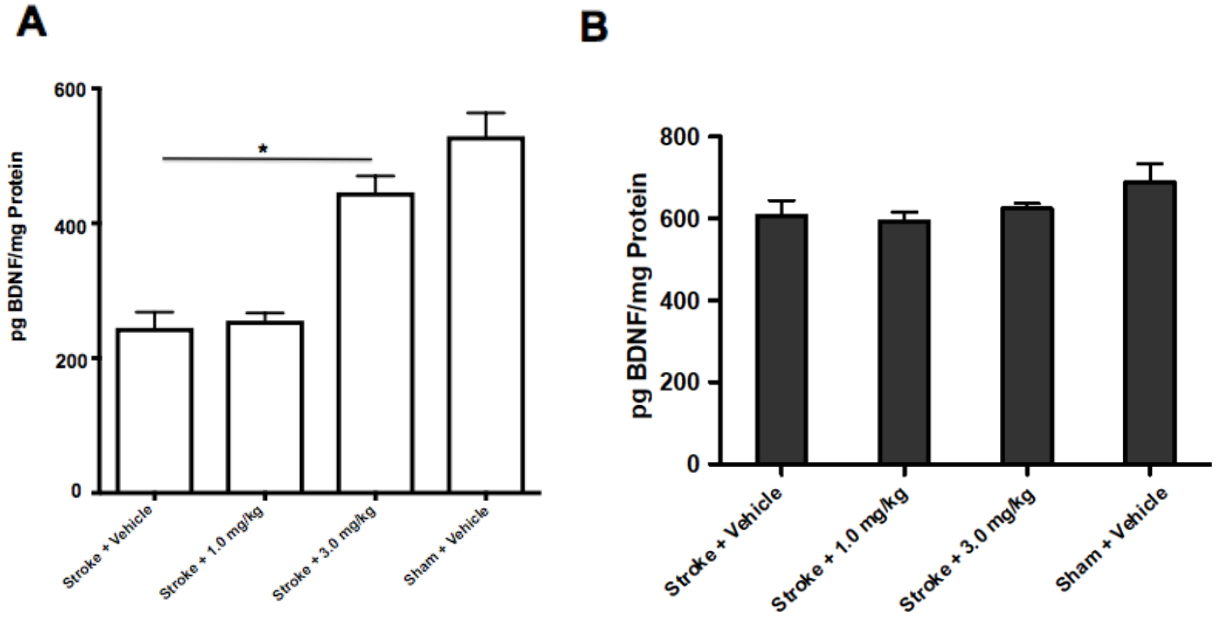

Figure 2: (A) BDNF expression levels measured with tissue ELISA showed a decrease at 3 weeks post-striatal stroke in striatal tissue, ipsilateral to the infarct. Treatment with TAK- 063 resulted in a significant increase in BDNF levels in the Stroke + 3.0 mg/kg versus Stroke + Vehicle group in ipsilateral striatum; P = 0.005, corrected for multiple comparisons. Error bars represent mean ± SEM for n = 10 per group. (B) Treatment with TAK-063 resulted no differences in BDNF levels in Stroke + Vehicle compared to other treatment groups in contralateral striatum. Error bars represent mean ± SEM for n = 3-4 per group.

Figure 3: Motor connections post-striatal stroke and TAK-063 treatment

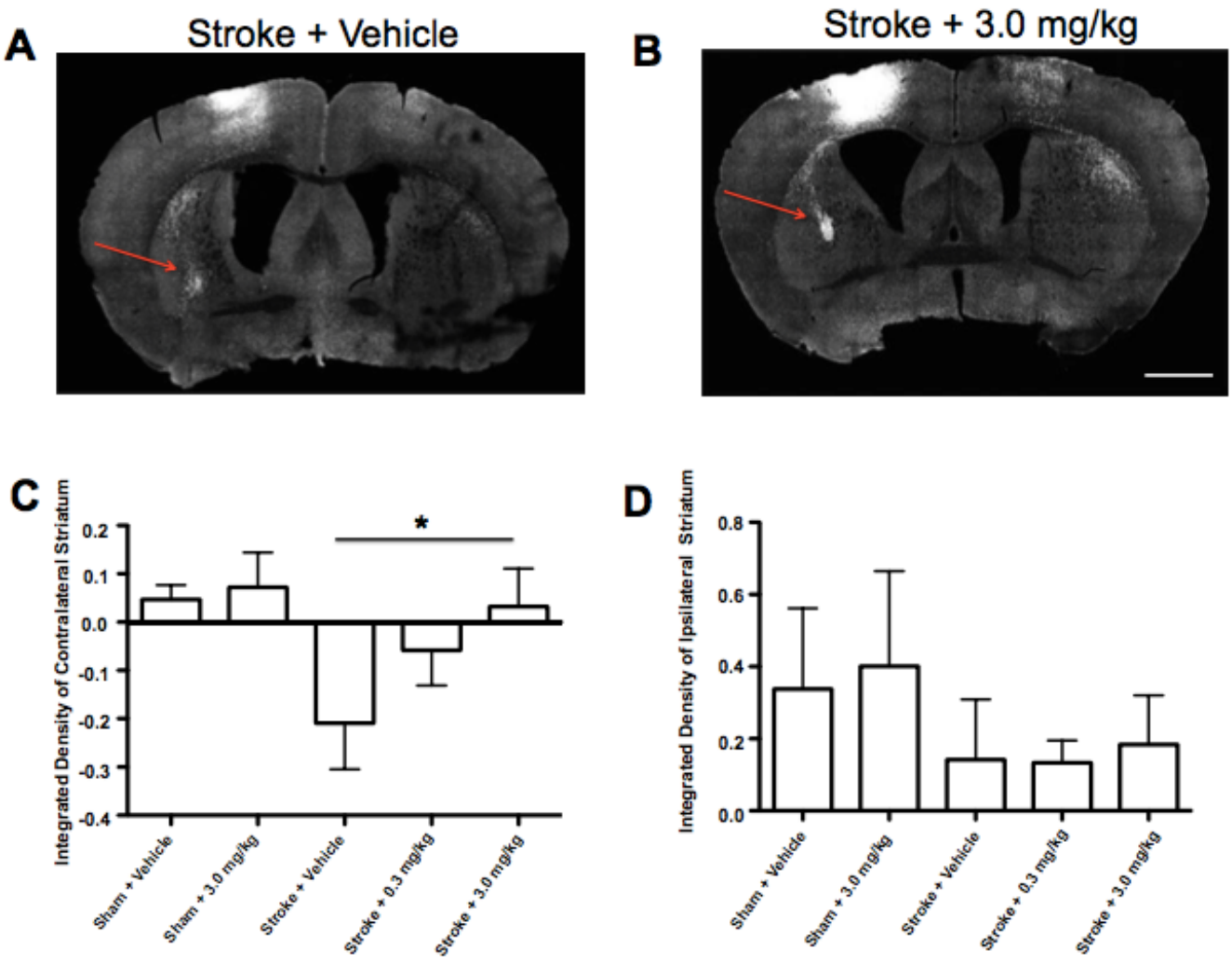

Figure 3: (A) Image of BDA-labeled connections from the motor cortex of Stroke + Vehicle and (B) Stroke + 3.0 mg/kg mice. Arrow shows labeled axonal fibers. Data were normalized to integrated density of the BDA injection site for each treatment group. (C) Total BDA-positive fibers were increased at 6 weeks post-striatal stroke in the contralateral cortical tissue in Stroke + 3.0 mg/kg versus Stroke + Vehicle; *P = 0.0009. (D) Ipsilateral striatum showed no differences between groups. Error bars represent mean ± SEM for n = 4-5 per group. Scale bar = 50 μm and applies to all photomicrographs.

Figure 4: Effects of PDE10A inhibition on angiogenesis, neurogenesis, and gliogenesis in post-striatal stroke

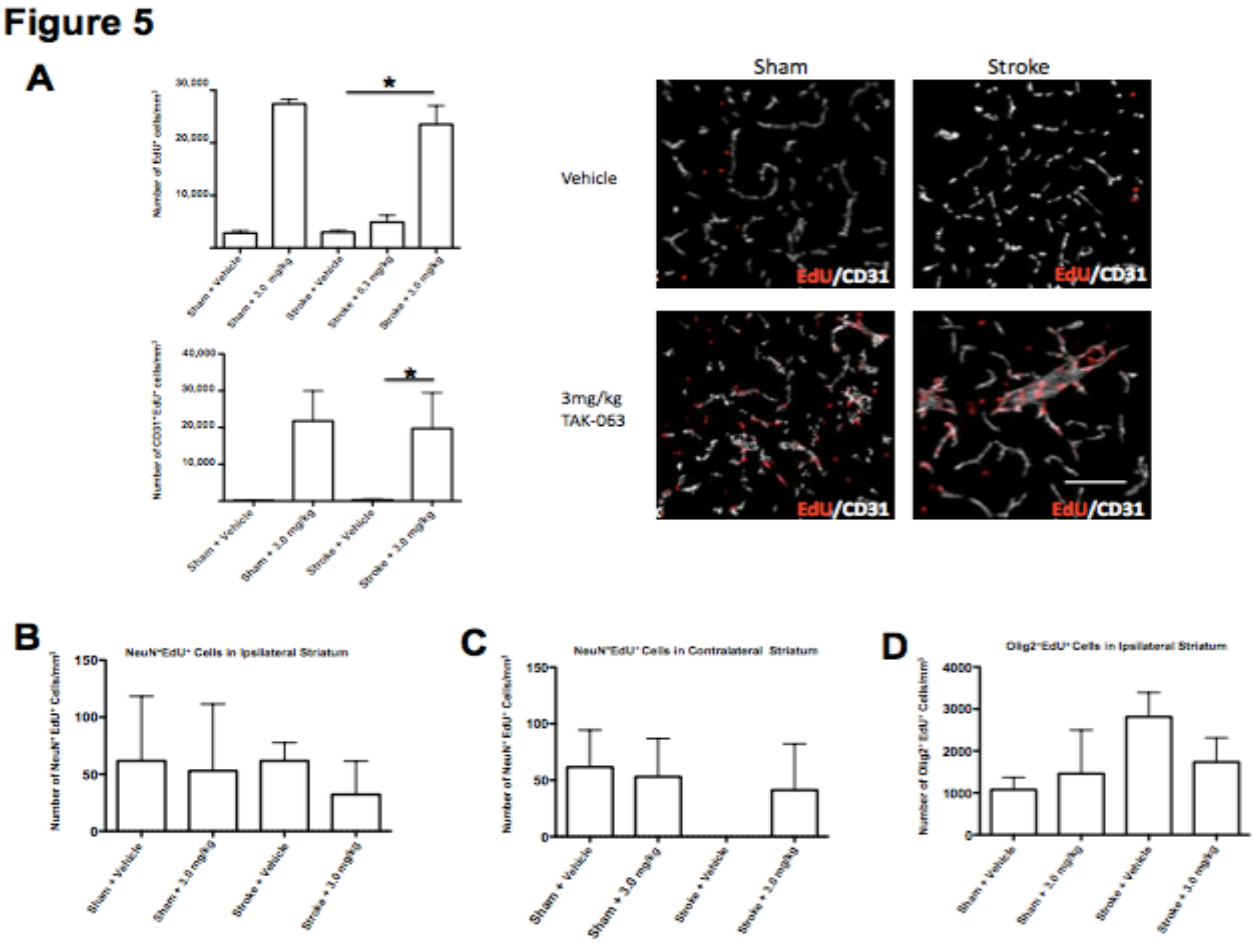

Figure 4: (A, upper panel) Total EdU$^+$ cells were increased at 6 weeks post-striatal stroke in contralateral striatum in Stroke + 3.0 mg/kg versus Stroke + Vehicle; *P < 0.0001. (A, lower panel) EdU$^+$ CD31$^+$ cells were increased at 6 weeks post- striatal stroke in striatal tissue contralateral to the infarct. Representative images of EdU$^+$CD31$^+$cells in contralateral striatum. Top left: Sham + Vehicle, bottom left: Sham + 3.0 mg/kg, top right: Stroke + Vehicle, bottom right: Stroke + 3.0 mg/kg. (A, lower panel) Treatment with TAK-063 resulted in a significant increase in EdU$^+$CD31$^+$ cells in the Stroke + 3.0 mg/kg versus Stroke + Vehicle group, *P = 0.0201. No differences were observed in Sham + 3.0 mg/kg and Stroke + 3.0 mg/kg. Error bars represent mean ± SEM for n = 3-5 per group. (B, C) NeuN$^+$ EdU$^+$ cells showed no differences between groups 6 weeks post-striatal stroke in contralateral and ipsilateral striatum. Error bars represent mean ± SEM for n = 3 per group. (C) Olig2$^+$ EdU$^+$ cells showed no differences between groups in ipsilateral striatum. Error bars represent mean ± SEM for n = 3-4 per group. Scale bar = 50 μm and applies to all images.

Figure 5: Degree of infarct size in TAK-063-treated mice

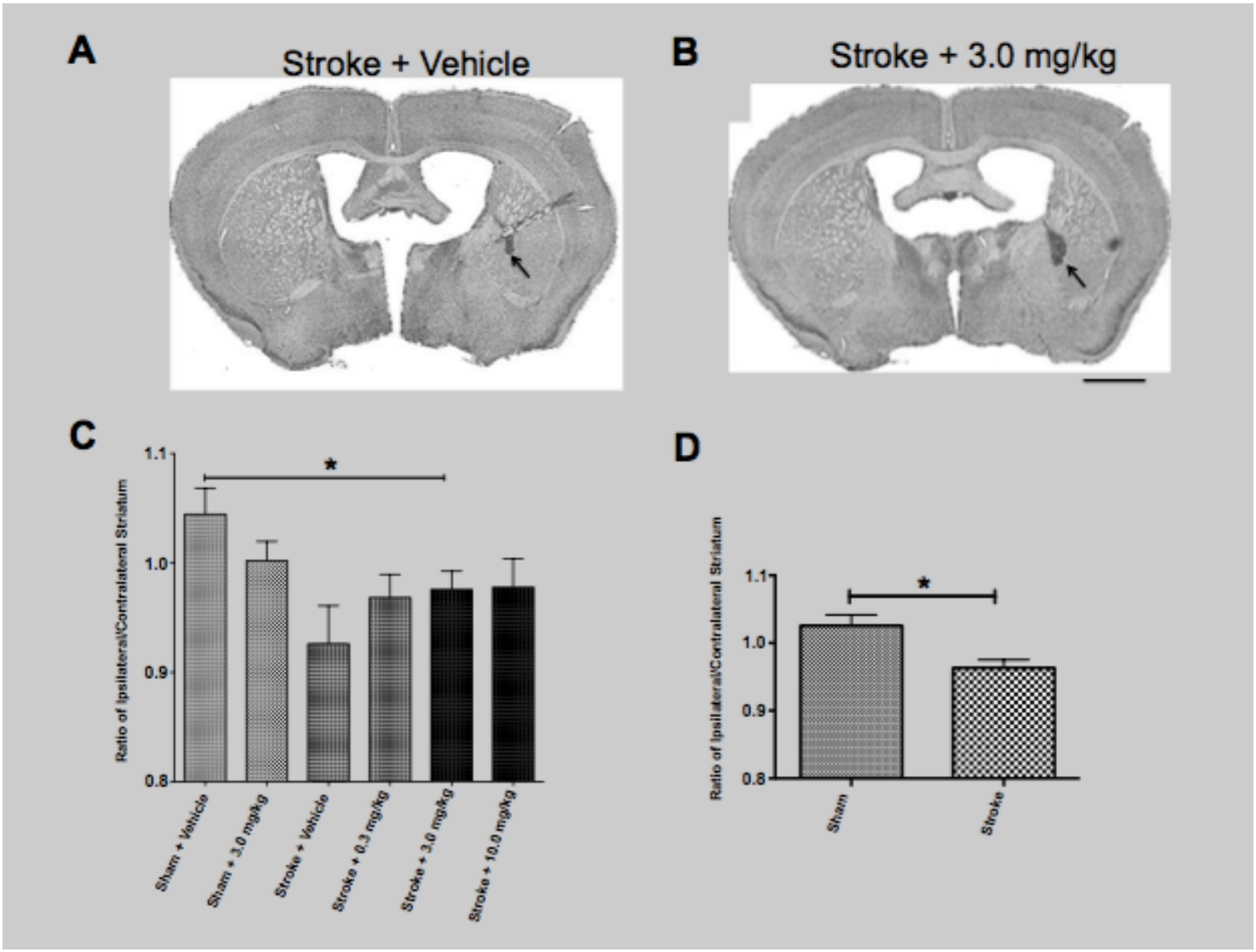

Figure 5: (A, C) Top panel shows Nissl-stained brain tissue section from representative stroke groups at 10 weeks post- stroke. The arrow indicates the area of striatal infarct, with no observable differences between groups. The lower panel quantifies the degree of stroke. The Y-axis indicates the normalized ratio of the ipsilateral stroked hemisphere over the contralateral non-stroked hemisphere. Data is shown are measurements at -0.22 mm to -0.82 mm from Bregma. Stroke leads to a loss of tissue volume in the hemisphere ipsilateral to the stroke. Lower values indicate loss of tissue volume. Data show that there was no effect between the treatment groups; however (B, C) Stroke + 3.0 mg/kg and Stroke + Vehicle group shows a significant difference compared to Sham + Vehicle; *P = 0. 0146; *P = 0.0053; n = 8- 10 per group. (D) The combined stroke groups versus the combined sham control groups (n =18 and 32, *P = 0.001). All p-values corrected for multiple comparisons. Error bars represent mean ± SEM. Scale bar = 50 µm and applies to all images.

Figure 6: Functional effect of TAK-063 on gait post-striatal stroke in aged mice

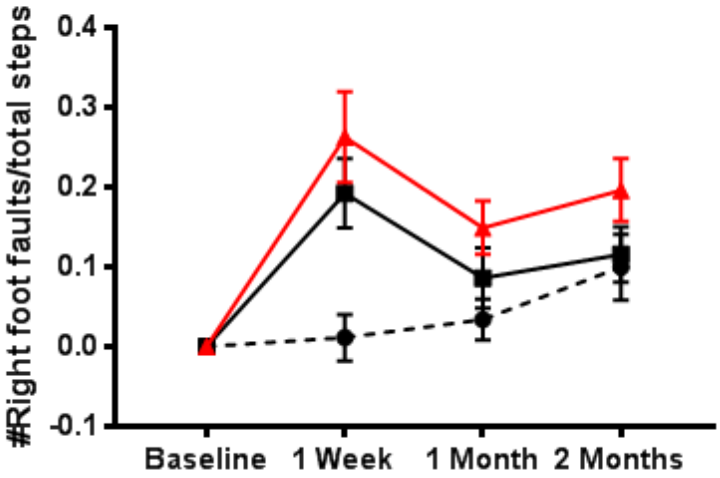

Figure 6: In aged mice, PDE10A inhibition via daily TAK-063 injection does not improve gait abnormalities made with the affected forelimb after stroke ($p = 0.4121$ and $p = 0.2359$ for 1 month and 2 months after stroke, respectively). Two-way ANOVA with Tukey multiple comparisons test. Data shown are means and SEMs, 12-17 animals per group.

Figure 7: Functional effect of PDE2A-T1 on gait, spontaneous forelimb use, and skilled reach after cortical stroke in young adult and aged mice

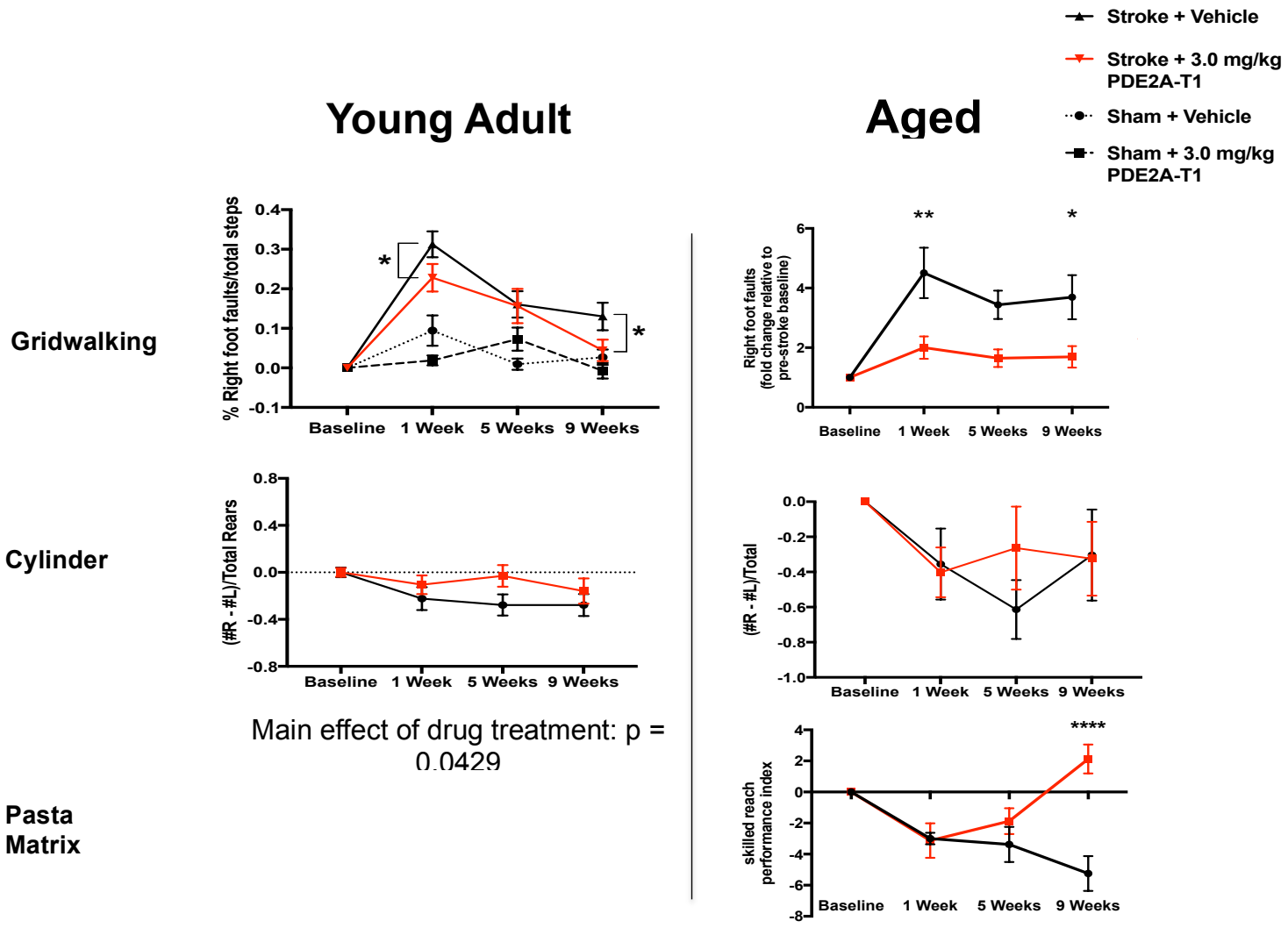

Figure 7: (A) In young adult mice receiving cortical stroke, PDE2A-T1 reduces gait abnormalities made with affected forelimb 1 week and 9 weeks after stroke ($p = 0.0478$ and $p = 0.0452$, respectively) and increases spontaneous use of affected forelimb during exploratory activity ($p = 0.0429$). (B) In aged mice receiving cortical stroke, PDE2A-T1 reduces gait abnormalities with affected forelimb 1 week and 9 weeks after stroke ($p = 0.0031$, $p = 0.0555$, and $p = 0.0255$, respectively), and increases the number of successful reaches through a skilled reach matrix ($p < 0.0001$). PDE2A-T1 does not improve spontaneous forelimb ability in aged animals 1, 5, or 9 weeks after stroke ($p > 0.9999$, $p = 0.5183$, and $p > 0.9999$, respectively). Two-way ANOVA with Tukey and/or Sidak multiple comparisons test. Data shown are means and SEMs, 12-15 young adult animals per group, 8-9 aged animals per group.

Figure 8: PDE2 inhibition increases peri-lesional axonal connections in aged mice after cortical stroke

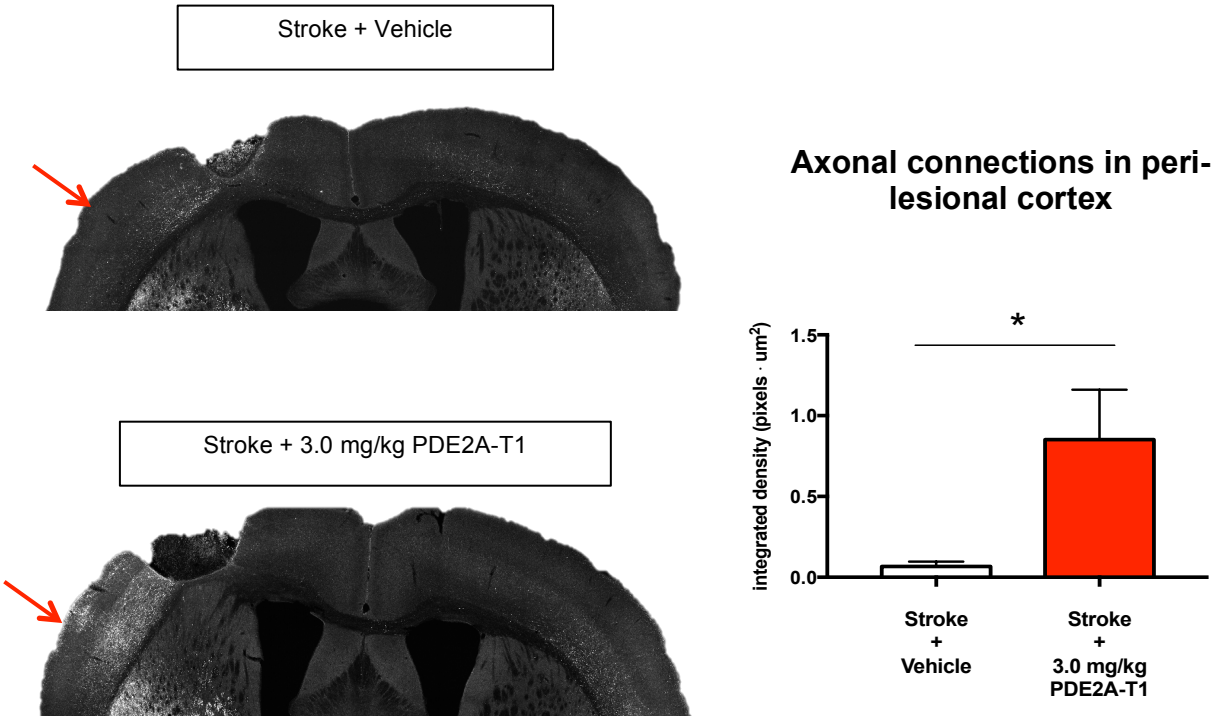

Figure 8: Amount of BDA-labeled axons in ipsilateral cortex is increased following cortical stroke and daily PDE2A-T1 treatment ($p = 0.0299$). Integrated density, a measure of fluorescence intensity, of BDA-labeled axons was quantified using ImageJ. Data were normalized to integrated density of BDA injection site. Data shown are means and SEMs, n = 4-5 animals per group.

Figure 9: PDE2 inhibition does not enhance early-phase cell progenitor proliferation in peri-lesional cortex after stroke in aged mice

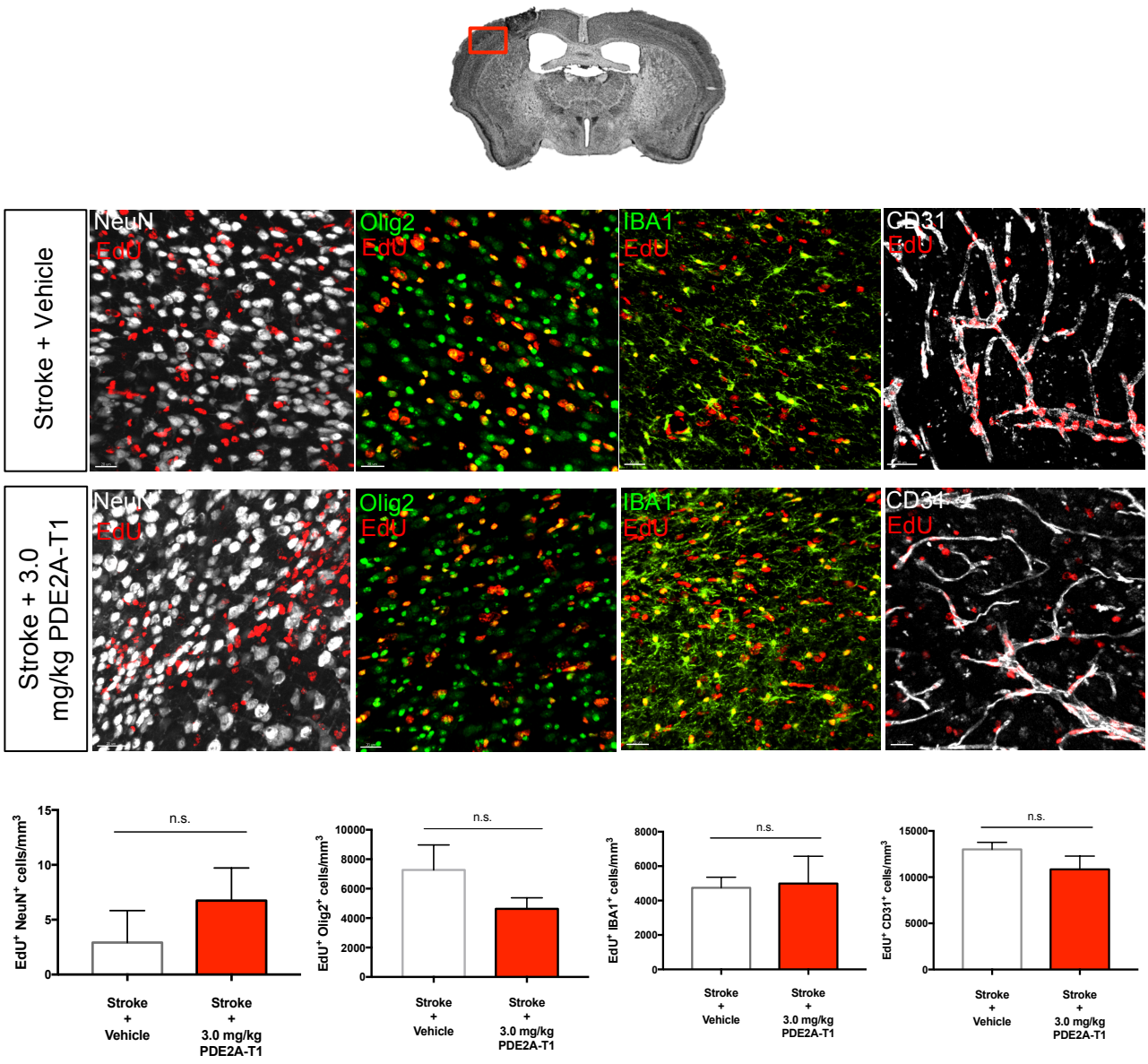

Figure 9: Mice were dosed with EdU 3-10 days after stroke. Number of EdU-incorporated cells co-labeled with NeuN, Olig2, IBA1, and CD31 in peri-lesional cortex does not change with daily treatment of PDE2A-T1. Data shown are means and SEMs, n = 4 animals per group.

Figure 10: Degree of infarct size in PDE2A-T1 treated aged mice

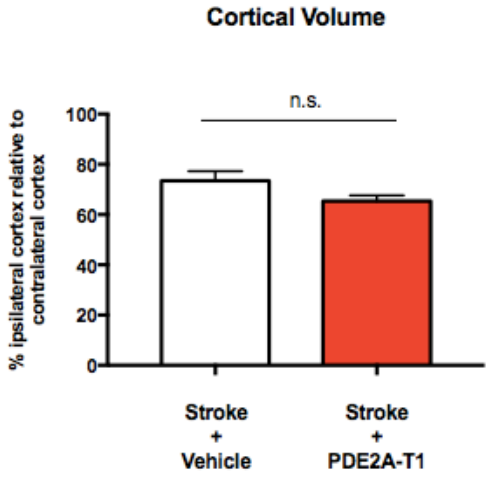

Figure 10: PDE2A inhibition via daily PDE2A-T1 injection does not alter the size of cortical infarcts after stroke in aged mice. The Y-axis indicates the normalized percentage of the ipsilateral stroked hemisphere over the contralateral non-stroked hemisphere. Data shown are means and SEMs, n = 5-7 animals per group.

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