TARGETING CHOLINERGIC NEUROMODULATION IN STROKE RECOVERY

APPROVED BY SUPERVISORY COMMITTEE

NOTE: All signatures must be original and in ink.

Goldberg, Mark P., MD

Meeks, Julian, PhD

Huber, Kim, PhD

Kilgard, Michael, PhD

Rennaker, Robert, PhD

DEDICATION

I would like to thank my supervisor, Mark Goldberg, for giving me the resources, opportunity, guidance and encouragement to approach the complex challenges I wanted to face. I want to thank my committee chair, Julian Meeks, for always giving me the best and most frank guidance and advice on every level that a student could hope for. I want to thank each member of my committee for being a continually open and available resource to me and for giving me a wide spectrum of advice on this project. I do not just owe this project to their guidance; I owe my doctoral training and what I may produce in the future to their relentless support.

TARGETING CHOLINERGIC NEUROMODULATION IN STROKE RECOVERY

by

APRIL MELISSA BECKER

DISSERTATION / THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August, 2017

TARGETING CHOLINERGIC NEUROMODULATION IN STROKE RECOVERY

Publication No. _____

April Melissa Becker, PhD

The University of Texas Southwestern Medical Center at Dallas, 2017

Supervising Professor: Mark P. Goldberg, MD

After ischemic stroke, patients can have significant deficits that limit their daily function. Most patients experience some degree of spontaneous recovery in the weeks after stroke. However, this recovery is often incomplete, and there are no treatment approaches which have been established to substantially restore lost function. Better functional assessment in mouse models and investigation into controlling functional plasticity in the injured brain could each be key to producing better recovery in stroke patients. To work toward better functional assessment of stroke deficit and recovery in mouse models, I developed an automated reach task that produces a longer lasting behavioral deficit after cortical infarct than most tests. A modified version of this test demonstrates that cortical ischemic stroke in

the mouse recapitulates human-typical patterns of precise distal forelimb muscle control deficits. After developing, validating, and characterizing this task I used it to investigate the role of neuromodulation on stroke recovery. The results of these studies show that NB cholinergic cells in the mouse are necessary for typical recovery from stroke, and increasing their activation during successful rehabilitation movements may improve recovery.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Baker MC, Becker AM. Mobbing calls of black-capped chickadees: effects of urgency on call production. Wilson Bull 2002; 114; 510-516.

Ortu, D., Becker, A. M., Woelz, T. A. R., & Glenn, S. S. An iterated four-player prisoner's dilemma game with an external selecting agent: A metacontingency experiment. Revista Latinoamericana de Psicología 2012; 44(1); 111-120.

Becker AM, Meyers E, Sloan A, Rennaker R, Kilgard M, Goldberg MP. An automated task for the training and assessment of distal forelimb function in a mouse model of ischemic stroke. J Neurosci Methods 2016; 258: 16–23.

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

Ach – acetylcholine

ABC – avidin-biotin complex

BDNF – brain-derived neurotropic factor

BSA – bovine serum albumin

ChAT – choline acetyltransferase

CNO – cloazapine n-oxide

CNS – central nervous system

DREADD – designer receptor exclusively activated by designer drugs

DMSO – dimethyl sulfoxide

EEG – electroencephalogram

EYFP – enhanced yellow fluorescent protein

GABA – gamma-aminobutyric acid

GFP – green fluorescent protein

HPA – hypothalamic-pituitary-adrenal axis

LTP – long-term potentiation

LDT – laterodorsal tegmental nucleus

LTD – long-term depression

MCAO – Middle Cerebral Artery Occlusion

NB – nucleus basalis; herein used to refer to all cholinergic basal forebrain cells

NGS – “normal” goat serum

NMDA – n-methyl-d-aspartate

PBS – phosphate buffered saline

PFA – paraformaldehyde

PPT – pedunculopontine nucleus

STDP – spike timing dependent plasticity

STAIR – stroke treatment academic industry roundtable

STEPS – stem cell therapies as an emerging paradigm in stroke

TMS – transcranial magnetic stimulation

VNS – vagus nerve stimulation

Preface

This dissertation includes two major bodies of work that have been developed in parallel during my doctoral studies. In the first three chapters, I will discuss the need to further the development and understanding of functional behavioral assessment in rodent ischemic stroke research and will present original contributions thereto. In the second three chapters, I will review the cholinergic diffuse modulatory system as a potential driver of guided functional behavioral recovery from stroke and present my contribution toward investigation of that hypothesis. In Chapter 7, I will review the implications of both bodies of research and discuss complimentary projects and future directions currently planned or underway.

CHAPTER ONE
Challenges for the functional assessment of brain injury in stroke

GENERAL CHALLENGES FOR MODELLING IN TRANSLATIONAL SCIENCE

Translational science in ischemic stroke seeks both understanding and potential clinical application. Preclinical work seeks predictive validity of treatments while translational science seeks conceptual validity of processes. Not all translational studies are preclinical, but all seek to better understand phenomena that occur in humans without actually experimenting with humans. In order to be an accurate model of a problem, the models need only bear similarity to the human case in terms of variables relevant to the specific question at hand. For preclinical studies, one of those variables is treatment and successful modeling leads to clinical translation. Especially early on in a translational science, animal models cannot be assured to share appropriate similarity with humans because relevant variables, particularly contextual variables that may change the relationships between directly tested variables, are unknown. That is why, all things being equal, an animal model is preferred when it recapitulates as many aspects of the modelled system as possible, not because every aspect will be important but because we have no idea which will be important to our questions. Unfortunately, there is only one way to avoid modelling guesswork: experiment identically in both the model and the modelled system and see if the results differ. If we could easily and ethically run such comparative experiments with humans in order to validate our models, we would not need the models at all. However, we do make such comparisons when we attempt to translate a body of preclinical research. Each attempt can serve as a

natural post-hoc experiment on model validation. Such validation may be context specific; it does not follow that an animal model that translates in one problem or context will necessarily translate in another. But absent better standards for prediction, we can get a rough idea of how well stroke models in rodents reflect processes that occur in humans by looking at treatments developed in animal models that went on to clinical trials. Such a test will only be directly applicable to preclinical model validity, yet the success rate of such research may reflect on modelling methods used more broadly; after all, if animal models produce poor predictive validity for treatment, they may be critically flawed in conceptual validity as well. However, it is important to note that predictive validity of preclinical research, which requires model fidelity along a greater set of variables than basic translational work, is more difficult to achieve than conceptual validity of stroke-related processes. Thus, failures to translate treatments using particular stroke models may present an opportunity to improve the quality of a model as an accurate depiction of particular basic processes.

I have developed an automated reach task for use in my doctoral experiments in an effort to address these considerations. In order to explain the goals and rationale behind this work, I will use this introduction to review and discuss aspects of stroke modelling in rodents according to the standards outlined above with particular emphasis on modelling of behavioral function, deficit and recovery in stroke. I will begin with an assessment of clinical translation in preclinical rodent research, then I will discuss particular aspects of rodent stroke models that may impact functional impairment. I will then discuss the current state of functional assessment itself, the reasons why it is vital for all preclinical work and much

translational work, and the opportunities for development and improvement that will underlie the experimental work of the next several chapters.

THE SUCCESS OF RODENT MODELS IN PRECLINICAL STUDIES

Translational animal research has been helpful in describing the mechanisms of ischemic injury and improving diagnosis [1]. Many processes relevant to the understanding of human stroke have been discovered in animal models, such as the timescales and mechanisms of injury during and after stroke [2]. However, attempts at treatment development in animal models have been disappointing. Many promising pharmacological, restorative or cell-based treatments thoroughly studied in rodents have failed to translate [1, 3, 4]. Examples include Caffeinol as a neuroprotective agent [5], transplantation of cultured neuronal cells [6] the administration of granulocyte-colony-stimulating factor to induce stem-cell based restoration [7] or the application of an Phosphodiesterase-5 Inhibitor to induce neurogenesis, synaptogenesis, and angiogenesis [8]. Despite a large amount of progress in translational understanding, animal studies have only produced one FDA approved pharmacological treatment for human stroke: acute administration of thrombolytic tissue plasminogen activator, which is useful for breaking up a clot only when administered closely after initiation of stroke [9]. In fact a promising drug-based treatment currently undergoing investigation with potential positive effects on stroke recovery, fluoxetine [10], was developed largely outside rodent models of stroke even though an effect of fluoxetine on cortical plasticity had previously been established in rodents without injury (i.e. [11]).

Another stimulation-based approach, VNS, may constitute a break in the pattern; the efficacy of VNS in stroke recovery in rodent models has thus far translated to small human trials [12]. While some stroke models appear to have conceptual validity applicable to stroke recovery, preclinical research utilizing these models is disappointing thus far in achieving effective clinical translation.

DO ANIMAL MODELS MATCH CLINICAL FEATURES?

Methodological rigor

Failures of predictive validity of treatments in rodent research on ischemia have been discussed at length, and analyzed with particular attention to methodological quality of the pre-clinical animal research. In 1999 and again in 2009, the Stroke Therapy Academic Industry Roundtable (STAIR) established important standards for future research in order to address the problem. Many of these recommendations constitute common sense standards of preclinical modelling that were violated in past research; these included using therapeutic time windows that are possible in the clinic, using effective doses or modes of administration that are possible in humans, failing to use both sexes in experiments despite established sex-based differences in stroke injury and recovery, and failing to use older mice despite the clear fact that ischemic stroke is largely an age-specific problem [13-15]. Other recommendations insist upon basic methodological rigor including “eliminating randomization and assessment bias, a priori defining inclusion/exclusion criteria, performing appropriate power and sample

size calculations, and disclosing potential conflicts of interest" [15] (see also [16]). Obviously, these points constitute the minimal criterion for a good model. However, they are not the only parts of such models that hold potential to improve translation.

Species Selection

Perhaps the most obvious approach to model improvement is to use a species more closely related to humans. Many animals share more brain-injury relevant variables with humans compared to rodents such as connectivity, proportional white matter, corticospinal wiring, cortical folding, immunology, etc., and these differences may well contribute to problems in translating brain injury research [17] [18]. Cats, ferrets, and rabbits have all been used as viable experimental models before. However, they are less convenient than rats and mice since they cost more, breed slower, and do not always fit the equipment readily available to scientists.

In addition to these logistical pressures, there are scientific reasons to improve the mouse model rather than abandon it. Decades of tool development in mouse models has produced a ripe environment for well-controlled circuit manipulations. The genetic manipulations in this dissertation would not currently be possible or accessible in other animals. In short, mice carry a sunk cost effect (or initial investment that modifies the value of change) that leads to real, practical reasons to use them first. In order to capitalize on the advantages of the rodent, strategies for improvement other than the species selection itself must be considered. The weaknesses of using mice could then be addressed by assuring

systematic extension of approaches developed in the mouse using other species, assuring that evidence between them converges before moving on to human trials.

Improvements in functional behavioral assessment in the context of stroke may also benefit from utilizing more nonhuman primates since they have musculoskeletal anatomy and motor patterns that more closely match humans than do rodents or even cats and ferrets. However, for the same reasons mentioned above, improving mouse behavioral assessments rather than abandoning the species will enable scientists to use a wider array of tools in stroke research as well as take advantage of shorter generation lengths and ready-made supplies.

Injury induction

No lab utilizes naturally-occurring strokes to study ischemic injury, and all procedures for inducing stroke differ in some way from injuries seen in the clinic. The physical damages that occur after stroke are not simple nor universally modeled; the initial ischemic insult that occurs on the scale of minutes to hours is followed by a number of different processes that cause further damage including inflammation, excitotoxicity, apoptosis, scar formation, microglial activity, etc. that progressively increase the size of the structural lesion over a longer period of time [2]. Functional deficits depend on the regions of CNS affected by this progressive damage [19], and the vascular architecture of the rodent locates damage related to specific artery blockage to different anatomical regions than in humans.

The question of which aspects of injury are the most important to reproduce in animal models has been an area of ongoing attention in the field. Different methods have been developed that allow research to target functional anatomy [20], disrupt white matter and connectivity [21], simulate particular patterns of permanent or temporary artery blockage [22], or simulate thrombolysis and pharmacologically induced reperfusion [23]. The diversity and versatility of convention in injury induction reflects the extensive model development that has occurred in this area, and STAIR and STEPS include guidelines on how best to select injury models for the particular question at hand [13, 24]. In short, opportunities for model improvement in injury induction have already been explored. Differences between the various models and the human case are well described and taken into consideration, and most aspects of human injury can be modelled using one strategy or another.

Importantly for the purposes of this paper, the procedures for injury induction will affect the optimal targets for functional behavioral assessment of stroke deficits. The anatomy and connectivity affected by the damage determines the kinds of behavioral impairments that occur as a result of injury, therefore the development of the functional assessment must occur in the context of the particular anatomical damage that the question at hand seeks to address, and the model of injury induction should be chosen to reflect this. Since the disruption of cortical motor neurons has been determined to underlie a great deal of relevant functional damage in humans [25-27] and since white matter damage and resulting disruption of connectivity has also been implicated [28, 29], the injury induction model used in these studies will be a large photothrombotic ischemic stroke targeted to forelimb representations and large enough to disrupt subcortical white matter.

BEHAVIOR

Other than the selection of animal itself and the method of injury, two obvious aspects of the model present themselves for improvement that may enhance both translational and conceptual validity: the method of measuring functional injury and recovery. Not only have these procedures undergone less thorough thought and development than injury induction (although significant contributions thus far will be discussed below), they ultimately constitute the keystone of successful preclinical science. All the myriad variables in stroke research that can be measured or intervened on the cellular, tissue, organ or organ system level including infarct volumes, network connectivity, neurogenesis, and plasticity claim clinical significance only when directly related to behavioral function. Indeed, the treatments that currently have the best measurable impact on post-stroke patients are largely behavioral or stimulation-augmented behavioral approaches, including constraint-induced movement therapy and transcranial magnetic stimulation (TMS) [30-36]. What matters to a patient is the ability to behave as before their injury. No treatment for recovery is useful therefore unless it can prevent or rehabilitate functional losses, and even non-behavioral treatments should be tested with a close eye to quality behavioral modelling.

Importantly, optimal behavioral assessment will depend on the model of injury induction. As mentioned in the previous section, a variety of ischemic models in the rodent differentially affect different brain areas and connectivity. When developing better behavioral assessments, it makes sense to do so in the context of those injury models

designed to impact the same anatomical areas and connections as in the human case rather than those designed to recapitulate cellular processes, etc. In particular, motor cortex, basal ganglia and internal capsule are affected by common middle cerebral artery occlusion [37], and corticospinal damage is considered specifically important in both typical human deficits and in prognosis for recovery [25-27]. Thus, the review of functional assessments below will be limited to those particular functions affected by inducing motor cortical stroke in rodents. Despite this limited scope, the concepts discussed below could be applied to broader functional assessment in the context of other modes of injury induction. For example, if a different mode of injury such as MCAO is selected in order to reproduce infarct damage gradients, functional assessment may make the research stronger but may also need to utilize different behavioral testing than those used in focal cortical ischemia simply because the brain regions in question are not the same in the model. This will limit the inferences that can be made in such research, but such limitations are always present since models never achieve complete fidelity.

STRUCTURE VS. FUNCTION

Two distinct approaches exist to the measurement of behavior. First, the structure of behavior can be measured, and much stroke research focuses on this strategy. Behavioral structure includes how that behavior physically proceeds and how it is arranged through time. Sometimes behavioral structure is inferred through outcomes such as the pressing of a lever or picking up a pellet. Another approach is to focus on behavioral function, which cannot be

assessed without also measuring structure. Behavioral function is not defined as the form of the behavior but rather by the relationship of particular forms to controlling variables in the environment. Different behavioral structures can be part of the same function. Functional aspects of the environment can potentially change behavior as well as holding it in equilibrium. Thus, behavioral function can underlie both dynamic and static behavioral structures. These points are acknowledged occasionally in stroke research with reference to motivation or learning, but by and large the structure of a motion constitutes a greater point of focus for the field and behavioral functions are left out. There is a good reason for this in the field of motor recovery from stroke; the ultimate locus of clinical interest is in the structure of very particular behaviors, and motor learning is usually intact if not unaltered. If teaching and feedback are consistent and well-designed, the lack of functional measurement should be a non-issue. However, where behavioral function has been perturbed, structure will likely follow, and this point is considered further in Chapter 7. For now, I will focus on the important point of behavioral structures in the context of stroke recovery.

SELECTION OF TASK

Brain injury does not evenly affect all things that people do, so performance assessments need to be targeted. Post-stroke human motor assessments such as the Fugl-Meyer, Motor Assessment Scale, and many more have undergone a great deal of development, so the replication of those assessments in mice may seem intuitive. Yet these assessments, while they have proven useful and reliable clinically, do not by consensus

represent a valid generalized measure of stroke-relevant motor function, nor have they been established to correlate with the ease of basic daily living activities [38, 39]. Thus reproducing human assessment in animals seems likely to carry over problems and to create new ones. On the other hand, directly reproducing and measuring in mice similar tasks and motions for which humans claim impairment have constituted a viable alternate approach.

This approach generates a second problem; mice simply do not do many of the things that humans do in the ways that humans do them, including many behaviors that are commonly affected by human middle cerebral artery occlusion. In general, upper limb function and walking or gait issues constitute the motor impairments that are most commonly problematic for humans [40]. Mice do not use their forelimbs in the same dexterous way that humans do, and the bipedal gait of humans differs from quadrupeds. The early approach to mouse behavioral modelling was therefore to select easily measureable rodent behaviors that shared some aspect of form with human behavioral targets, establishing similarity in kind if not in particulars. For example, the cylinder test, a common rodent stroke assessment tool, seeks to model hemiplegia or lateralized damage to forelimb function [41]. This rodent test uses postural support of the front paws during an upward rear as a behavior with which to study changes to lateralization of function after single-hemisphere stroke. Since humans do not generally use their forelimbs for postural support and since the ability to lean broadly on either arm is not typically lateralized as a post-stroke chronic motor impairment in humans, the mouse model obviously is not measuring the same kind of laterality that humans experience and may not be measuring a long-lasting laterality at all. The question of whether this mouse-specific laterality is a good model for human-specific laterality is an empirical

one, to be discussed below. Similarly, gait problems in brain injury are modeled in tests like the rotarod in which a mouse walking on an accelerating rotating bearing is thrown off if forward locomotor acceleration is not maintained [42]. Another gait test in mice involves skilled placing of all four limbs on ladder rungs or wire grids (also called foot fault test) [43]. Humans do not generally walk or run in a manner that requires constant acceleration as in the rotarod nor do they face such a dire consequence for poor locomotor performance as a drop equal to several times their height. Human gait generally does not involve rung placement and bipedal gait is different in physical demand than quadrupedal. Thus, the models are different in particulars yet still measure behaviors with intuitive similarity.

In the end, only empirical observations can say if these conceptual extensions of human functional injury in the mouse constitute a useful model of stroke impairment. If the rodent behavior task suffers similar perturbation in response to brain injury as do the human movements targeted for rehabilitation, it follows that the task may make a good model. Of course, "similar" is not a dichotomy, and these tests would not have been utilized as widely as they are if they were not perturbed by stroke. However, some clues indicate that the similarity may not be sufficient for either clear signal or good translation. First, impairment is often very subtle on the group level in these tests; of the two experiments in this thesis that utilize cylinder tests (Chapters 4 and 5), only one produced a statistically significant group-wide impairment after cortical stroke (Chapter 4). Additionally, these tests do not seem to stay impaired after cortical stroke; typically the mice are back to normal within a matter of a few weeks (i.e. focal cortical lesions in the cylinder [41]). In the experiment reported in Chapter 5 of this work, rotarod behavior returned to normal at week 2 after focal cortical

stroke, and footfault tests can have a similar timecourse [44]. This is the rule rather than the exception in stroke literature; some of the best demonstrations of longevity of injury last only a matter of weeks after focal cortical ischemia [45]. Finally, these tests do not seem to scale with the anatomy of the stroke as we would expect. For example, as mentioned above one-sided forelimb dexterity deficits in humans are sensitive to motor cortical stroke, yet the cylinder test is more sensitive to the MCAO that spares more motor cortex than it is to focal cortical ischemia [41, 45]. Thus, while these tests do model post-stroke behavioral injury, they do not model the long-term injury that is the subject of our concern in humans. Particularly limiting is the lack of chronic deficit measured in these strokes, which makes it impossible to use them to establish that a particular intervention can create recovery where it would not independently occur: the goal of chronic stroke intervention research. Thus, these tasks severely limit the usefulness of animal research.

These limitations might be due to the fact that these tests simply do not capture motion patterns injured in humans well enough; the conceptual similarity may not translate to stroke-specific functional similarity. One way to address this is to directly train the mice to do things in a more human-like way or to utilize motions that are most human-like, particularly those motions that are important in the context of brain injury. As mentioned before, one deficit that tends to remain chronically impaired in humans is dexterous forelimb use; only 5% of stroke survivors regain full use of their affected forelimb(s) and 20% make no gains at all [46]. Tasks falling in this category usually require some pre-training in order to achieve stable baseline performance in these skilled motions. They include the single-pellet reach task, the staircase task, the pasta matrix task, the pole task, the wire hanging task, the

forelimb force grip task, and the brand new pronation/supination task [47]. The single-pellet reach task, the staircase task, and the pasta matrix task require the animal to pick up food using dexterous forelimb motions rather than primarily using the mouth as preferred by the species. The pole task requires animals to right themselves and climb down a vertical pole, a task requiring forelimb use. The wire hanging task and force grip task require animals to grip with their forelimbs as either the experimenter or their own weight provide a resistance. The pronation/supination task requires rats to turn a knob as a human would open a door. While all these tests utilize forelimb movements, they do so in importantly discrepant ways. In the case of the wire hanging and force grip tests, the animals grab and hold the wire under aversive conditions in order to keep from falling or being snapped backward. That the maintaining consequence for this behavior is avoidance is seen in the recommendations for wire hanging, which instruct that a mouse not be allowed to escape the situation by letting go and instead be forced to hang to avoid manipulation [48]. The motion needed to meet this avoidance task is simply tension; no coordinated motions of the forepaw such as rotation or temporally distributed differential contractions of various muscles are necessary. Because of this criteria, these tasks may not as effectively test precision of forelimb use but rather minimal strength of grip. Perhaps for this reason, the wire hanging task does not produce deficits that last more than a few weeks (i.e. [45]). The pole test has also carries an implicit escape function; the criteria for escape involves acrobatics rather than sheer grip, however it barely shows any deficit after cortical stroke [45]. The food manipulation tasks, on the other hand, carry an implicit positive consequence for hungry rodents. While they may differ in the amount of forelimb muscular coordinated precision required, they all have in common that

they do require such precision; the food must be gripped hard enough not to drop at first but soft enough not to crush or break, then released at the right position with sufficient coordination to transfer to the mouth or to adjust as eating continues. The onset, duration and offset of the grip must be coordinated with arm and mouth movements. Perhaps for this reason, these tests generally produce longer-lasting injury, with some studies showing a deficit lasting up to 25 days after large cortical lesions [49, 50]. Finally, the pronation/supination task has been developed very recently in the same timeframe as this dissertation and may be even longer-lasting than tests that came before it, but thus far it has only been tested out to 6 weeks [51].

In Chapter 2, I will discuss the development of an automated reach task for mice that was extended from a rat version [52], which shows significant group-wide impairment up to 22 weeks after stroke, making it a successfully advanced model of stroke impairment and a good paradigm from which to build further improvements.

Though these more human-like tasks better model the longevity of injury, they suffer from a potentially major deficit; even when animals can be directly trained to emit more human-like motions, they may remain divergent from humans in the learning history that produced that motion. In short, some of these motions are not typical for a mouse and are unlikely to be incorporated into their everyday behavior outside the training/experimentation paradigm. In animals, these skills will not be acquired in a developmentally typical way as they are in a human, they will have less generality of use, they will have a shorter and less habitual history of use, and post-injury practice opportunities will be narrower. Additionally, though they produce decent longevity of functional loss, that longevity remains conditional

in a contradictory way. These tasks are affected less by stroke and may recover more readily when trained more thoroughly during the pre-lesion period; this point is widely recognized in explicitly trained forelimb motor assessments and has led to lab convention of avoiding "overtraining". For example in Chapter 6, we saw that animals trained in the automated reach task for 100 days or longer recovered to a greater extent than animals who received less experience on the task before stroke. The human context of injury stands diametrically opposed to this conditionality. Not only are human forelimb deficits long-lasting without intervention, they are usually composed of activities performed thoroughly and with mastery throughout pre-stroke years such as holding a glass of liquid, buttoning buttons, or writing. It has long been understood that long-mastered behaviors, particularly species-typical behaviors, respond to brain injury differently than other behaviors and likely depend differently on particular neurological substrates and networks (see [53], particularly chapters 9, 11 and 13). We may take the effects of overtraining on recovery in our current tasks as a warning that the kinds of behaviors we are using to assess post-stroke function, even in our most developed models, may still fundamentally be of a different kind than the ones we seek to treat in humans. They may involve different physical brain substrates than the same motions do in humans, and they develop from a different pattern of historical behavioral processes. Whether this consideration is relevant to accurate behavioral modelling is again an empirical question. The question can be answered by developing a chronically-impaired task for modelling functional recovery that mimics the human pattern of acquisition, use, and mastery rather than just form, then comparing it to other behavioral tasks to see if they

produce different results in the context of intervention. I will expound on this idea in the final chapter of this dissertation.

SUMMARY AND CONCLUSION

Translational animal modelling in general, and behavioral modelling in the context of ischemic stroke in particular, require only relevant variables to be in common between the animal and human cases in order to be useful. However, sometimes it is difficult to determine *a priori* which variables need to be in place for a model to be optimally representative either of preclinical concerns or translational conceptual questions. Tasks used to assess functional damage and recovery after stroke generally do not produce a long-lived deficit that can then be experimentally boosted in the context of cortical stroke. Moving from behaviors that conceptually model human stroke deficits to behaviors that are explicitly trained to more closely resemble actual, injury-relevant human motions has provided some much-needed improvements, particularly in longevity of injury. This progress could be further developed by refining tasks to reflect the natural learning history of rodent species in the same way that human injury occurs in the context of human-specific learning histories.

A NEW ASSESSMENT TASK

In order to contribute to the development of functional behavioral modelling of ischemic stroke in rodents as reviewed above, I have developed an automated task for mice

that requires precise distal forelimb use and responds chronically to focal cortical stroke. The next chapter will describe this task and its validation. The following chapter will utilize the task to assess the mouse model itself, testing focal cortical stroke to see if it really produces similar forelimb deficits in mice compared to humans. This task will then be applied to translational questions regarding ischemic stroke recovery in Chapters 5 and 6.

CHAPTER TWO
An Automated Task for the Training and Assessment of Distal Forelimb Function in a Mouse Model of Ischemic Stroke

The following chapter was published in the Journal of Neuroscience Methods in 2016 and is co-authored by Eric Meyers, Drew Sloan, Robert Rennaker, Michael Kilgard, and Mark Goldberg.

ABSTRACT

Background: Behavioral models relevant to stroke research seek to capture important aspects of motor skill typically impaired in human patients, such as coordination of distal musculature. Such models may focus on mice since many genetic tools are available for use only in that species, and since the training and behavioral demands of mice can differ from rats even for superficially similar behavioral readouts. However, current mouse tests are time consuming to train and score, especially in a manner producing continuous quantification. An automated assay of mouse forelimb function may provide advantages for quantification and speed, and may be useful for many applications including stroke research.

New Method: We present an automated assay of distal forelimb function. In this task, mice reach forward, grip and pull an isometric handle with a prescribed force. The apparatus partially automates the training process so that mice can be trained quickly and simultaneously.

Results: Using this apparatus, it is possible to measure long-lasting impairment in success rate, force pulled, latency to pull, and latency to success up to 22 weeks following photothrombotic cortical strokes in mice.

Comparison with Existing Method(s): This assessment measures forelimb function as do pellet reach tasks, however it utilizes a different motion and provides automatic measures that can ease and augment the research process.

Conclusions: This high-throughput behavioral assay can detect long-lasting motor impairments, eliminates the need for subjective scoring, and produces a rich, continuous data set from which many aspects of the reach and grasp motion can be automatically extracted.

INTRODUCTION

About 80% of people who suffer ischemic strokes incur motor deficits that interfere with quality of life [54]. Developing and refining measures of functional motor impairment and recovery after stroke in mouse models could therefore contribute to improved relevance of mouse research. The most promising current motor assays require extensive scoring and subjective evaluation that makes efficient, high-throughput, flexible research challenging.

Since functional impairment of the distal forearm is an important cause of disability in stroke patients, an ideal rodent assay will capture the important aspects of such movements [55]. It is advantageous to develop such assays particularly for mice given the extensive availability of genetic and pharmacological mouse models. However, assays for mice need to be developed independently from those utilized in rats since neither training nor performance

patterns necessarily overlap between the species. Existing tests meeting these criteria such as skilled pellet retrieval reaching tasks [56-58] can be time consuming to train and score, especially in a manner producing continuous quantification. When behavior is automatically measured rather than scored visually, it can be more easily quantified to a finer degree than is practical with visual scoring. We have developed such an automated assay of skilled forelimb use for mice based on versions published for use in rats [59, 60]. Here, we describe this assay, which requires the coordination of several forearm muscles. Like existing skilled reach tasks, this assay requires a mouse to reach through a slit to grasp an object in a manner amenable to automated or hand-scored motion analysis [56], however unlike existing tests the subsequent force exertion on the object is highly constrained and isometric. The task precludes behavioral compensation, shows lasting deficits as a result of photothrombotic cortical stroke, allows for flexibility in different aspects of behavioral measurement, can be trained in a partially automated fashion without close attention, and can be consistently applied in large numbers of mice with efficiency and precision. We present and validate this assay primarily for use in a photothrombotic mouse model of stroke and demonstrate its sensitivity to that injury. However, this assay could be useful for any application that requires a sensitive assay of forelimb function.

METHODS

Apparatus and Procedure

Enclosure, Behavior, Measurement and Analysis

The apparatus has been developed in collaboration with Vulintus, Inc. (Dallas, TX) and resembles a similar design optimized for rats6 (Figure 1). It consists of a plexiglass enclosure 5.5" high x 5" wide x 8" long. A pattern of square holes in the floor allow waste to fall to the level below. A slot in the right side of the front wall provides access to a vertical handle 3 mm tall, 1 mm wide and 1 mm thick, connected to a force transducer that measures unidirectional horizontal force exerted in the direction of the mouse. The position of the handle and the directionality of the required force constrain the behavior; the mouse cannot succeed by pushing the handle from the sides, top or bottom and must use paw musculature to grasp around the back of it. Additionally, the handle is most easily grasped from the side since a grasp from the top would allow fewer digits to exert force on the back of the handle, providing a natural constraint to the top-down raking motion normally considered compensatory in similar assays. The calibrated transducer measures up to ~70 g with 1 g precision. Accuracy of the force signal is assured through regular calibration and testing with precision weights. Mice can generally pull a maximum of about 35 g on this apparatus. The front edge of the handle is positioned 1 cm from the inner edge of the chamber. Between that edge and the handle, an infrared (IR) slot detector is positioned vertically across the slit to detect reach attempts. Adjacent to the slot, a bracket recessed in the plexiglass wall presents the blunt tip of a feeding needle controlled by a pinch valve. Following a successful pull, the pinch valve emits an audible click and releases approximately 2 µl of peanut oil at the end of the feeding needle. Signals from the infrared beam and the force transducer are sampled every 10 ms using a custom control board and recorded permanently during adjustable trial

windows. Trials are initiated by either a break of the IR beam or by a force exerted on the handle greater than an adjustable initiation threshold of 2 g. A trial ends upon the longer of two seconds (also adjustable) or when the IR beam has been unbroken and less than 2 g has been exerted on the handle for at least 1 second. Trial data is written continually during a behavioral session, preventing incidental data loss. Data is streamed by custom MATLAB software, which displays and stores the data as continuous traces. The raw data is used to derive five different measurements, summarized in Table 1 and Figure 2. Only the first 50 responses of a session are considered in this analysis.

Program

The apparatus is controlled by custom MATLAB software. This software presents a user interface as seen in Figure 2. A drop-down menu allows the user to select from variable, customizable program settings. The program specifies the initiation force required to begin a logged pull, the force that must be exceeded to trigger reinforcement (peanut oil delivery), and the manner in which the force required to trigger reinforcement changes throughout the session. “Static” sessions retain a constant force requirement. Adaptive “Linear” sessions increase the requirement by a customizable increment every time a successful pull occurs. Adaptive “Median” sessions set a force criterion as the lower half or quartile of the previous n pulls. For experiments reported here, “Linear” was used for training and “Static” for baseline and post-stroke sessions. While a session is running, the MATLAB interface provides a real time list of logged pulls and a plot of trial vs. grams of force, with successful

trials in green and unsuccessful in red (Figure 2 shows a black-and-white image; the on-screen graphical user interface is in color).

Validation of Apparatus in the Context of Stroke

Subjects

Thirteen adult C57-Bl6 mice weighing approximately 20-30 g were used to assess the behavioral effects of photothrombotic stroke on this forelimb task. Mice ranged in age from 25 to 35 weeks old at the time of stroke; four were female and nine were male. All mice were housed in a temperature and humidity maintained facility on a reverse light cycle to assure that their high-activity periods would occur during working hours. All mice had food and water available to them ad-libitum in their home cage and also in their reach chambers if subjected to long sessions. All procedures involving these mice were approved by the UT Southwestern Institutional Animal Care and Use Committee.

Training and Baseline Procedure

Mice for this validation were trained using automated sessions to pull the handle with a force greater than 20 g. First, mice were exposed to peanut oil in the home cage. After acclimation to the reach chamber, they received random deliveries of peanut oil every 3-8 minutes until they responded to the sound of the pinch valve by approaching the feeding needle. The handle was then introduced through the slit in the chamber wall, baited at first with a small drop of peanut oil. At first, any detectable force exerted on the handle triggered

delivery of peanut oil. The handle was slowly removed from the chamber until located at its final position relative to the opening, at which point the force criteria for peanut oil delivery slowly increased until it reached 20 g. Baseline sessions then began and continued until three consecutive sessions showed stability in all measures; stability was defined as a variance that was equal to or less than the average variance of the final three sessions of three mice who had run for 2-3 months without injury and no longer showed any performance trends. Overall, this procedure takes ~10-20 sessions (one session/day) for training and another ~5-10 for baseline. The range in duration for the final three baseline sessions for these subjects was .48 hours to 9.75 hours (mean 3.06, standard deviation 2.39). The longest durations were due to logistics and not due to slow response rates; during baseline animals were often allowed to pull well more than 50 times if it was not convenient to check on them often. Training sessions were usually longer, ranging from approximately 5 to 24 hours, also depending on logistics. In this group 4 of 17 mice were eliminated because they were taking too many training sessions to progress due to human error or unknown factors. Mice were safely left alone in the chambers for spans of several hours with food and water available in longer sessions. However, ideally the mice were monitored every hour or two in order to assure that sessions ended at approximately the target number of pull attempts or to assure ideal training progress.

Stroke

After training and baseline, stroke was induced surgically according to the procedure described in Chapter 2.

Behavioral Recovery, Sacrifice, and Perfusion

Sessions of automated reach occurred three and seven days after stroke, then weekly for another 21 weeks. Post-stroke session duration varied to allow the mouse to reach at least 50 pulls. Due to low pull rates, some mice were given very long sessions or multiple chances through a period of days to reach their minimum pull count. Of 299 sessions, 88 were multi-day sessions (35 of these were eliminated from analysis, see below), 41 were overnight, and non-overnight sessions ranged in duration from 0.18 to 11.45 hours (mean = 3.94, SD = 2.52). The average amount of practice that an individual mouse experienced in one week was 134 pulls, though this varied (SD = 138). Variation in pull rate tended to be higher between mice than within mice; mice who required longer sessions did so consistently (5 of the 13 mice accounted for 65 of the 88 multi-day sessions). The first three pilot mice continued to be tested weekly after their 22nd week in order to probe possible longer-term patterns of recovery before being sacrificed and perfused at 5 ml/min with 20 ml chilled PBS and 0.1% heparin and then 40 ml of chilled paraformaldehyde. Other mice were sacrificed and perfused after 22 weeks. Three mice, after an extended period of recovery, ceased to pull at a sufficiently high rate to confidently quantify their performance. This may have been due to apparatus repairs (and thus potential accidental environmental changes) that loosely coincided with these performance disruptions. Since these mice produced viable data until the point of their disruption, their data is included in graphs. However, for any week where less than 20 pulls occurred (a total of 35 sessions between the three of them), their data has been removed and they are not included in recovery statistics.

Histology

Brains from perfused mice were extracted and stored for one day in paraformaldehyde at 4˚C and for at least two days in 30% sucrose at 4˚C. Coronal slices 30 µm thick were collected with a freezing microtome and stained with cresyl violet to visualize and quantify the stroke size and location. Six sections from each mouse were first mounted onto slides; the first section was located at approximately 1.7 mm anterior of bregma, and subsequent sections were 720 µm apart. Slides were incubated for 20 minutes in cresyl violet, developed for 5 minutes in 70% EtOH, 3 minutes in 95% EtOH, 3 minutes in 100% EtOH, and 5 minutes in Xylene. Slides were then coverslipped with permount and visualized with a Hammamatsu Nanozoommer bright field slide scanning microscope after drying.

Analysis and Statistics

For behavior data, each session's success rate, average highest force within trial, and average latency to pull measurement were calculated using only the first 50 pulls of each session. For average latency to success, analysis was restricted to the first 50 successes. If a session yielded no successes, a value for latency to success was determined by averaging the previous two non-baseline sessions and the subsequent two sessions for that mouse; the same method applied to rare sessions in which equipment malfunction yielded inaccurate latency measures. Impairment on each derived measure was assessed using a two-tailed paired t-test between the average of the last three stable baseline sessions and the 7-day post-stroke time point. Recovery data was normalized to individual baseline averages before analysis. To

assess global recovery, each behavioral measure was evaluated using a two-tailed paired t-test between day 7 post-stroke and day 154 post-stroke. One way ANOVAs were used to assess changes over time, and each time point was assessed individually by performing a Fisher's LSD analysis comparing each post-stroke time point to the final time point of baseline.

To determine stroke volumes, the healthy area of each hemisphere of cresyl-violet stained sections was determined using Image J, excluding ventricles. A mouse's stroke volume was calculated by subtracting the area of the ipsilesional hemisphere of each section from the contralesional, then multiplying the sum of the differences by the space between sections.

RESULTS

General

The apparatus and software produced a reliable force signal with a resolution of 1 g of force and an easily discernable infrared break signal due to paw extension (Figure 2). Mice performed the reach task in the desired physical form, reaching and grasping with the right paw from the right side of the handle (Figure 2). Three mice without a stroke performed the task for three months and showed no trends in performance.

Stroke impairment

After baseline, thirteen mice were subjected to photothrombotic stroke of the forepaw representation of the left motor cortex. Most mice had lesions larger than 10 mm3, and all but one had lesions larger than 5 mm3 (Figure 3). The infarct included the forelimb representation for all mice, and the subcortical white matter below the target was eliminated in all but two. The variability of photothrombotic stroke volumes (coefficient of variation .43) falls within the range of some of the most recent examples of mouse photothrombotic stroke experiments in the literature (coefficitions of variation .459, .2410, .0511, .2212) despite the fact that we did not eliminate subjects based on stroke volume. Variability in stroke volume can be due to slight differences in laser scattering, rose Bengal uptake, or individual differences in physiology.

After stroke, measures of success rate, highest force within trial, latency to pull, and latency to success all showed significant impairment (2-tailed paired t-tests between the average of the final 3 baseline sessions and the 7 day post-stroke session p=0.001, 0.002, 0.002, and 0.008, respectively). The number of attempts before success did not change after stroke (p=0.737). Impairment was not equal among all mice, and impairment in success rate and highest force in trial was correlated with stroke volume (Spearman's correlation success rate r = .67, p = .01, highest force in trial r = .66, p = .02) (Figure 3). While mice with smaller stroke volumes had less impairment, all animals had statistically significant impairment at day 7 following stroke as compared to baseline.

Recovery

Success rate and highest force in trial improved significantly through the course of 154 days of recovery (paired two-tailed t-tests between day 7 post-stroke and day 154, each normalized to individual baselines p= .04, .02, respectively), but latency to pull and latency to success did not (p= .83, .36, respectively). Though variance in the latter two measures was too high to show statistical differences between days 7 and 154, averages returned to approximate baseline performance around week 8 and Fisher's LSD comparisons to baseline no longer showed consistent differences. Performance in success rate and highest force in trial continued to show averages below baseline and statistically significant differences from baseline throughout the 22 week period (Figure 4). One-way ANOVAs show significant changes over time for these measures (p = .05, .03) but not for latency measures (p = .17, .22), indicating recovery in the latter. Individual recovery data (Supplementary Figure 1) shows that reliable baseline performance, defined as returning to the individual's baseline 95% confidence interval at least twice, was never reacquired in the 22 week period for 4 mice in this group (31%). Standard deviation of performance at week 28 (a typical recovery period in the literature) indicates that for some effect sizes, fewer than 10 mice should be needed to determine differences in improvement when using this assay to test variables affecting functional recovery (Table 2).

Age and sex differences

Mice ranged in age from 25 to 35 weeks at the time of stroke, and these age differences did not correlate with either impairment or recovery after 22 weeks (all Spearman's correlations $p>0.2$). This experiment included both sexes, but not in the numbers necessary to determine potential sex differences.

DISCUSSION

This study establishes a valid measure of functional forelimb impairment and recovery for this mouse model of ischemic stroke. A relatively small, 5-20 mm3 cortical stroke produced an impairment of one to two standard deviations in four of the five derived measures examined here. Impairments in these measures were long-lasting, and 4 of 13 mice never returned reliably to baseline performance in a 22 week period. Attempts before success did not change as a result of stroke, even though a similar measure in rats did show impairment6.

Some researchers are interested not only in outcome measures of forelimb behavior (such as success rate) but also in the physical form of the motion. Video scoring remains the primary methods for performing this kind of analysis. While our setup allows for video, it also captures near-continuous quantitative measurements that permit many more derived measures than those directly examined here, some of which may serve as indices of physical motion. For example, particular movements could influence the shape of the force waveform,

slope, local maxima, duration or could limit rate or latency. Investigators could also easily modify aspects of the behavior itself via reinforcement contingency, requiring different particular forms of force, timing, etc. for reinforcement. The apparatus could even easily be adapted to measure different forms of motion, requiring the mouse to push, displace, or even twist the handle13.

Many aspects of this apparatus provide benefits for the research process. Most trained behavioral assays require an experimenter to closely shape the initial behavior in each animal and sometimes to individually monitor subsequent sessions, which can be prohibitively time-consuming when many mice are needed for statistical analysis. In this apparatus, numerous mice can be trained and evaluated concurrently. Shaping is largely automated, which eliminates the need to closely monitor, permits the simultaneous training of multiple mice and decreases the time to run experiments. Shaping and training require ~15-30 sessions. Overnight sessions are possible but not necessary. Two derived behavioral measures are directly related to the operant requirements of the task (success rate, highest force) and two are not (latencies); thus, indirect training effects can be evaluated along with overtly trained/rehabilitated motor patterns without extra data collection requirements. The long-lasting deficits determined here present the potential to test interventions that change the extent as well as the speed of recovery. Finally, a dynamic range of one to two standard deviations enables the clear evaluation of experimental impacts with manageable group sizes (Table 2).

This task and pellet reach tasks are designed to measure forelimb motor function, although they involve different mechanical motions and reinforcement parameters; thus they

are not directly comparable. Pellet reach tasks also show long-lasting deficits following ischemic injury. In one case those deficits lasted up to three months in rats14; however in that study the stroke was considerably larger. Photothrombotic strokes in mice of a size comparable to that reported here produce faster recovery of approximately 16 days3, 14 days4, or at least 28 days5 whereas our task detected deficits up to 154 days. The performance change resulting from injury measured via single-pellet reach tasks in these studies was approximately 30%3, 25%4 and 65%5 of baseline; success, peak force, pull and success latency measures of this assay compare well at approximately 69%, 40%, 132% and 122% of baseline, respectively.

This task, along with most operant tasks, is taught and maintained using food reinforcement. Unlike many tasks, this assay requires no food restriction for either acquisition or maintenance; the novel innovation of using peanut oil as a reinforcer maintains high levels of responding without dietary constraints. Since caloric restriction influences stroke impairment and recovery15, and since most patients are not calorically restricted, the possibility of training and maintaining this task without deprivation adds strength to the model and could potentially help with translational validity. However, in scenarios where this consideration is not paramount, this task still could be maintained using food restriction. Such restriction could potentially decrease training time or session time, which could increase throughput and efficiency even further.

Key genetic manipulations are often possible or readily available only in mice; this assay of forelimb function should be particularly useful for studies utilizing models such as gene knockouts, modifications, or insertions. Genetically encoded tools such as activity

sensors and optogenetically controllable ion channels may prove especially valuable for the study of stroke and stroke recovery.

Mice in this study were 25-35 weeks old at the time of stroke; while age was not a factor in impairment or recovery in this study, potential differences between wider age ranges remain possible. It is also possible that strains other than the C57/BL6 mice examined here may differ in training, performance and post-injury performance.

In summary, we have developed a new task for assessing upper forelimb function in mice. The task requires mice to grasp a small handle and pull it in the direction of their body while the extension of the paw and the force exerted on the handle are automatically measured. The task setup is mechanically constrained to minimize compensation. Mice can be trained quickly and simultaneously, with most or all of the shaping process unattended. This high throughput behavioral assay is capable of quantifying multiple aspects of the reach and grasp motion such as success rate, force dynamics, and more that may be important in the context of stroke research. The test can be administered efficiently, analyzed automatically, and produces reliable, precise, and richly informative data while requiring relatively little time investment.

Recovery from stroke is ultimately a functional behavioral issue; any relevant biological phenomenon will be accompanied by behavioral effects. Rodent models of ischemic stroke recovery have produced many promising approaches to stroke treatment that failed to show similar effects in humans16. While we do not know the exact reason for this trend, one potential approach to improving the translatability of mouse models is to develop behavioral assays that model the details of problems experienced in the clinical setting more

closely without imposing prohibitive logistical strain on the research process. The automated reach task described here therefore provides one potential step toward increasing the clinical impact of rodent stroke research.

ACKNOWLEDGEMENTS

A version of this chapter was published with my co-authors Eric Meyers, Andrew Sloan, Robert Rennaker, Michael Kilgard, Mark P. Goldberg in the Journal of Neuroscience Methods. This study was financially supported by a National Institute of Health training grant (T32 NS069562 Cellular Biophysics of the Neuron Training Program, AMB), the Patrick and Beatrice Haggerty Foundation (MPG), the Beatrice Menne Haggerty Center for Research on Brain Injury and Repair in Stroke (MPG), the Texas Institute for Brain Injury and Repair, and National Institute of Health research project grants R01NS085167 and R44NS086344 (MK). We are grateful for help provided by the Neurorepair lab at UTSW and the Rennaker lab at UTD's School of Behavioral and Brain Sciences.

TABLES AND FIGURES

Derived Measure	Description
Success Rate	Number of successful trials divided by number of total trials analyzed
Highest Force in Trial	Highest force measurement within each trial, averaged over trials
Latency to Pull	Time between the first moment that the IR beam was broken until the moment that the force reading exceeded the initiation threshold, averaged over trials
Latency to Success	Time between the first moment that the IR beam was broken until the moment that the force reading first exceeded 20 g, averaged over trials
Attempts Before Success	Number of local force peaks above initiation threshold within a trial before the force reading first exceeded 20 g. These "attempts" are caused by grasp-and-pull motions that fall below force requirements.

Table 1
Derived Behavioral Measures. This validation experiment used these five transformations of the raw data returned by the apparatus, though many others are possible.

Effect Size	Approximate Sample Size (# of Mice)			
	Success Rate	Highest Force in Trial	Latency to Success	Latency to Pull
50%	9	2	3	3
40%	15	3	5	5
30%	26	5	8	8
20%	59	12	18	18
10%	236	48	72	72

Table 2
Power Analysis. These are estimates of sample sizes needed to distinguish a difference of certain magnitudes in each of four measures at day 28 after stroke. Power analysis used 5% alpha, 50% beta and the standard deviation and mean of day 28 data normalized to baseline.

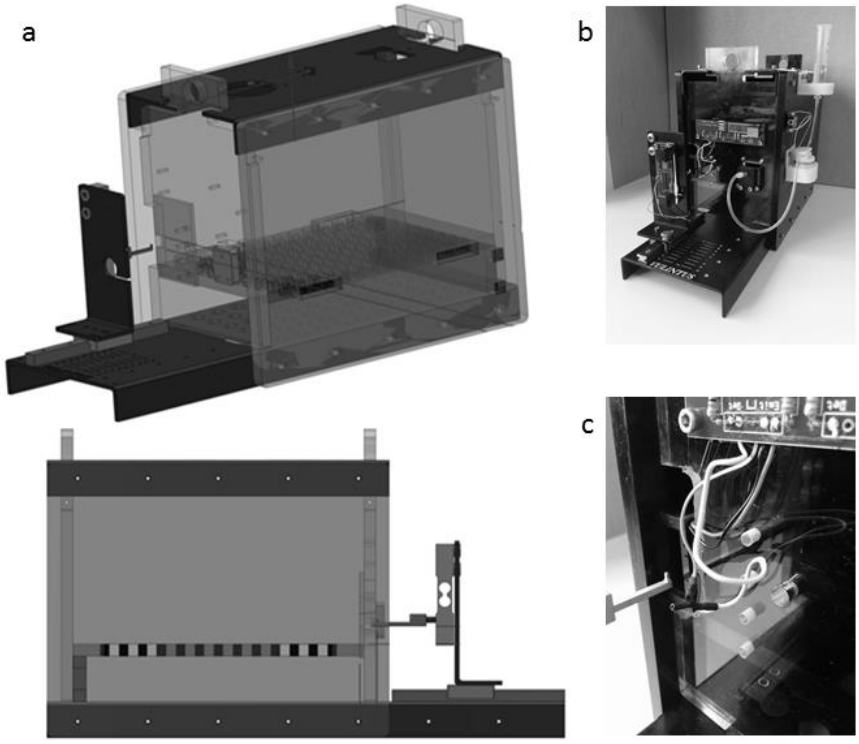


Figure 1
Apparatus a. Schematic representation without peanut oil dispenser. b. Wide-angle pictures of complete apparatus. c. Close up of handle and positioning relative to apparatus.

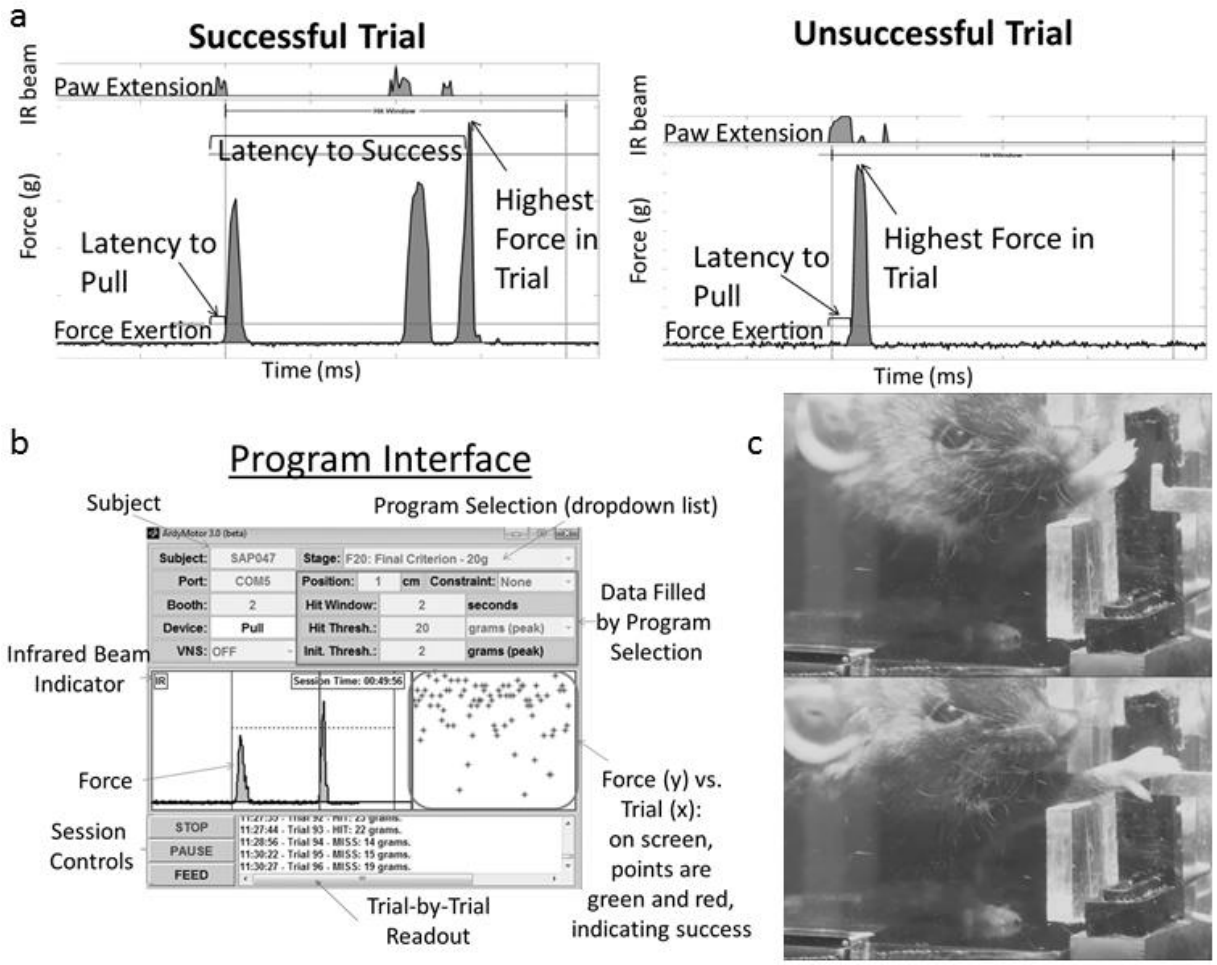


Figure 2

a. Example Trials. Data traces for sample trials showing raw measurements (paw extension and force) and derived measurements (latency to success, highest force in trial, latency to pull). Both signals are stable while the mouse is not interacting with the slot or handle, and both signals change upon behavioral performance with a clear signal relative to noise. The left trace shows 3 attempts before success. b. Program Interface. An experimenter can control and monitor a session via this GUI. Individual trails can be seen in a list on the lower right, and a maximum trial force through time for both hits and misses can be seen in a graph at the middle right. Raw data for the past several seconds can be seen in the middle left, and settings for the session at the top. Sessions are controlled by buttons on the lower left. The GUI is displayed in color on computer screens. c. Photos of a mouse reaching for the handle. The mouse first brings its nose close to the opening then extends the right forepaw with the long axis of the wrist close to vertical. The first digits then wrap around the handle and exert force in the direction of the mouse's body.

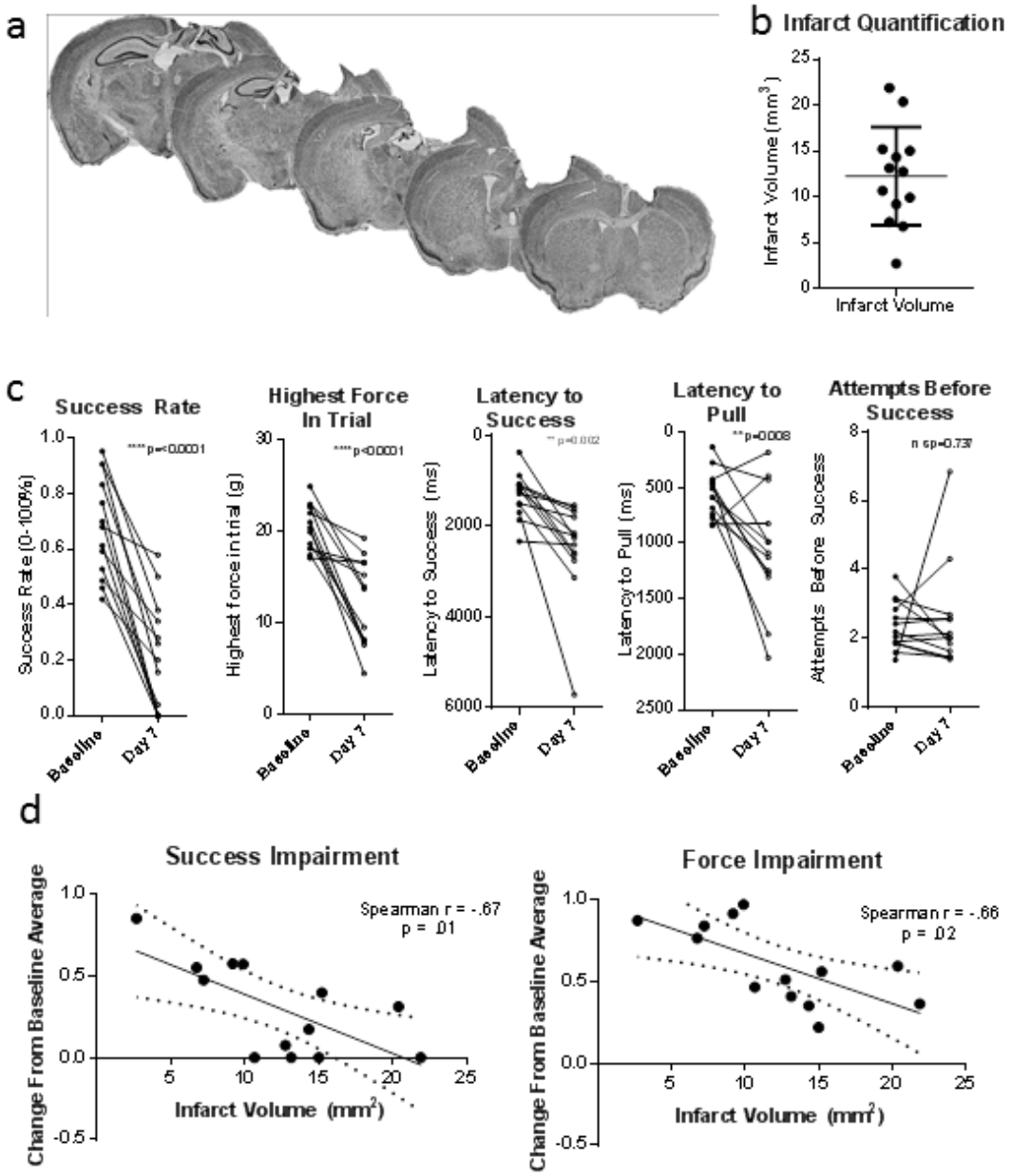


Figure 3

a. Cresyl Violet stains of 6 serial sections 720µm apart from one mouse who received photothrombotic stroke. Stroke included the forelimb representation of the motor cortex and disrupted subcortical white matter tracts in all but two animals. b. Infarct volumes between 1.7 mm anterior to bregma to 2.6 mm posterior to bregma. c. Baseline and post-stroke performance in each of 5 measures, p values from paired two-tailed t-tests. After stroke, the average success rate and maximum force were nearly two standard deviations below baseline, while latency measures were one standard deviation below baseline. d. Decrease from baseline average in success and force on day 7 following stroke is correlated with infarct volume.

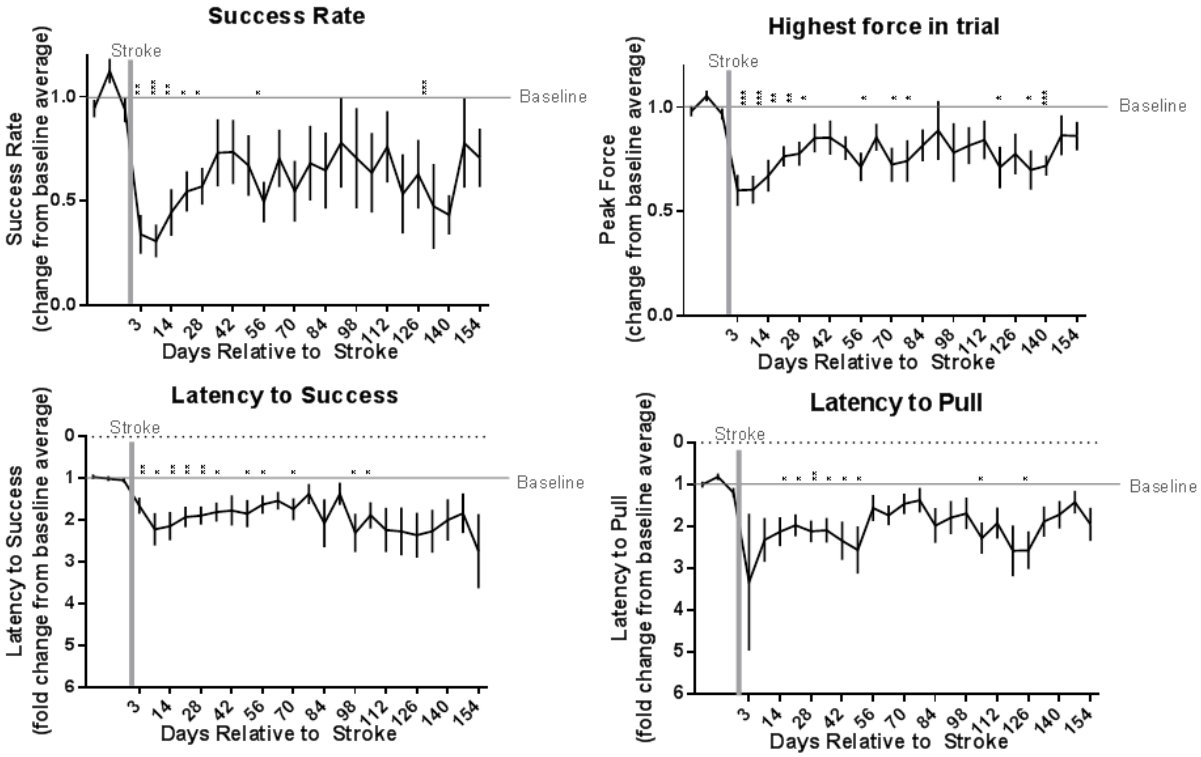
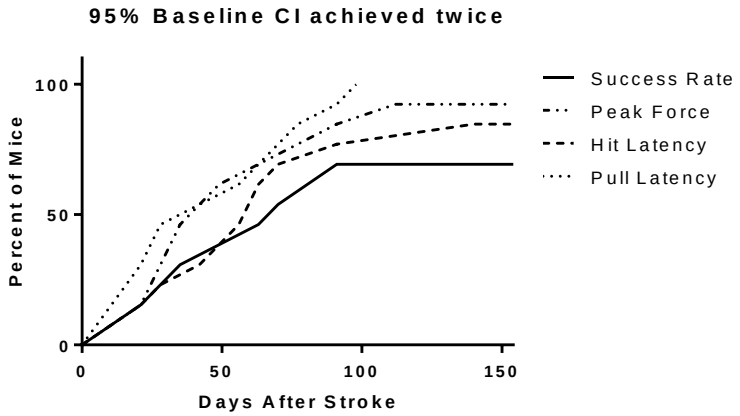


Figure 4
Recovery data (n=13) collected at days 3, 7, and weekly until day 154 after stroke: error bars represent SEM. Stars above individual time points represent Fishers LSD individual comparisons between that time point and the last baseline time point: $p \leq .05$ (*), $p \leq .05$ (**), $p \leq .005$ (***), and $p \leq .0005$ (****).



Supplementary Figure 1
Individual Recovery. Cumulative plot showing percent of mice at each timepoint that had performed within that individual's baseline 95% confidence interval at least twice.

CHAPTER THREE
Motor Forelimb Cortical Stroke Causes Loss of Precise Muscle Control in Mice

ABSTRACT

Rodent models of stroke impairment would ideally share key features of injury and recovery dynamics with human impairment. We previously reported on a new automated assay of distal forelimb function in mice for use in research assessing motor impairment and recovery from stroke. The musculature utilized, the target motion, and the long-term impairment caused by stroke in this automated assay are similar to common human impairment. We used a modification of this assay to determine if it reflects impairment in maximal strength or motor precision.

INTRODUCTION

We have previously developed and validated an automated assay to efficiently test forelimb function in the mouse (see Chapter 2 [61]). The assay utilizes the distal musculature of the forepaw; homologous movements in humans show lasting impairment after ischemic stroke [40]. The assay automatically collects rich and precise data from a reach-and-grasp motion, utilizing an infrared beam to mark the initiation and termination of reach attempts and a load cell to measure the unidirectional isometric force exerted using digital pressure on a pull handle [61].

This assay can be used to experimentally evaluate the effects of numerous variables on forelimb function. It can also be used to evaluate models of brain injury, and in this study we sought to do so. In human motor cortical stroke, loss of precision in distal muscle movement often underlies forelimb impairment [40]. Unlike rodents, humans and other primates have monosynaptic cortical-motoneuron connections, the number of which correlate with species-specific digital dexterity [62]. We might therefore expect that functional impairment of the distal forelimbs after motor cortical stroke might differ between mice and humans. We used our automated assay to ask the question: does the forelimb deficit measured in mice after motor cortical stroke of the forelimb representation represent a loss of precision of muscle movement as in the human case?

METHODS

General

In validating our original assay, we required mice to pull at the upper end of their strength range: a required 20g for mice that weigh around 20-35g. We discovered that after stroke, the force exerted on the handle decreased significantly and never recovered to pre-stroke levels even after 22 weeks of recovery. The impairment that we observed in this task may have been due only to a loss of strength or endurance. It may also have stemmed from a loss of ability to precisely control the force exerted; in the latter case, we would still expect the impaired mice to decrease their overall force exertion simply because the force

requirement was close to the natural limit of a healthy mouse's strength, so errors in force exertion would be more likely to fall on the weak side. For the following experiment, we modified the requirements of the task slightly so that the mouse could more easily err by pulling too hard as well as too soft. We trained mice to pull with a peak force in the middle of their strength range; the peak of the force exertion had to exceed 8 g but not 11g. This gave us the ability to measure a functional deficit more dissociated from strength than in our previous setup.

Apparatus, Procedure and Analysis

Our enclosure, program, measurement, and analysis procedures are identical to those described in Becker et al. [61] with the following exceptions:

During training, after mice were able to pull at least 1g with the handle at the final position, the training proceeded using progressive target force windows rather than progressive minimal force criteria. To earn reinforcement, the mouse had to pull harder than minimum force of the window without exceeding the maximum force. The mice started with a window of 1-15g, and after each successful pull the minimum force was increased by .3g until 8.2-15 g was reached. The mice then switched to a window of 3-13g, during which the lower threshold was again increased by .3 g per successful pull until reaching 8.2-13g. The final training window was 5-11g, again with the minimum threshold increasing with each success. The final window was then set at 8-11g and baseline measurements began.

In addition to the highest force pulled per trail, the local peak that was closest to 9.5g (the exact middle of the target range) within each trial as well as an average of all pulls within and across trails were extracted during analysis.

Stroke

After reaching a steady baseline performance, photothrombotic stroke was induced in the left forelimb cortical representation as described Chapter 2.

Post-stroke Behavioral Recovery, Sacrifice and Perfusion

On the third and seventh day after stroke, then weekly until 6 weeks after stroke, animals were assessed in the reach task using the same behavioral criteria as during baseline: an 8-11g window. After the 6th assessment, animals were sacrificed with an overdose of isothesia and perfused with 20ml chilled PBS and 0.1% heparin at 5ml/min, then 40ml of chilled 4% paraformaldehyde (PFA) at the same rate. The brain was then extracted and soaked in PFA overnight. It was then fixed in 15% sucrose for at least 24 hours, 30% sucrose for at least another 24 hours, and sliced into 30 μm coronal sections on a freezing microtome. The section closest to approximately 1.7mm anterior of bregma and 5 slices every 720 μm moving posterior were identified and collected. Slices were mounted onto a slide and stained with cresyl violet, developed for 5 minutes in 70% ethoanol, 3 minutes in 95% ethanol, 3

minutes in 100% ethanol and 5 minutes in xylene. Stained slices were coverslipped with permount and visualized with a Hammamatsu Nanozoomer (model 2-HT) after drying.

Analysis

Analysis of each reach session considered only the first 50 pulls. For each trial, we recorded success rate (successful trials/total trials x100), closest force (the average across trials of the local peak in that trial that was closest to 9.5g), maximum force (the highest peak within a session), all forces (peaks of all pulls in all trials), and the root mean square of the difference between the closest peak and the target (9.5g).

Animals were considered to be behaviorally impaired if, at day 7, their successful pull rate in the first 50 attempts was significantly lower than the average success rate of the last three sessions of baseline. Six of the nine animals were considered impaired.

For impaired animals, we conducted a linear regression analysis to see how closely the impairment (the difference between day 7 and baseline success) was related to the change in average closest force exerted per trial or to the change in the error of that closest force relative to 9.5g.

Stroke volume was calculated by measuring the area of healthy tissue of each hemisphere for the 6 cresyl violet stained sections. The area of the hemisphere with ischemia was subtracted from the area of the healthy hemisphere to determine the area of the stroke. Each area was multiplied by the 720 μm distance between slices and the volumes from the 6 samples added to reach total volume within the target area.

RESULTS

Whole-group measures after stroke and recovery

Animals showed a decrease in success rate in this task after stroke that lasted the entire 6 weeks of recovery (Figure 1). The average pull closest to the target range decreased slightly but nonsignificantly after stroke, while the highest force pulled per trial and the average of all pulls in a trial showed no meaningful change (Figure 1). The average error from the target increased more clearly after stroke and remained steady during recovery (Figure2). Average errors separated into negative and positive showed trends as well, but less pronounced than the overall trend (Figure 2).

Individual Variation

Our group results represent many different distinct patterns in individual animals. Some animals showed increases in closest force pulled to target, some showed decreases, and some showed a spread of the distribution after stroke to include many pulls both higher and lower than baseline. Some animals shifted from a normal distribution to a bimodal, with a new population of pulls close to the minimum pull requirement. Changes occurred in these patterns during recovery as well, with some distributions stable and others trending. Figure 3 shows example individual data of these basic patterns.

Assessment of impairment

All animals showed a lesion in the target motor cortex that ranged from 5.94 to 9.11 mm^3 (Figure 4), however only six of the nine animals also showed significant functional impairment of their success rate in the automated reach task seven days after stroke. The data from those 6 animals along with stroke volume data is considered separately in Figure 4 to characterize only the data underlying functional impairment. Of the six animals that were functionally impaired at day 7, all but one showed an increase in the error from the target force that would be expected to accompany a decreased success rate (Figure 4e). However, only half showed a concomitant decrease in the closest force pulled to the target (Figure 4c), only a few showed a decrease in the average force of all pulls (Figure 4d), and as many increased as decreased the values of their highest force pulled per trial (Figure 4b). When positive and negative errors (errors above or below 9.5g, respectively) are considered separately, both show a frequent increase after stroke (Figures 4 f-g). Finally, the change in the closest force pulled to the target showed no relationship to the change in success rate between day 7 and baseline, while a significant relationship did exist in the error of those closest forces from 9.5g (Figure 4h).

DISCUSSION

Based on the results of this study, we can conclude that motor cortical stroke has an impact on performance in the automated reach using a small, mid-range precise force requirement, and that this deficit lasts at least 6 weeks (Figure 1). We found that this deficit for individual animals sometimes represented an increase in the forces exerted, sometimes a decrease, and sometimes both an increase and a decrease (Figure 3). Group data therefore showed mixed results and nonsignificant p values when analyzed in terms of closest force to target, highest force within session, or average force of all pull attempts (Figure 1). However, the deviation in the closest pull from the middle of the target range was significantly related to the decrement observed in success rate (Figure 4 h). This error was heavily influenced by errors below 9.5g, however many animals also showed an increase in errors pulled over 9.5g. We can therefore conclude that motor cortical stroke in rodents does impact the precision of muscle movement rather than just the strength of those movements *per se* because deficits occur through both weak and forceful movements. We cannot conclude from our data that loss of absolute contractile capacity or endurance capacity did not occur in at least some animals; however it cannot account for the entirety of performance decrements in this task.

Despite the disparate wiring of mouse and human corticospinal tract pathways, and despite the apparent relationship between these differences and the differences in distal muscle control between the species, we still see a functionally similar deficit of loss in precise distal muscle control after similar injury in both species. Recovery of skilled reaching performance after such strokes has been shown to involve rewiring of cortical areas in both

mice and humans [63]. Taken with our results, this implies that cortical damage and rearrangement underlying behavioral deficit and recovery in ischemic stroke can operate with functional similarity in animals with different variations of corticospinal wiring, lending support to the strength of the mouse model of cortical stroke.

The data obtained in this study showed a rich amount of variation from individual to individual. Not only were overall patterns of impairment different, but even the increase of error from the target – here closely related to loss of success rate – only occurred in 5 of the 6 impaired mice. In addition, despite having clear strokes, three animals were still able to perform on day 7 after stroke in a manner statistically indistinguishable from baseline. This variation may be frustrating for researchers who prefer precision and clear signals, but it does not imply that the automated reach is a bad model of stroke impairment. Stroke impairment among humans is also highly variable. The automated reach is thus not only able to clearly detect long-lasting behavioral impairment, but it is capable of distinguishing between various patterns of deficit that naturally occur. This means that this tool may be not only useful for the purpose of testing the effects of treatments on central tendencies of animals with ischemic injury but also for the purpose of characterizing differences between individual impairments and potentially addressing subsets of behavioral injury where relevant. Still, the 20g reach criterion from our previous study generated a wider dynamic range within which to probe for the effects of post-stroke interventions, making it logistically more convenient for the assessment of post-stroke forelimb function. However, we can conclude that it does not simply reflect loss and recovery of strength alone, but also of precision of muscle control.

FIGURES

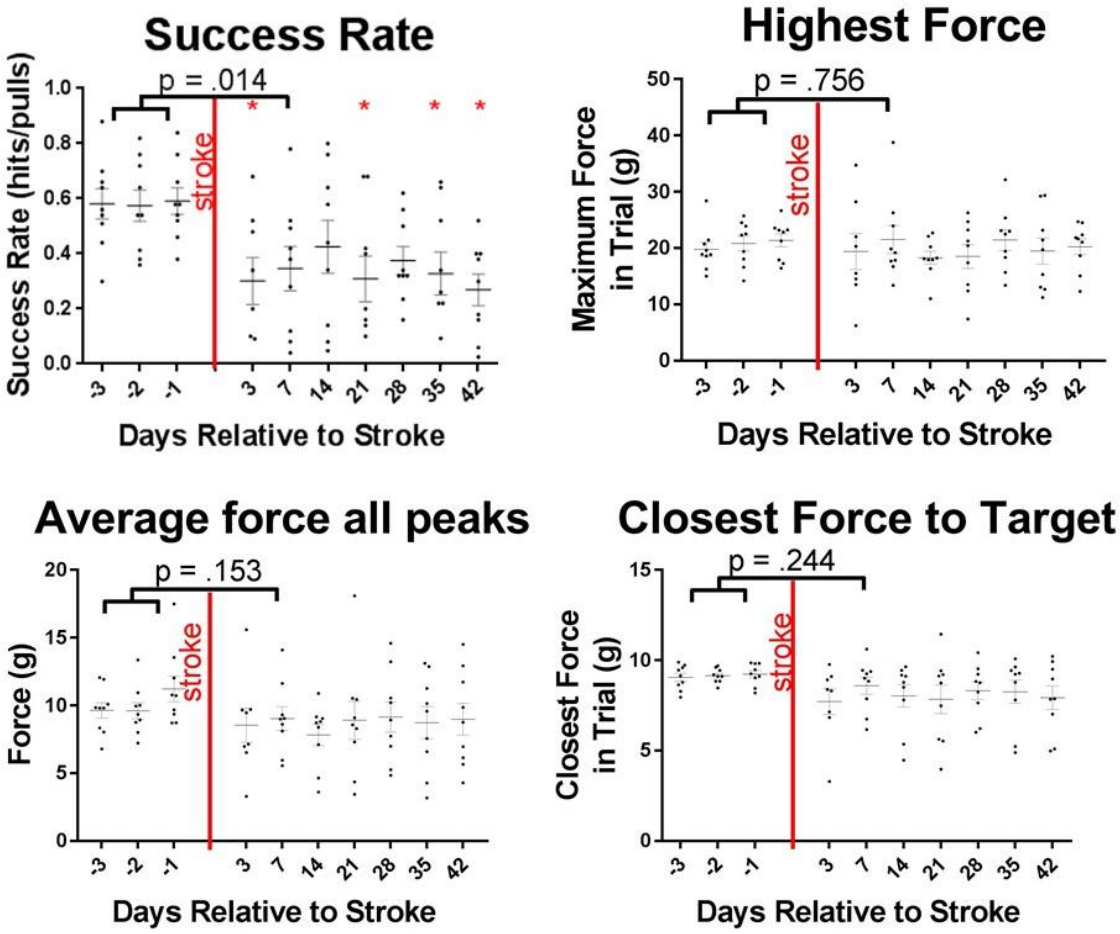


Figure 1

Stroke-induced performance deficits do not coincide with overall decrease in force exertion. Upper left: Photothrombotic stroke caused a significant loss of function in the precision version of the automated reach task. No similar decrease is seen in the maximum force exerted (upper right) or the average overall force exerted (lower left). The force within each trial that was closest to the middle of the target range did not significantly change after stroke either; however, more of the closest errors fell below rather than above the target range after stroke (lower right).

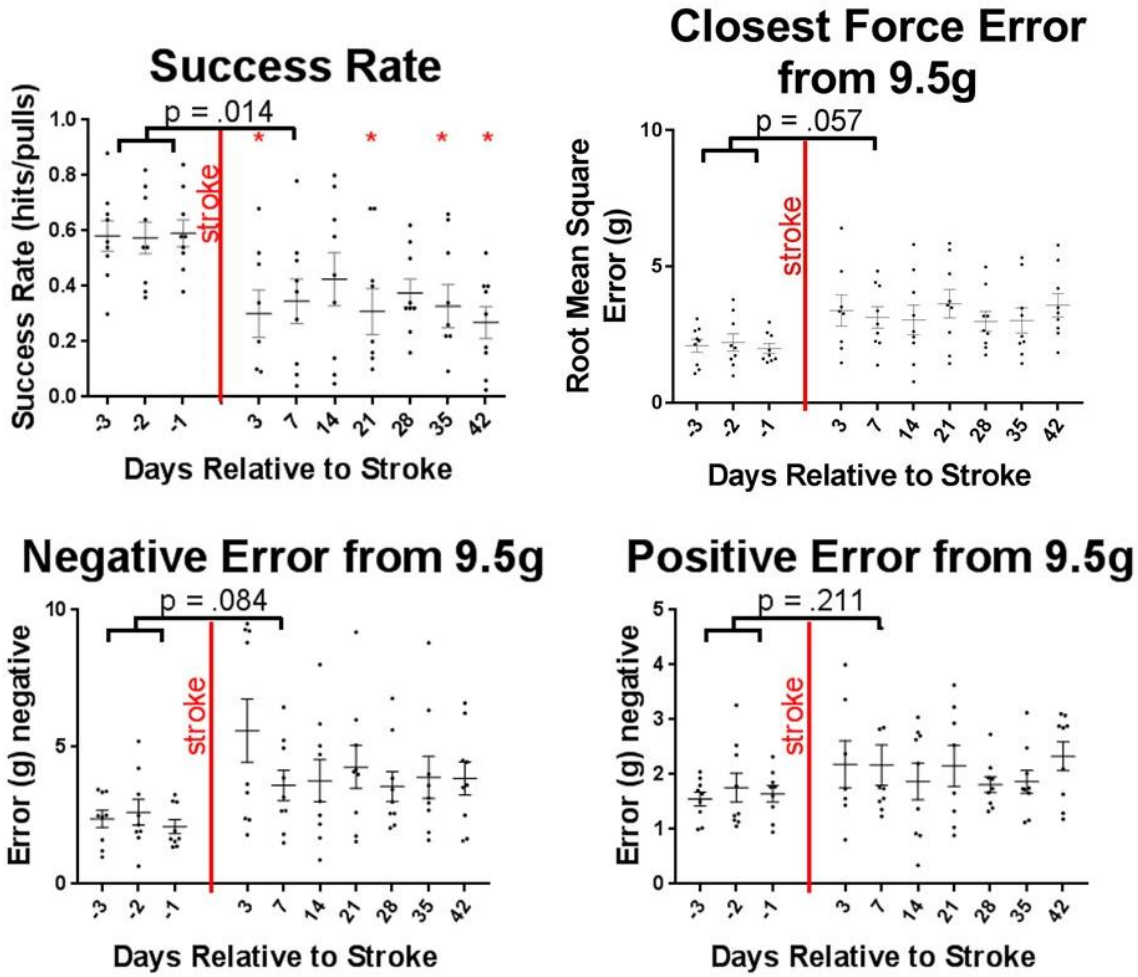


Figure 2
Stroke-induced performance deficits coincide with increases in root error from target of closest forces. Increase in root error (upper right) is more pronounced than changes in force values (Figure 1). Increases are larger for negative deviations (undershooting, bottom left) but also individually present for positive deviations (overshooting, bottom right). Though these individual increases are present, they are not significant with this small group. See Figure 4 for individual impairments.

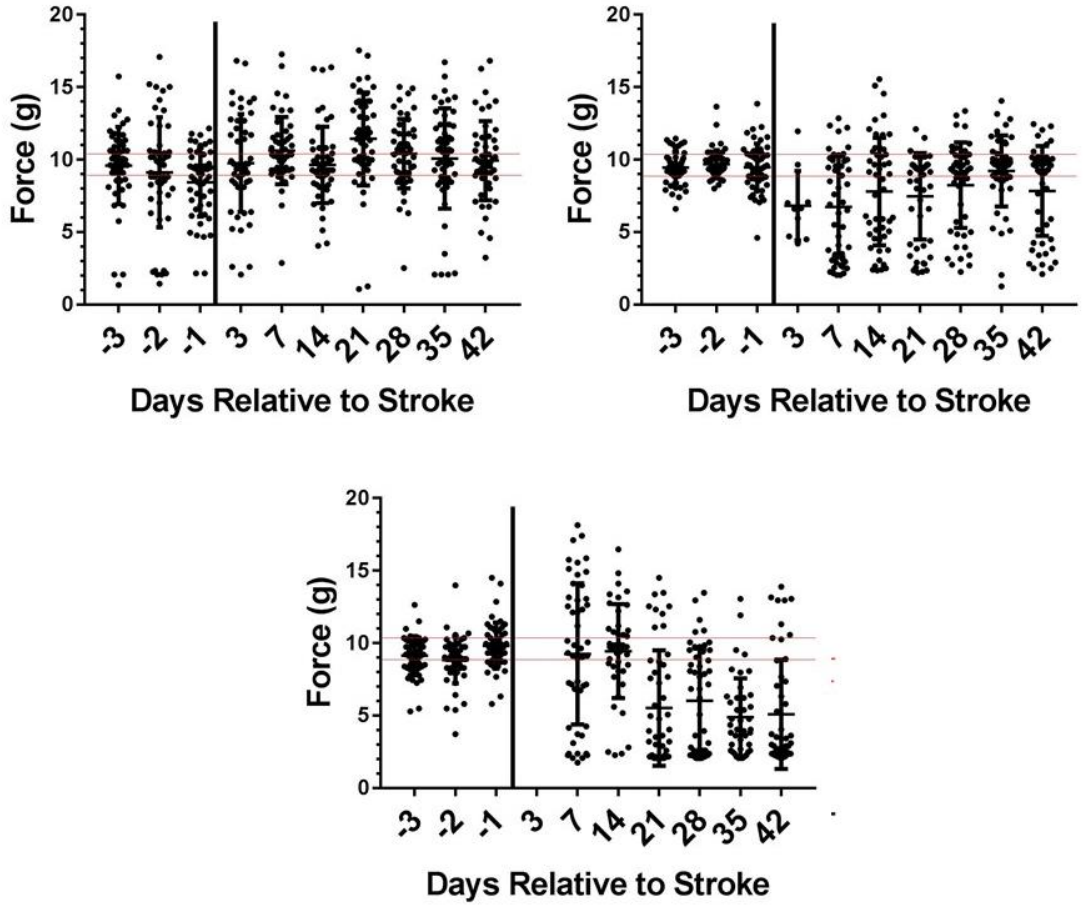


Figure 3
Individual data show different patterns of deficit after stroke. Three individual animals here show three different patterns of behavioral injury after stroke. Each data point is the value of the closest peak to the middle of the target range (red lines) within each trial. Some animals tended to overshoot their targets (upper left), some to undershoot (upper right), and others to both over-and-undershoot (below). Post-stroke force distribution did not always remain normal (upper right) nor was the pattern of initial deficit always maintained throughout recovery (below).

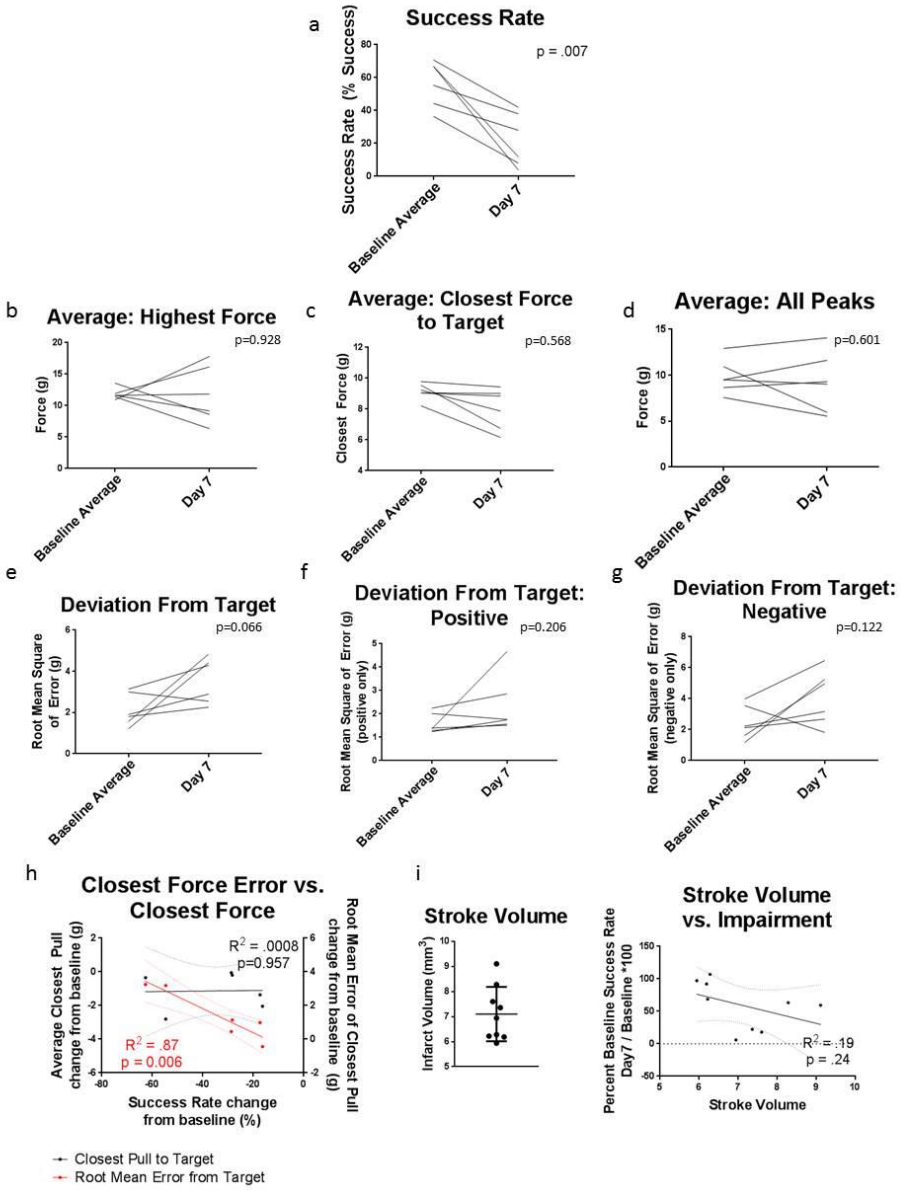


Figure 4

Error of the closest force pulled in both directions from the target accounts for changes in success rate. Impairment data are shown for six of the nine animals in the study that had significant performance deficits at day 7 after stroke. Force readings did not account for the loss of success rate (a-d). Deviation of the closest force per trial from the target range (mean error from 9.5g) increased in most animals after stroke (e), and individual increases in this measure can be seen in both positive (>9.5g) and negative (<9.5g) deviations (f-g). The change in error correlates better with the loss of success than does the closest peak per trial (h). Stroke volume (distributed from 5.94 – 9.11 mm^3) were not tightly correlated with success rate changes (i).

CHAPTER FOUR
Is the Cholinergic System a Potential Therapeutic Target for Recovery from Stroke?

THERAPY TARGETING RECOVERY HOLDS THE GREATEST POTENTIAL FOR GAINS

Stroke is a leading cause of death and disability, especially among the aging population; advances made in stroke research hold great promise for substantial impact on public health. Since it is not always possible to predict imminent strokes or to gain access to patients during the critical earliest periods during or after stroke, research focused on preventative or acute treatments is naturally limited in impact. The only treatment strategies which can be universally administered to all stroke victims are recovery strategies, especially those focused on the most common impairments such as motor control. As discussed in Chapter 1, recovery strategies heavily involve re-learning processes, the efficacy of which depends on the quality of the rehabilitative therapy independent of other neurological interventions. Further advances in the quality and efficacy of rehabilitative therapy alone may be possible via direct research on that level. Currently, though, for many patients complete recovery is not achievable via rehabilitative therapy alone; other neurological treatments that may augment therapy therefore could have substantial impact on public health.

SUCCESSFUL RECOVERY RE-ESTABLISHES PARTICULAR FUNCTION, NOT PARTICULAR NEURAL STRUCTURE

On the neural level, recovery does not aim to preserve or re-establish a patient's previous exact cellular arrangement and connectivity; a functionally recovered, reorganized brain is not identical in these respects to an uninjured brain, though it may produce robustly recovered behavior. (Please note: "functional" here is used in the neurological sense and not in the sense of functional behavioral measurement as reviewed in Chapter 1). Thus, one promising approach to developing neural treatment strategies is basic investigation into processes of guided plasticity, particularly that subset of processes that can produce behavioral recovery. This approach by definition implicates basic as well as clinical aims in stroke research since elucidating plasticity-guiding processes in general will be part of identifying therapeutic targets. Importantly, potential plasticity modulating target systems may have a role in the uninjured brain as well as in response to injury; the mechanisms for change in the two scenarios may be different but may also overlap.

REHABILITATION AND LEARNING INDUCE PLASTICITY THAT MAY BE TARGETED THERAPEUTICALLY

Effective learning and rehabilitation alone guides many aspects of functional and structural plasticity [64, 65] including changes in dendritic spine number [66, 67], BDNF secretion [64], expansions of task-specific peri-infarct motor cortical representations [68],

and – interestingly – changes in contralateral motor cortical representations [69]. All but the last of these factors correlate with better functional outcomes after stroke [63, 70-72]. Axonal outgrowth [73] and establishment of new functional connections [74] also occur after stroke, and post-stroke rehabilitative conditions can affect the genesis of new synapses [75].

From this information we may deduce that the same ultimate variables that guide adaptive behavioral change may also guide the neural plasticity that underlies those changes. If this is the case, than both maladaptive plasticity and maladaptive learning (reviewed in Chapter 1, also see [76-78]) may in some cases be guided by off-target feedback, and on-target feedback may be vital for recovery. However, since this on-target feedback may be insufficient to produce full recovery, it may be fruitful to augment those systems that tie plasticity processes to the critical environmental feedback signals that guide behavior and neural plasticity toward feedback-matching function in the natural environment.

Many plasticity-modulating interventions are possible since so many subcellular, cellular, synaptic, and systems processes have been thoroughly studied on the topic. Promising plasticity-based interventions may include increasing BDNF secretion [79] or increasing excitability of target cortex in order to encourage change in that cortex's functional connectivity [35], or increasing neurogenesis [80]. However, the plasticity-modulating systems that have perhaps the most obvious connection with environmental signaling and feedback, and are thus likely to favor the subset of plasticity that leads to functional improvement, are the diffuse neuromodulatory systems. These systems, which include the diffuse cholinergic, dopaminergic, seratonergic, noradrenergic, histaminergic and other systems, have a few qualities in common. First, all originate as small nuclei or cell

clusters whose phasic activation correlate with important environmental signals such as reinforcement, punishment, or discrepancy. They all project broadly to the entire brain rather than to one or a few targets. They all have been shown on the cellular level to affect neural plasticity via a wide array of mechanisms and receptor subtypes, and they have all also been connected to system-level plasticity processes and learning [81]. The adaptive or maladaptive nature of neural and behavioral changes may therefore be partially determined by the neuromodulatory signals elicited by the therapeutic environment and thus naturally paired with stimuli, responses and feedback. Isolating and manipulating such signals could open a pathway toward directing plasticity and optimizing therapeutic outcomes.

VAGUS NERVE STIMULATION (VNS) AS EVIDENCE OF PRINCIPLE

When an electrical cuff is placed on the vagus nerve and energized during the most successful rehabilitative movements after brain injury, the rate and efficacy of rehabilitative therapy is increased, and functional maps reorganize to a greater extent in both injured and uninjured animals [82-85]. This approach works in the context of a variety of functional injury including stroke [83-85], traumatic brain injury [86], and spinal cord damage (unpublished data). The approach was first developed with the intention of activating neuromodulatory systems; VNS activates the cholinergic, noradrenergic, and seratonergic systems. As would be predicted by the idea that neuromodulatory signaling underlies VNS and guides plasticity based on learning signals and feedback, VNS stimulation loses its efficacy when not paired with successful rehabilitative movements [83] or when the

cholinergic system is selectively ablated [87]. However, activation of those nuclei constitute only a small subset of the effects of VNS. Large networks both in the brain and throughout the periphery are also activated by such stimulation such as the amygdala, thalamus, hypothalamus, orbitofrontal cortex, viscera, HPA axis, the parasympathetic component of the autonomic nervous system, and peripheral and central networks that modulate neuroinflammatory processes and metabolic homeostasis [88]. Thus, while neuromodulatory activation may be necessary for the well-established ability of VNS to augment the efficacy of learning and feedback, we cannot say definitively that the effects of VNS are owed solely to neuromodulatory activation.

Clinically, the efficacy of VNS does not require mechanistic understanding. However, such understanding could impact future development of the stimulation approach to therapy. If one or a few neuromodulatory systems alone underlie the VNS effect, then therapies that more narrowly target those minimally necessary subsystems could be developed. Such targeting would serve a clinical purpose if off-target VNS downstream effects either dull the positive gains of the stimulation or cause unwanted side-effects. Thus far, no significant adverse side-effects have been identified as a result of VNS, but potential gains of narrow targeting cannot be fully known without empirical testing. Additionally, specifically elucidating the role of each neuromodulator in plasticity processes may lead to technological advancement in guided plasticity and greater basic understanding of neural dynamics. Such understanding could also lead to the development of biomarkers for therapeutic fidelity. Thus, clear clinical and basic science potential exists in the effort to investigate

neuromodulatory systems in parallel with the clinical development of VNS as a treatment for neurological damage.

THE CHOLINERGIC NUCLEUS BASALIS CONSTITUTES A PROMISING TARGET FOR CONTROLLING PLASTICITY

Among the neuromodulators affected by VNS, the cholinergic diffuse modulatory system has perhaps the largest body of pre-existing literature establishing its potential independent efficacy in modulating plasticity. It originates in the basal forebrain. In humans, these cells are clustered into a tight nucleus, however in rodents cholinergic cell bodies are spread out among the substantia innomata, horizontal diagonal band nucleus, magnocellular preoptic area, medial septal nucleus, nucleus ansa lenticularis, vertical limb diagonal band nucleus, ventral pallidum, and the nucleus basalis magnocellularis [89-91]. Different regions of this cell cluster project to different parts of the cortex, hippocampus, and olfactory bulb, and within the cortex-innervating cluster a rough spatial map may correspond to cortical targets [91, 92]. For the purposes of this dissertation, these cell groups will be referred to as a single unit, abbreviated Nucleus Basalis (NB).

Phasic activity of the NB is temporally related to behaviorally relevant stimuli

The NB includes cholinergic, GABAergic, glutamatergic and peptidergic cells, the former three of which at least include projection neurons [93, 94]. Cholinergic projection

neurons are the predominant cell type in the nucleus, though the exact proportion of cell types is still debated. GABAergic projection neurons destined for the cortex generally synapse onto cortical inhibitory interneurons, so they have a complimentary net activating effect to cholinergic projection neurons [95]. The activity of these neurochemically distinct cell populations has been difficult to distinguish until recently due to the lack of reliable methods for identification *in vivo*, however one study tentatively differentiated cell types based on correlation of the cell's activity to EEG desynchronization [96]. More recently, cholinergic cells of the NB have been independently investigated for the first time using optogenetic methods [97]. The results of these studies are congruent; the phasic activity of neurons in the basal forebrain, and particularly cholinergic projection neurons, correlate with learning feedback in both Pavlovian and operant paradigms, where behaviorally relevant events such as aversive, reinforcing, discrepant, or conditioned stimuli heavily recruit NB activation [96-101]. Those cells that show reverse correlation to such stimuli are likely mostly interneurons, the silencing of which would increase activation of the projection neurons of the nucleus [96]. Although the basal forebrain system includes many subpopulations of cholinergic cells that project to different cortical and subcortical structures through distinct pathways and whose cell bodies can be far apart, the phasic activity of these cells appears to be coordinated throughout the nucleus [97]. This indicates that they act as a unit despite their distance and wiring, although potential exceptions to this rule have not been completely ruled out in all cholinergic substructures. While it is well established that cholinergic cells respond to and temporally follow behaviorally important stimuli, the importance of cholinergic activity preceding such stimuli to increase attention to them has

long been postulated. While the activity of some basal forebrain neurons predict more accurate responses when they fire before onset of cues, those neurons are not cholinergic [97]. Thus, the priming of cortex to respond to stimuli on short timescales may be more a function of GABAergic and glutamatergic projection neurons rather than cholinergic neurons, with cholinergic neurons playing a more important role post-stimulus or in priming on a longer timescale.

Learning, rehabilitation, and artificial activation of NB controls network plasticity

New motor pattern acquisition changes cortical maps, notably in the primary motor cortex; however, practice of a previously acquired motor pattern does not [102], indicating that motor map rearrangement is involved in acquisition or reacquisition but not in performance. Motor rearrangements occur in a manner specific to the task learned; for example, animals trained in skilled reaching show plasticity in the representations of their wrist and digits but not elbow or shoulder as opposed to animals trained in a leverpress [103, 104]. Acquisition of new stimulus discrimination similarly changes cortical sensory maps along the behaviorally relevant dimensions of the stimuli in question; for example rats trained to respond based on auditory stimulus intensity showed increases cortical sensitivity to the target intensity range but did not show changes in tonotopic maps, whereas rats trained with the same stimuli to discriminate based on frequency showed changes in tonotopic maps [105]. Changes in cortical maps likely reflect larger network changes that can be directed by training, including thalamocortical connections [106].

Such motor remapping also occurs in the context of post-stroke recovery, as would be expected if motor recovery from stroke paralleled a motor acquisition process. Not all motor remapping has been associated with optimal functional recovery. Changes in the cortex contralateral to ischemic injury has been associated with decreased motor recovery [77, 107] (although see [65] for natural changes in ipsilateral cortex during learning, [108] for a contrast between short and long-term deficits, and [109] for a review of bilateral activation and its relationship to recovery). Particular dimensions of motor map plasticity such as complex movement representations [110] and ipsilateral primary motor maps [109] have been correlated with better functional recovery.

Similarly, electrical stimulation of the NB increases functional plasticity of auditory cortex when paired with particular auditory frequencies in order to increase representation of those frequencies [111, 112]. The sensory plasticity enhanced by NB is particular to the parameters of the sensory input in the behavioral paradigm with which it is timed [113]. Pairing NB stimulation with visual stimuli has a similar plasticity-modulating effect, which is again specific to the particular parameters discriminated in the behavioral paradigm [114, 115]. Cholinergic agonists also enhance motor map plasticity when active during motor training [116] and acetylcholine is necessary for some experience-related plasticity normally seen during rehabilitation [66].

It should be noted that while cortical map expansion may be involved in producing new pathways for newly acquired behavior, it does not last as long as the behavior. Expansions that occur during acquisition tend to renormalize while behavioral performance endures [65].

Thus, while such plasticity may be critical for acquisition of a new behavioral pattern, expanded representations should not be conceptualized as producing the behavior.

NB cholinergic signaling underlies behavioral acquisition in injured and uninjured animals

In rodents with selective lesions of the NB cholinergic systems, many parameters of learning and behavior are affected. Loss of the cholinergic system tends to increase locomotion in some contexts [117, 118] and affects anxiety tests by either mildly [117] or markedly [118, 119] making animals less likely to avoid open spaces and bright lights. Relative to behavioral performance and learning, acquisition of many navigational tasks are negatively affected such as in the Morris water maze ([120-122] but see [117]), passive place avoidance [117, 119], and the barnes maze [120]. However, performance in a delayed non-match to position task is either not impacted or only slightly impacted at long delays by loss of NB cholinergic neurons [117] as are other types of short-term memory tasks such as discriminating one aspect of a complex stimulus after a single trial and a delay [123]. These tasks, though, can be conceptualized as performance rather than acquisition tasks since they were pre-trained before cholinergic lesions. Croxen et al. [123] demonstrated that while loss of cholinergic cells did not affect the performance of this pre-trained memory task alone, it did prevent the recovery of that task after brain damage to the fornix – again supporting the idea that the cholinergic system modulates behavioral acquisition or recovery-related plasticity. Fine et al. [124] demonstrated that loss of cholinergic NB neurons in monkeys

prevented discrimination learning but not performance. Similarly, and more importantly for the topic of motor recovery, NB lesions prevent acquisition of new motor tasks [68, 125, 126] and prevent motor recovery of a reach task after electrolytic cortical lesions [68].

Acetylcholine-enhancing drugs have positive impact on recovery when pharmacologically active during learning

Many drugs affect acetylcholine along with other neurotransmitter systems such as amphetamine [127, 128] and flouoxetine [10, 129]. While potential effects of those drugs on recovery from stroke may be linked to cholinergic action, such an assertion is difficult to make given the variety of effects that these drugs have. Acetylcholine-specific drugs such as the acetylcholinesterase inhibitor Donezepil have also been examined in the context of recovery from brain injury, and results show potential augmentation of recovery especially when the drug is active during rehabilitation [130-134]. However, the system-wide effects of drug treatment does not establish that positive effects of cholinergic drugs are due to augmentation of basal forebrain cholinergic activity alone since the functions of acetylcholine throughout the nervous system and the body are so numerous and so varied. Questions about optimal therapeutic value and mechanistic learning and plasticity processes related to cholinergic substructures require selective rather than pharmacological augmentation alone.

Acetylcholine is linked to many potential mechanisms that could underlie guided plasticity

Acetylcholine changes activity patterns that affect synaptic plasticity

Broadly speaking, synaptic plasticity occurs when the effect of a presynaptic action potential on a postsynaptic membrane potential is changed. This can occur with our without cholinergic or other diffuse neuromodulation, depending on the location and type of plasticity. The relative timing of the action potentials of pre- and post-synaptic cells has long been established as a basic determinant of synaptic plasticity; therefore changes in the activity patterns of either or both cells on either side of a synapse can change their relative timing and thus the patterns of synaptic plasticity that can occur. Acetylcholine (Ach) has long been linked to overall increases in net potentiation throughout networks [135-138], and this link was originally ascribed to its ability to increase excitability or responsiveness of cells [139], thus increasing the chances for long-term potentiation (LTP). Current understanding reserves a much larger and more complex role for acetylcholine in plasticity processes, however the direct effects of nicotinic depolarization and the shorter-term excitability-related effects of muscarinic activation in cortical neuron activity still remains as one of the mechanisms by which it can affect plasticity dynamics. For example, cortical pyramidal neurons respond to a cholinergic pulse with a fast inhibition followed by a long excitation. The inhibition is modulated in part by nicotinic depolarization of GABAergic interneurons [140], while the slow depolarization results in part from muscarinic activation of the pyramidal neurons themselves and the resulting closure of M-type potassium channels

[141]. Meanwhile, nicotinic receptors on thalamocortical circuits increase the glutamatergic signal from that pathway onto sensory cortex [142] and muscarinic input onto subsets of interneurons decreases GABAergic inhibition to these circuits, again making pyramidal neurons more excitable by thalamocortical connections [143]. At the same time, muscarinic activation in pyramidal cell axon terminals [144], together with muscarinic inhibition of a different subset of GABAergic interneurons [140] decreases the activity of cortico-cortical connections. The resulting bias of cortical networks to respond to direct feed-forward effects of thalamic inputs rather than cortico-cortical loops constitute a potential mechanism for biasing both behavior and plasticity. Such biasing increases the probability that potentiation will involve increasing connectivity between particular sensory stimulation and active downstream networks. This mechanism is thought to underlie the relationship of acetylcholine with the concept of attention since it tunes the cortex toward outward responsiveness.

Cholinergic-dependent plasticity depends on astrocytes

The activity of astrocytes and gliotransmitters can contribute to plasticity processes ([145-147] but see [148]). In V1 cortex, astrocytes show calcium responses to acetylcholine that is dependent on muscarinic receptor activation and independent of neuronal activity. When these calcium responses are attenuated, the slow depolarization of cortical neurons in response to cholinergic release is attenuated as well [149]. The astrocytic activity influences slow neuronal depolarizations in an NMDA-dependent fashion, likely via gliotransmitter release [149, 150]. Mice with deficient calcium responses in astrocytes show deficient

stimulus-specific cortical plasticity when nucleus basalis stimulation is paired with visual stimulation [149] or whisker barrel stimulation [150]. Modulation of hippocampal plasticity by acetylcholine shows a similar dependence on astrocytes [151]. Thus, astrocytic responses to acetylcholine and downstream NMDA-mediated effects on cortical neurons could play a part in the mechanism of cholinergic mediation of cortical plasticity.

Acetylcholine changes STDP rules

Plasticity may be changed by altering the firing patterns of constituent cells, but it may also be changed by altering the rules by which relative firing of those cells modify the synapse (i.e. spike timing dependent plasticity or STDP). Relative firing is not sufficient to induce plasticity in all brain regions; for example in visual cortex, layer 2/3 pyramidal neurons show no plasticity under typical pre-post synaptic timing protocols unless neuromodulators are added; specifically noradrenaline gates LDP in these neurons and acetylcholine LTD [152]. Once any plasticity is gated at all, the outcome of particular timings of pre-post activation can be targeted using different levels of neuromodulators; for example in the aforementioned visual pyramidal neurons, application of both modulators produced bi-directional STDP [152]. In the prefrontal cortex, nicotinic activation causes normally LTP-inducing protocols to induce LTD, and changes the threshold for plasticity in general [153]. In other contexts, acetylcholine has instead been shown to bias cortical networks toward LTP [138].

While all mechanisms for these effects are not yet fully known, it is clear that the relative and absolute concentrations of acetylcholine and other neuromodulators and their

receptors affects the temporal windows over which the relative timing of pre and post-synaptic activation can result in LTP or LTD, and that the mechanisms and particulars underlying this fact are dependent on region, cell type, receptor types, geometry of modulator release and receptor location, and many other factors [154]. These effects are not simply additive; composite receptor activations have a nonlinear effect on spike-timing curves [152, 154-157]. Thus, Ach could modulate plasticity by controlling complex and momentary changes in plasticity rules as well as altering or biasing the firing patterns of target neurons in coordination with other diffuse neuromodulators.

Conclusion: mechanisms of cholinergic control of plasticity

Much more experimental work may eventually lead to a comprehensive understanding of the mechanisms underlying cholinergic neuromodulation throughout the brain and may elucidate how exactly acetylcholine helps to direct plasticity toward functional outcomes. However, such a mechanistic understanding is not necessary to establish that on the systems (i.e. cortical maps) and behavioral levels, phasic activation of NB cholinergic pathways concomitant with environmental events results in plasticity adapted to that environment. Current work on acetylcholine establishes that many cellular and synaptic mechanisms are in place that connect acetylcholine to plasticity and may form a bridge between these levels of analysis; the connection is sufficient to justify further work on the influence of cholinergic modulation on stroke recovery.

THE CHOLINERGIC SYSTEM MAY BE AFFECTED BY STROKE

Although NB dysfunction is not thought to underlie behavioral impairment after stroke, the ascending cholinergic pathways of the NB do pass through regions that are commonly affected by stroke and could thus be disrupted [158-160] and some evidence indicates that the system is necessary for stroke recovery [66, 68]. That cholinergic damage is not generally considered important in the context of stroke may be a result of lack of evidence rather than evidence of irrelevance. If the NB plays a necessary role in modulating rehabilitation-associated rewiring processes, stroke-related damage to its ascending pathway may make an NB-targeted stimulation approach even more efficacious.

SUMMARY AND HYPOTHESIS

Recovery strategies to therapeutic intervention in stroke hold great promise for public health impact since such strategies may be widely applied among stroke victims. Most patients experience some degree of recovery after stroke, which can be augmented by deliberate rehabilitative training. However, this recovery is often incomplete, and patients can have lasting motor, sensory, and cognitive deficits which limit their daily function. Controlling functional plasticity in the injured brain could be the key to producing better recovery in stroke patients; this might be accomplished by utilizing natural systems that underlie the modulation of neural plasticity to produce behavior adapted to environmental

cues and feedback. Neuromodulatory pathways originating from deep brain nuclei may constitute such target systems; they signal learning events widely to the entire brain and the process of cortical plasticity is influenced by their activation. The NB in particular fires in phase with the important stimuli to which behavior must adapt to be functionally recovered. The NB releases acetylcholine, which is linked to plasticity through a variety of mechanisms. Cholinergic modulation may therefore help to direct neural changes to produce functional behavioral changes. If this is the case, augmenting NB activity during rehabilitation could potentially support recovery in both individuals with compromised cholinergic ascending pathways and in those without such compromise. I therefore hypothesize that cholinergic neurons of the NB modulate behavioral plasticity by releasing acetylcholine while functional stimuli and responses are occurring, directing plasticity after brain injury toward motor improvement. To test this hypothesis, I determined whether NB cholinergic neurons are necessary for "spontaneous" behavioral recovery from motor cortex stroke in a mouse model of ischemic stroke (a systematic extension of previous related work [68]). I then determined whether increased activation of NB cholinergic neurons during motor rehabilitation is sufficient to increase the speed and extent of behavioral recovery following motor cortex stroke in the same model. These basic aims for the next two chapters can be seen in Figure 1.

METHODOLOGICAL CONSIDERATIONS

The nucleus basalis signals with both a tonic and phasic activity patterns [161] and likely controls processes other than environmentally-dependent plasticity modulation [162].

In addition, other cholinergic systems throughout the brain have their own role to play in learning and other processes; for example cholinergic striatal interneurons are momentarily silenced in response to the same stimuli that activate NB cholinergic projections and this silencing likely contributes to plasticity modulation [163, 164]. The PPT and LDT, other cholinergic brainstem nuclei, are vital for arousal and other functions [165-167], and the PPT also responds to behaviorally relevant stimuli and is involved in modulating other modulatory systems [168]. Thus, nonspecific cholinergic manipulations such as pharmacological activators or blockers would be expected to produce changes that could work both for and against learning and are not sufficient to answer my narrow question about the functional unit of the NB cholinergic system.

In addition, as discussed above, the timing of activity and function of cell populations within the NB with different transmitter identities are distinct. The nucleus itself is spread out over a scale of several millimeters in the mouse, and projection neurons are integrated with local interneurons. Thus, electrical stimulation or electrolytic ablation of the nucleus would also be insufficient to answer my questions since these manipulations would not selectively manipulate cholinergic projection neurons.

Conversely, targeted immunotoxin, optogenetic, and chemogenetic approaches allow for the interrogation of cholinergic projection neurons alone on a timescale relevant to the learning environment. Through immunological or genetic targeting of neuron identity and spatial targeting of the NB via toxin, virus or light fiber placement, these manipulations can target projection cholinergic NB neurons alone. Optogenetic approaches allow for the exact control of timing in the targeted neurons, while chemogenetic approaches allow for the

modification of cellular responses to naturally timed inputs. Thus, the following chapters utilize these tools to interrogate my hypothesis.

FIGURES

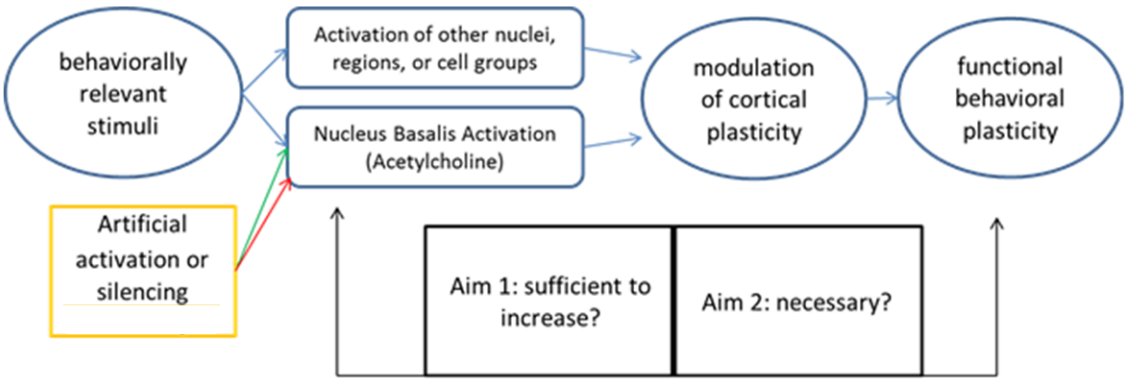


Figure 1
Two aims to study the relationship between the cholinergic neuromodulation system and stroke recovery

CHAPTER FIVE
The Cholinergic Nucleus Basalis System is Necessary for Typical Recovery of Forelimb Reaching after Stroke in Mice

INTRODUCTION

As reviewed in previous chapters, the goal of stroke recovery is not to produce an uninjured brain. The cell death that has occurred after a stroke cannot be undone at this point, yet that does not preclude full recovery. The brain can be modified and rewired in response to both normal learning and to injury, and we know that the set of possible brain organizational networks that can support sophisticated behavioral function are not limited to the original, uninjured structure. Rewiring healthy tissue rather than replacing damaged tissue therefore becomes an aim of chronic stroke treatment.

Much is known about the systems that influence brain rewiring on the cellular, synaptic, circuit and systems levels. The literature contains evidence that environment and learning are intrinsically connected with brain changes. As reviewed in the previous chapter, the cholinergic projection system of the basal forebrain, along with several other diffuse modulatory systems, constitute a potential link between functional brain plasticity and environmental signals. We can see from previous studies that these systems are also linked with recovery from brain injury. Indeed, Conner [68] has shown that both rewiring and behavioral recovery from electrolytic cortical lesions are prevented when the cholinergic system is lesioned. Studies thus establishing that the cholinergic system is necessary for certain types of learning and recovery make a clinical as well as a basic point; it may be more

possible to recover from brain injury when underlying plasticity-modulating systems remain intact, while a limit to rewiring and recovery may be imposed via injury of the modulatory systems themselves. Thus, establishing the necessity of cholinergic basal forebrain for recovery from ischemic brain injury can be useful for both basic and clinical reasons. Here we have systematically varied and extended Conner's [68] experiment by testing the necessity of the basal forebrain cholinergic system in mice for recovery from ischemic injury.

METHODS

Subjects

Twenty-two adult C57-Bl wild-type mice were used in this experiment; ten control and twelve with targeted lesions. At the time of stroke, each group ranged in age from 17-38 weeks old (Figure 6). Most were male, though each group included one female. Home cages were kept in a temperature and humidity maintained facility on a reverse light cycle to assure that behavioral testing would occur during normal waking hours. All mice had food and water available to them ad-libitum in their home cage and also in their reach chambers if subjected to long sessions. All procedures involving these mice were approved by the UT Southwestern Institutional Animal Care and Use Committee.

Automated Reach

All animals were trained in the automated reach chamber using the same procedure described in Chapter 1. After animals reached a stable baseline performance, they underwent injections of either saporin or PBS as described below. Animals were allowed to recover for several days before re-initiating baseline. Baseline was continued again until a second period of post-injection stability was reached. At that point, animals received strokes and were assessed in the reach chamber in the same manner described in Chapter 1 on day 3, 6, 14, 21, and 28 after stroke.

Rotarod

At least 3 days before injection surgery, animals were trained on the rotarod in six sessions over a period of two days. Two sessions occurred after injection and prior to stroke, then two sessions on days 3, 14, 21, 28 and 35 after stroke. For each trial, animals were placed on a stationary bearing. Rotation was initiated at 4 rotations per minute and accelerated constantly up to 40 rotations per minute in 2 minutes. We recorded the latency from the initiation of rotation until the mouse either fell off the rod or started to cling to it and spin. Each session consisted of four trials. In analyses, two sessions are averaged together in order to produce baseline and recovery data points.

Injection

Mice were anesthetized with a mixture of 30% O2, 70% NO, and 1-4% isoflourine. They were then affixed to a stereotaxic apparatus, their scalp injected with lidocane, and a small incision made along the midline of the scalp. The skin was pulled aside and the scalp dried. The levelling of the mouse's skill was checked by measuring the location of the surface of the skill 1mm directly to the left and to the right of bregma and assuring that the z-dimension coordinate of these points were within .03mm of one another. The same comparison was made between the z location of bregma and lamda. Once levelling was confirmed, a small, approximately 3/64" hole was drilled bilaterally .3mm posterior to bregma and 1.7mm lateral from bregma on either side. A Hamilton Syringe with a flat needle was then lowered at these same bilateral locations to a depth of 4.72 mm from the surface of the dura then raised again to 4.7 mm to create a small space at the tip. For experimental animals, 2000nl of mu p75-SAP (murine p75NTR-saporin) targeted toxin suspended in PBS (Advanced Targeting Systems catalog # IT-16) was injected at a rate of 200nl per minute. This immunotoxin recognizes the cholinergic-specific $p75^{NTR}$ receptor and uses it to gain entry to the cell. The conjugated saporin, a ribosomal inactivator, causes death in these cells. For controls, only PBS was injected. Seven minutes after the injection was complete, the syringe was withdrawn. After both injections were complete, the scalp incision was stitched shut and 0.2 cc subcutaneous saline was administered to restore any lost body fluids. Buprenorphine was used during and after surgery to control postoperative pain.

Stroke

Stroke was induced surgically according to the procedure in Chapter 2

Sacrifice, perfusion and histology

After their final behavioral tests were complete, animals were sacrificed with an overdose of isothesia and perfused with 20 ml chilled PBS and 0.1% heparin, then with 40 ml of chilled .4% paraformaldehyde all at a rate of 5ml/minute. Brains were extracted and kept in 4% paraformaldehyde for one day, then transferred to 15% sucrose in water for 1 day, then 30% sucrose for a final day. Using a freezing microtome, 30µm coronal sections were collected. Starting at 36mm anterior to bregma, every 6^{th} section (180µm spacing) was then mounted on a slide, allowed to adhere, and rehydrated with PBS. Sections were then incubated in .3% triton-x in PBS for 2 hours at room temperature, then .1% triton-x and 10% bovine serum albumin for another 2 hours. Sections were then rinsed thoroughly with PBS and incubated overnight for two nights in the dark at 4 degrees C in PBS with 10% BSA and 1:100 goat anti-choline acetyltransferase (ChAT) antibody (Millipore catalog #AB144P). On the second day, slices were rinsed thoroughly with PBS and washed with PBS for 10 minutes. Sections were then incubated in PBS with 1:500 donkey anti-goat alexa594 conjugated secondary antibody (Invitrogen A-110568) at room temperature for 2 hours. Slices were then washed with PBS for 10 minutes three times. Vectashield hard set mounting

medium was added and slides were coverslipped and stored in the dark at 4 degrees until visualization.

On separate slides, the section closest to approximately 1.4mm anterior to bregma based on atlas referenced anatomical structures was mounted along with every 24th subsequent slice for 6 slices total. These sections were allowed to adhere then rewetted with PBS and incubated in Cresyl Violet stain for 20 minutes. Slides were rinsed in ddH20 and developed in 70% ethanol for 3 minutes, 95% ethanol for 3 minutes, 100% ethanol for 3 minutes, then xylene for 3 minutes. Permount was added and slides were coverslipped and allowed to dry for two nights before visualization

Histological visualization and analysis

Slides were scanned at 40x with a Hammamatsu Nanozoomer slide scanner. Cresyl violet stains were scanned with brightfield settings, ChAT stains with fluorescent scanning using the TxRed filter.

Cresyl violet stains were analyzed to determine stroke volumes; the total area of healthy tissue from each hemisphere was determined by outlining those areas and excluding ventricles. Each measurement was made three times and the average accepted if the three varied by less than 5%. The area of the injured hemisphere was then subtracted from the uninjured hemisphere result multiplied by the distance between sampled sections (720µm). Results from the 6 sections were added together to produce an estimate of stroke volume between the edges of the sampled area.

The ChAT-labelled scans were quantified by blinded counting; all fluorescent-positive cell bodies located below the border of the striatum were counted on each side of the sampled slices by an individual blinded to group assignment.

RESULTS

Cholinergic lesion, stroke induction, and behavioral impairment

Pre-tests with mu-p75 saporin indicated that if subjected to doses above that in this experiment, noncholinergic cells may experience toxicity as assessed by Floro-J Gold staining (data not shown). We therefore had to use at most the dose mentioned above, which decreased but did not eliminate cholinergic cells from the nucleus basalis. Still, a significant reduction in the cholinergic cells of the target area was achieved (Figures 1 and 2). This reduction was more dramatic on the right side than the left.

The photothrombotic stroke procedure produced strokes in these groups that ranged from 0.92 to 6.64 cubic millimeters (Figure 5), which constitutes 2.1 – 14.5% of the hemisphere. The saporin group had a bimodal distribution of stroke volumes and the control group a more normal distribution.

Photothrombotic stroke caused a significant loss of function in all measures for all tasks: cylinder, rotarod (Figure 3) and automated reach (Figure 4).

Recovery from stroke

In the cylinder task, neither control nor saporin-injected groups recovered their loss of right paw use after 4 weeks of recovery. However, the saporin-injected group tended to get even worse than just after stroke. Still, the resulting difference between the groups did not reach statistical significance ($p = 0.22$, Figure 3).

In the rotarod task, animals recovered quickly by week two, as is typical of this task. Both groups recovered at approximately the same rate. On the fourth and final week of assessment, the control group was staying on the rod slightly longer than the saporin-injected, group, but not significantly ($p=.40$, Figure 3).

In the reach task, both success rate and highest force pulled showed similar post-stroke deficits, but the control-injected group improved well over the next four weeks while the saporin-injected animals did not (Figure 4 left, significant interaction effects on 2-way ANOVA shown below graphs, significantly different time points determined by uncorrected multiple comparison t-tests only on ANOVA-positive measures and indicated by asterisks). Through randomly assigned to groups, control animals had better pre-baseline performance in latency to initiating a pull (Figure 4, lower right) and that advantage maintained and widened slightly through injury and recovery. However, the difference between groups was not significant; the 2-way ANOVA did not show significant time-group interaction. In the measure of latency to successful pull, control animals performed slightly worse than saproin-injected animals just before stroke and that difference maintained after stroke, indicating an equal deficit. During recovery control animals surpassed saproin-injected animals in this

measure, however the 2-way ANOVA did not show a significant time-group interaction (Figure 4, upper right).

Relationship between stroke volume and recovery

As expected, there was a slight but not a tight relationship between stroke volume and final performance, with smaller strokes tending to correlate with higher performance (Figure 5). This relationship was not different between the groups. Importantly, the individuals in the saporin group had low performances at many different stroke volumes: both the largest and the smallest stroke volume of that group were included in the worst overall third of performers.

Age of mice at the time of stroke

Animals received stroke in this experiment at various ages ranging from 17 to 38 weeks. Both groups contained exactly the same range of ages, however the saporin injected animals had more older animals compared to the control animals. Linear regression of final performance in automated reach success rate vs. age indicated that the slopes of the saporin and control groups were not significantly different, while the intercept was (Figure 6).

DISCUSSION

This experiment demonstrates a small but consistent effect of loss of cholinergic nucleus basalis neurons on recovery from cortical stroke. Importantly, while the less developed, more canonical tests of motor function in mice (rotarod and cylinder) were not helpful in detecting performance differences 4 weeks out from stroke, the nonsignificant differences in this data consistently trended in the same direction as did the more sensitive reach data, indicating the possibility of generalized effects but also locating the relevant effects of saporin on the most model-relevant behavior.

The immunotoxin did not completely eliminate cholinergic neurons. It is possible that the effect on recovery would be stronger with a more complete destruction of this neuronal population. If these results scale with the magnitude of the loss of such neurons, we would expect a complete lesion to have an even greater impact.

Our study also established the strength of our results in terms of age: a commonly overlooked issue in mouse models of stroke. Older mice, like older humans, tend to have greater and longer-lasting deficits after stroke. For this reason, the typical usage of young mice in stroke research has been criticized. Any results in stroke recovery research would be strengthened by a demonstration that effects discovered in young animals are also strongly present in older ones. This study considered a similar range of ages in each group and found significant differences between the groups in all age ranges. At nearly 9 months of age, the oldest mice in our study greatly exceed the typical age of mice in stroke research. We found the expected relationship between age and stroke recovery overall to be consistent between

groups (Figure 6, same slope) and a similar effect of targeted immunolesion throughout this range of ages (Figure 6, different intercept).

Since our experiment only included one female in each group, we cannot show the same generality across sex as we do across ages. However, our two females did conform to the general trend, with the saporin-injected individual finishing out at 10% success rate and the control individual 84%.

This experiment supported the hypothesis that the cholinergic cells of the nucleus basalis are necessary for the typical recovery seen after cortical ischemic injury. While we presume that the mechanism for this necessity is cholinergic release in the cortex itself, this experiment was not designed to test hypotheses about target-specific effects. Cholinergic cells of this nucleus also project to subcortical regions such as hippocampus which may be involved in our results. Future experiments are required to investigate potential differential roles of cortical vs. subcortical cholinergic action.

The necessity of the cholinergic nucleus basalis for stroke recovery may be a clinically significant point that could lead to better, personalized treatments for stroke. While typical strokes generally do not directly impose ischemic injury on the basal forebrain itself, they often affect the areas through which the ascending cholinergic projections are travelling, as reviewed in Chapter 4. If biomarkers can be identified that indicate damage to this system, it may indicate a need for pharmacological or stimulation-based treatments that specifically target and augment NB cholinergic function.

FIGURES

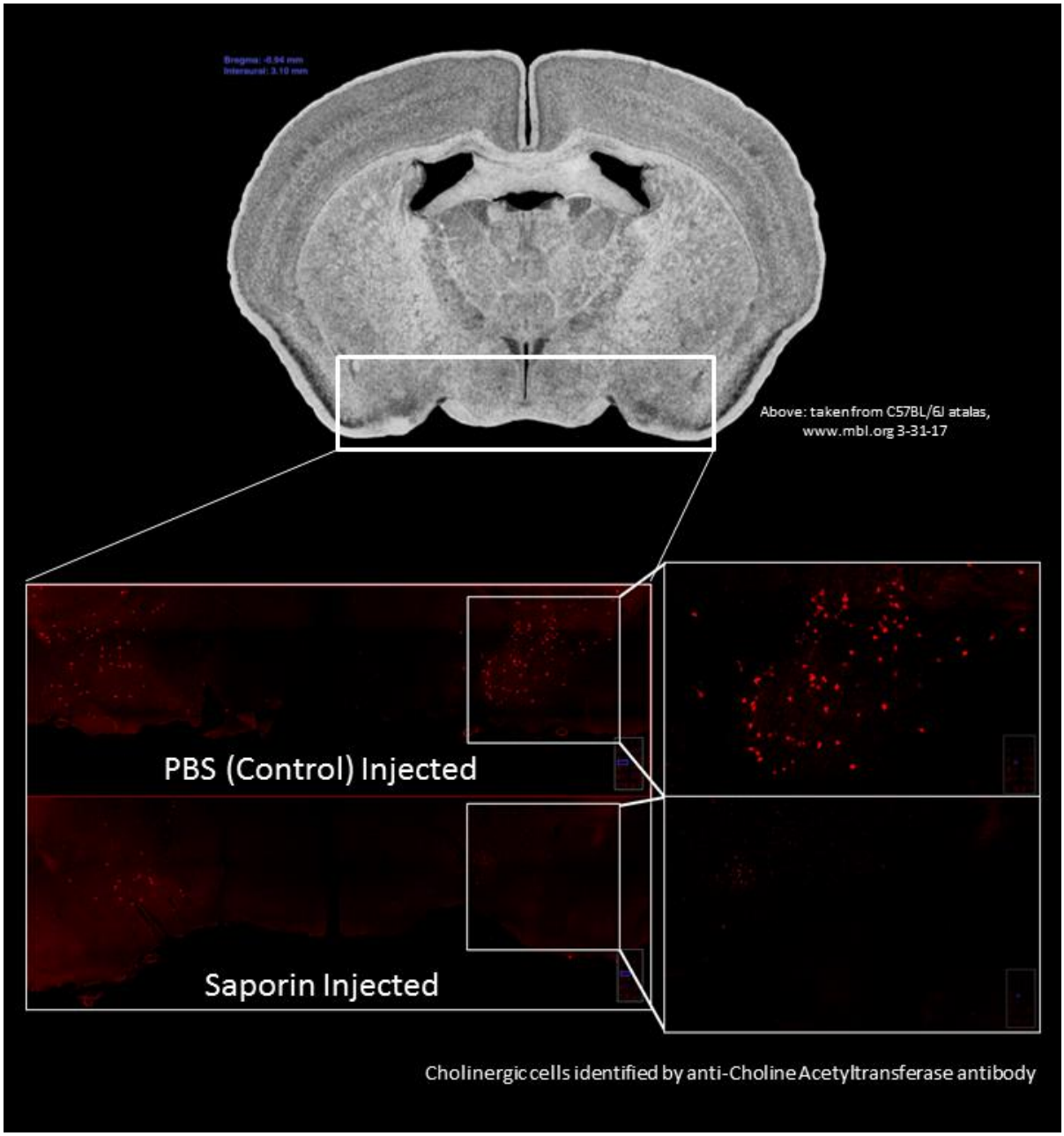


Figure 1
Sample stains showing partial loss of cholinergic cells after injection of mu-P75-saporin. Above: coronal section representing one of the target slices, taken from C57BL/6J atlas

(www.mbl.org) to demonstrate location of stain. Below, left: lower part of similar coronal section of an experimental and a control animal probed with an antibody to choline acetyltransferase (a marker of cholinergic cells, red). Below, right: close-up of NB for both animals.

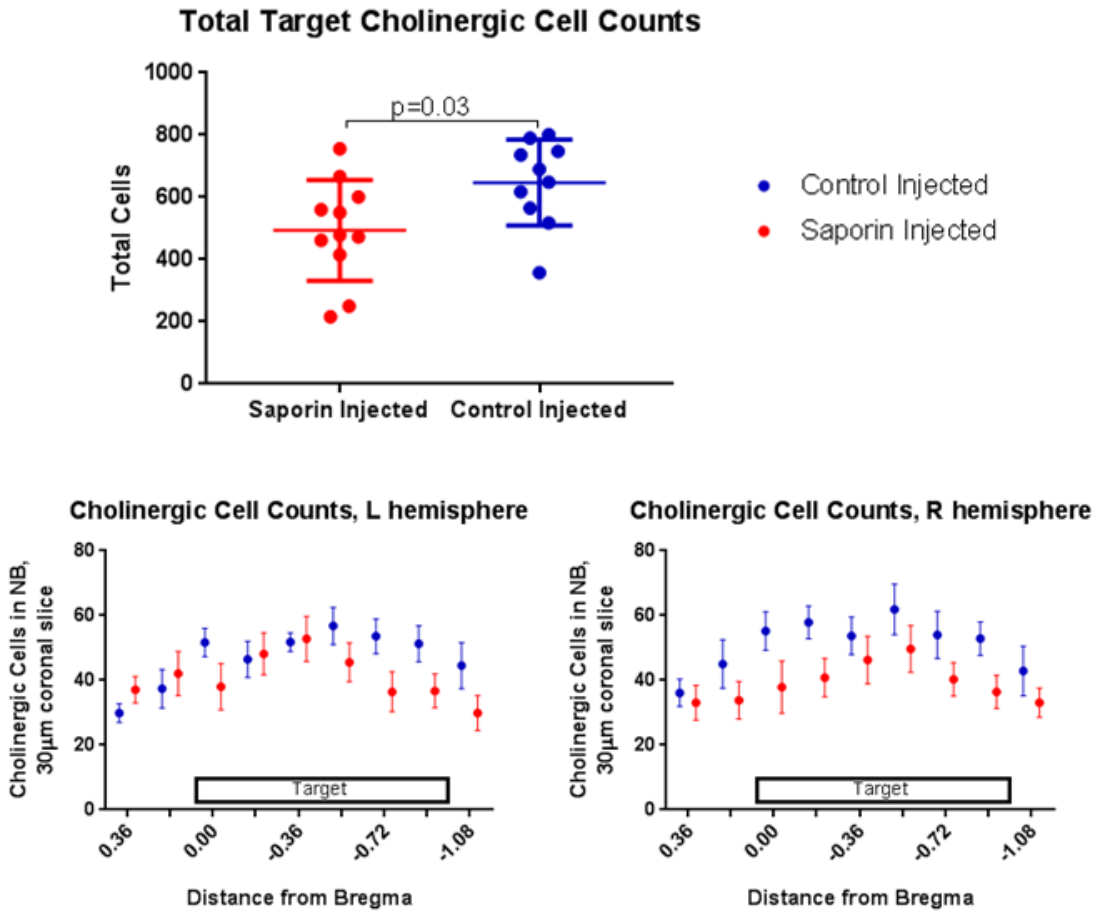


Figure 2
Quantification of cholinergic cell differences between the groups. Above: total cholinergic cell count within the target range (0.00 to -0.54mm posterior from bregma). Below: Distribution of differences across the anterior-posterior axis in left and right nucleus basali.

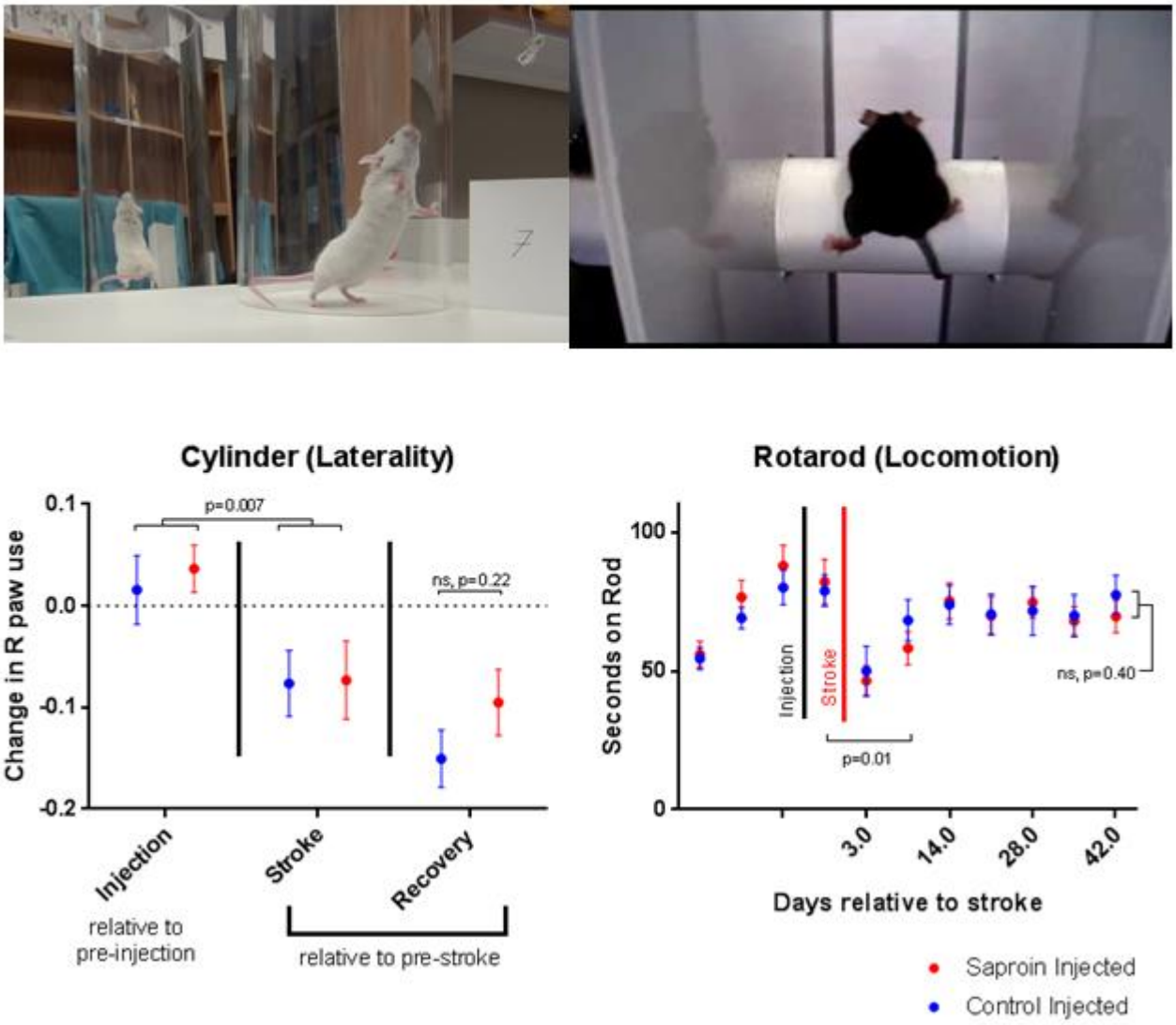


Figure 3
Classic motor assessments. Above: Pictures of animals performing in the cylinder (left) or rotarod (right) test. Below, left: change in paw use (right paw interactions with cylinder divided by total paw interactions) between pre- and post-injection, pre-and post-stroke, and pre-stroke post-recovery. Below, right: seconds of maintained locomotion on an accelerating rotarod before injection, before stroke, and during recovery.

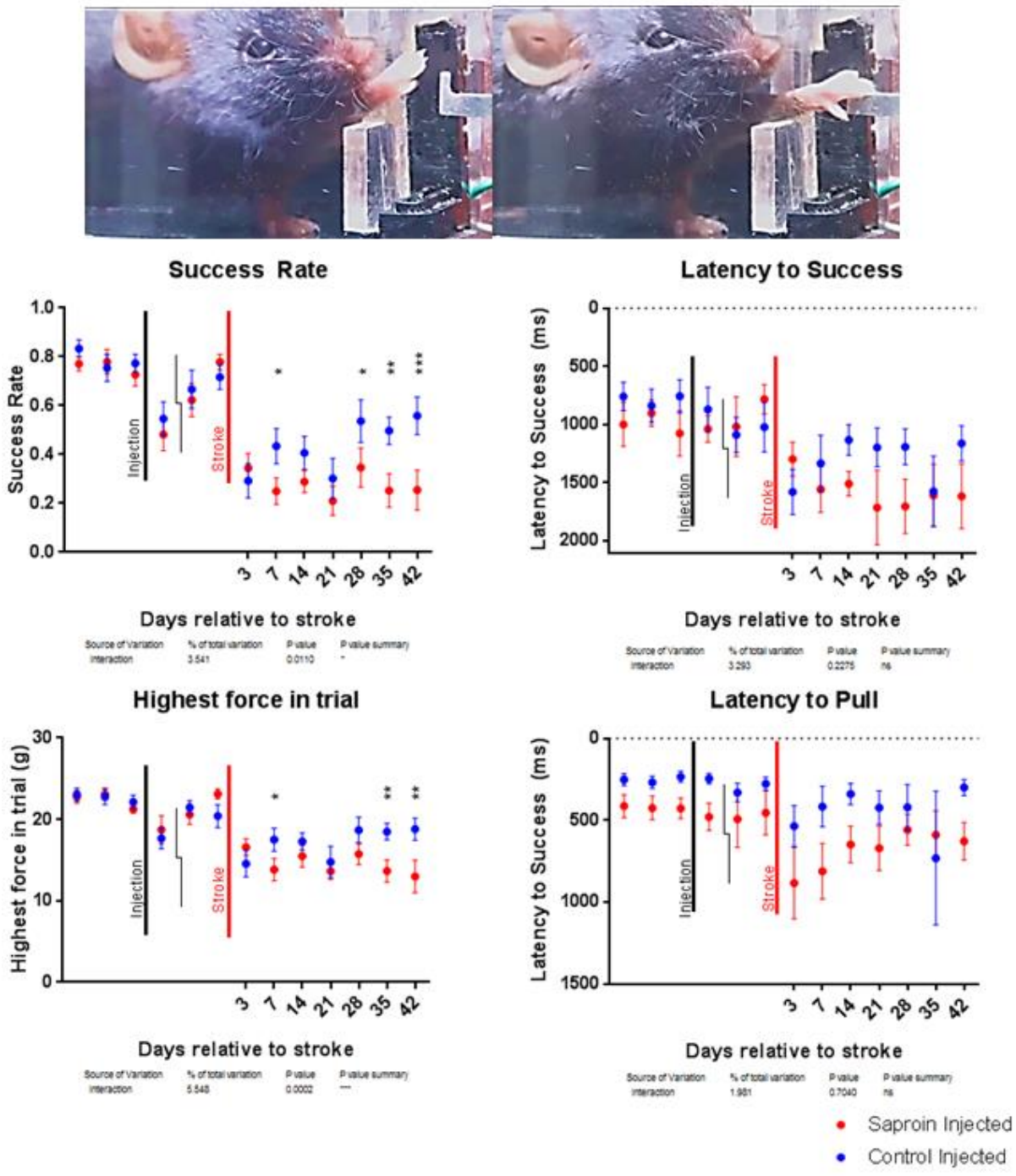


Figure 4
Performance data on automated reach task. Above: picture of reach task motion. Below: performance data graphs with Repeated-measures ANOVA analysis below x-axis label. Where ANOVA shows positive group-time interaction, multiple comparison t-tests were made for days 3-42 without correction (* = p<.05, ** = p<.001, *** = p<.0001)

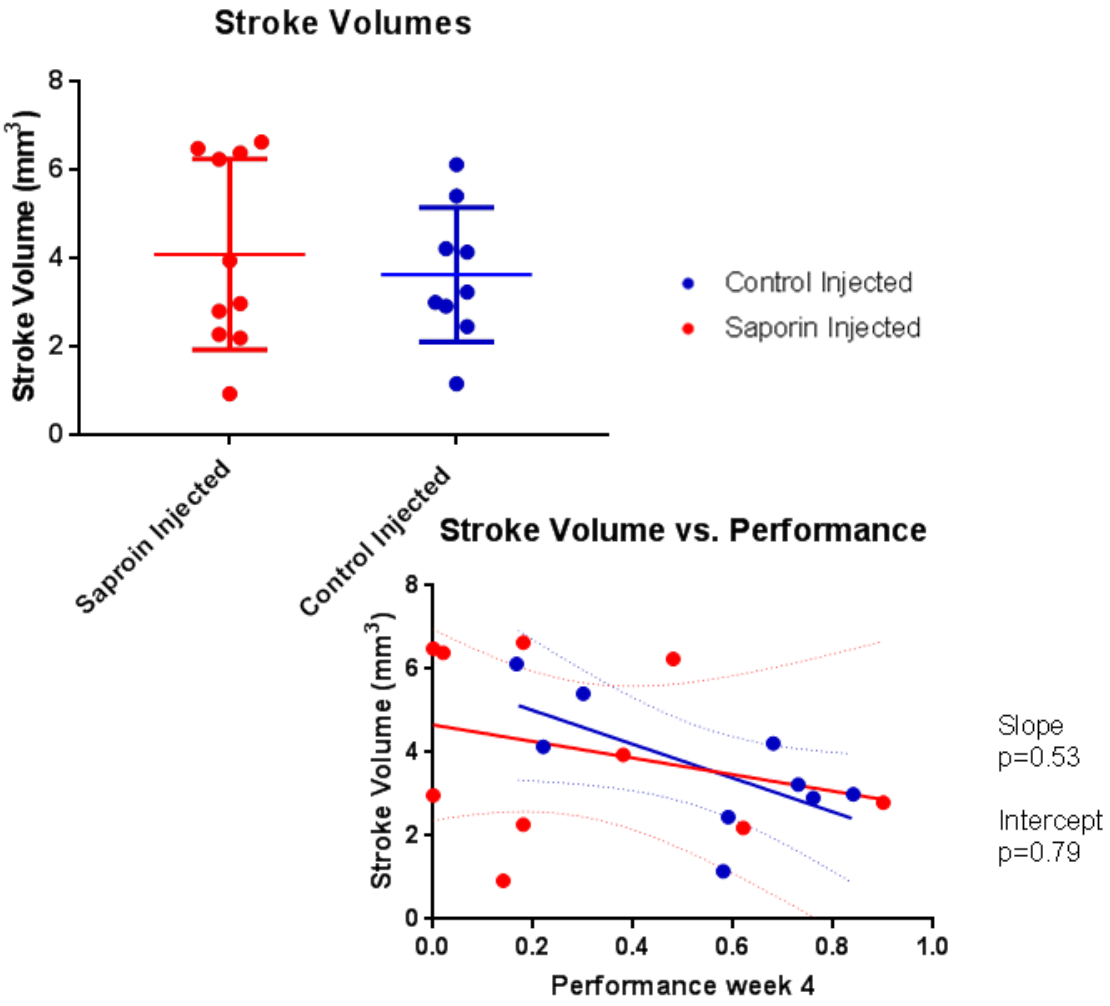


Figure 5
Stroke volumes and their relationship to recovery. Above: stroke volumes occupied roughly the same range between groups, with saporin injected animals in a more bimodal distribution. Below: the relationship between stroke volume and performance (proportion of pulls that were successful) was not different between saporin-injected and control-injected animals. Low-performing Saporin-injected animals (< 30% successful) included a balanced number of large and small stroke volumes.

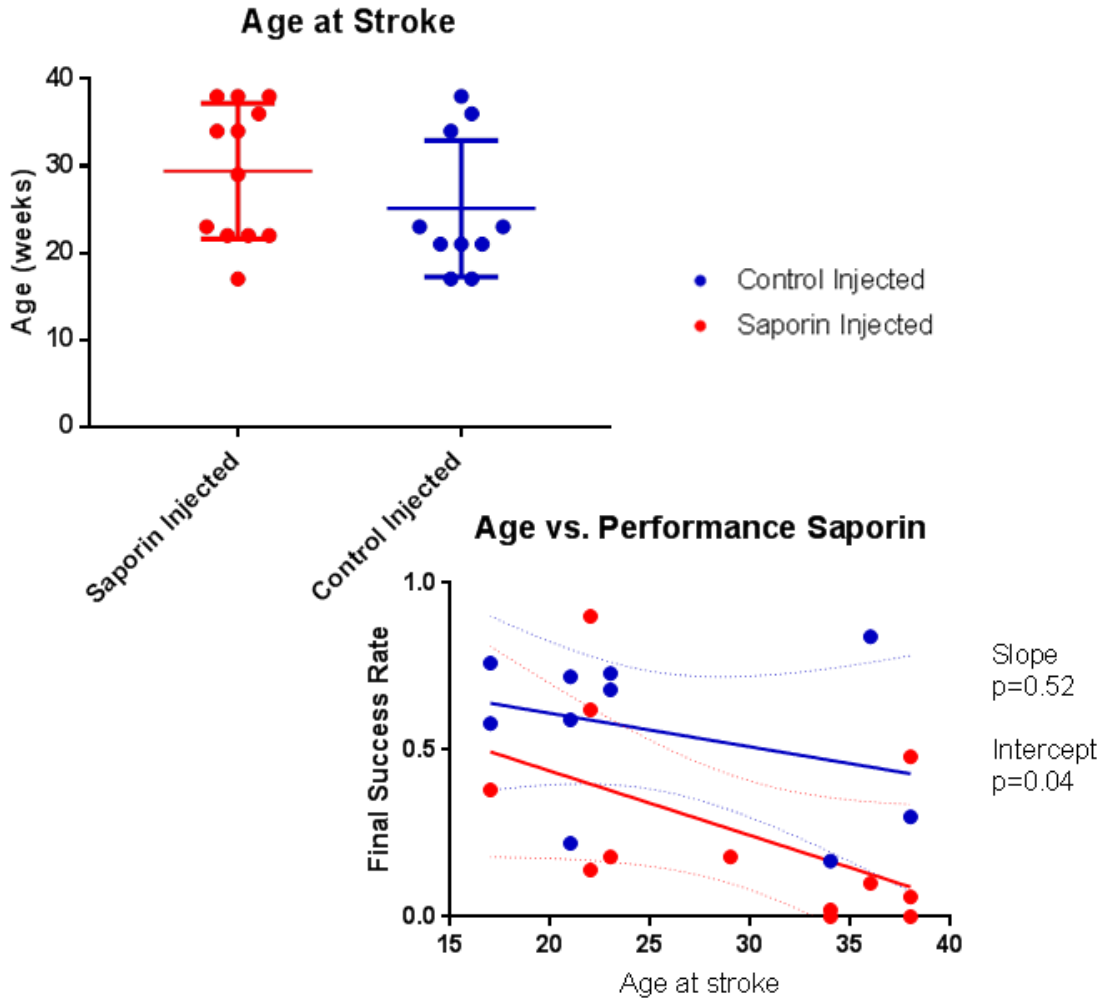


Figure 6
Age at stroke and its relationship to recovery. Above: both groups included the same range of pre-stroke ages, with the saporin group containing slightly more older animals. Below: the slope of the relationship between the age of stroke and final performance was not different between groups, but the intercept was; saporin-injected animals had a lower success rate than control animals across the tested age range.

CHAPTER SIX
Increasing Activity of Cholinergic Neurons in the Nucleus Basalis Increases the Extent of Recovery after Stroke

INTRODUCTION

In Chapter 4, I reviewed the reasons why the cholinergic projection system of the nucleus basalis constitutes a potential connection between guided plasticity and behavioral feedback signals. In the previous chapter, I established that the cholinergic projection system is necessary for behavioral recovery from stroke and discussed the reasons why this may be clinically relevant. I also proposed that this system may constitute a potential therapeutic target in ischemic stroke. In order to directly demonstrate such therapeutic potential, many approaches are available. As I discussed in the final section of Chapter 4, the cholinergic system may be augmented with pharmacological approaches or with electrical stimulation of the nucleus basalis. As previously explained, though, two other approaches, chemogenetic and optogenetic, carry major advantages over the former approaches in both their better potential elucidation of basic processes and in their ability to narrowly specify translational targets. I therefore used these tools to investigate the potential for cholinergic system augmentation in recovery from stroke.

Optogenetic tools have been developed that allow for precise spatiotemporal control of target neurons [169]. These tools involve genetically targeting light-sensitive ion channels to particular neurons and opening these channels using light delivered through fiberoptic implants. Chemogenetic approaches involve genetically targeting g-protein coupled receptors

to particular neurons. These receptors are engineered to be activated by an injection of the non-endogenous molecule cloazapine n-oxide (CNO) but not by any endogenous factor ([170], but see [171]). These g-protein coupled receptors can either increase or decrease the excitability of particular cells using a number of signaling pathways; they were engineered originally from muscarinic receptors [170].

I have developed the optogenetic approach to my question to a great extent, however this approach is currently still limited because chronic implantation of fibers together with other logistical considerations impose many opportunities for experimental error and failure that dramatically decrease the throughput of such an approach. The work to resolve these challenges is underway and can benefit future research requiring chronic implantation. However, the chemogenetic approach can produce faster results upon which future optogenetic experiments can build. For this report, I will briefly summarize the most important aspects of development in the optogenetic approach before presenting the chemogenetic experiment.

METHODOLOGICAL DEVELOPMENT

Expression of channelrhodopsin and verification of optogenetic cell activation

We bred two transgenic C57/Bl6 mice to produce mice that express channelrhodopsin in their cholinergic cells (Figure 1, top). One parent line has an insert that expresses cre under the choline acetyltransferase (ChAT) promoter. The other has a channelrhodopsin-EYFP

insert under control of the CAG promoter with a floxed stop cassette. When cre is transcribed, it excises DNA between flox sites, activating the chanelrhodopsin gene. We were able to confirm that animals heterozygous for each insert expressed channelrhodopsin in the desired pattern by co-localizing the EYFP tag with an immunohistochemical stain for choline acetyltransferase (Figure 1, bottom). A breeding scheme in which an animal with a homozygous insert of the channelrhodopsin gene is crossed with an animal with a heterozygous insert of the ChAT-cre gene produces offspring half of which express channelrhodopsin and the other half of which can serve as littermate controls who still contain the unexpressed channelrhodopsin insert (Figure 1, top).

In order to determine if the channelrhodopsin insert would allow us to control the activity of cholinergic NB neurons, animals received fiberoptic implants that would allow us to illuminate part of the NB with 473nm light. After 15 minutes of occasional trains of stimulation, the animals were sacrificed 90 minutes after light exposure and coronal sections stained for the immediate early gene, c-fos, which can serve as an index for recent activity. Under the fiber on the implanted side, cholinergic NB cells showed greater c-fos staining than the same cells on the opposite side of the brain (Figure 2, top).

To characterize the control that the channelrhodopsin afforded in these cells, we patched EYFP-positive basal forebrain cholinergic cells and monitored their membrane voltage potential during trains of light stimulation from a fiberoptic tip 500um away (thanks to Marina Maksimova and the Meeks lab for this work). Mice were anesthetized with isoflourane and perfused with artificial cerebrospinal fluid (aCSF), then decapitated into cold aCSF and brains extracted and sectioned coronally. EYFP-positive cells were clamped using

a glass electrode filled with cerebrospinal fluid. We found that action potentials could be invoked with precise timing based on patterns of light exposure (Figure 2, middle). We tested the efficacy of different frequencies and pulse widths to determine what parameters to use to maximally stimulate cells (Figure 2, bottom). No adaptation was detected in any cell after 24 trains of 20 pulses each as measured by a repetition of the parameters of the first train after the last was complete.

Expression of DREADD virus and verification of increases in activation

We used the same ChAT-cre animals for DREADD insertion that were used to breed the optogenetic animals. These animals, again, expressed cre in only cholinergic neurons. We then injected the nucleus basalis of these animals with an AAV8-hSyn-DIO-hM3D(Gq)-mCherry virus obtained thanks to the lab of Brian Roth. Injection of this virus caused expression of the m-cherry tagged receptor in cells that could be confirmed as cholinergic using a stain for choline acetyltransferase (Figure 3). Using test animals, we determined that we could achieve widespread infection of cholinergic cells throughout the nucleus basalis by making three 900nl injections along the anterior-posterior axis of the nucleus. This pattern of injection resulted in about a 35% infection rate of cholinergic cells throughout the nucleus, with targeted areas closest to the injections infected up to 50% (Figure 4).

We tested the functional effects of DREADD activation by unilaterally injecting one hemisphere's NB with DREADD virus, allowing 1 week for recovery and viral expression, injecting animals with CNO, then1 hour later placing animals in a novel homecage with

peanut butter spots; this novel environment and primary food reinforcers should activate NB neurons endogenously. Ninety minutes later, animals were sacrificed and coronal sections collected and stained for ChAT and c-fos. In cholinergic cells from the injected NB, c-fos staining was greater than those from the non-injected side. This effect was reliably replicable and clearly detectable (very statistically significant) over several animals (Figure 5).

METHODS

Subjects

Seventeen adult C57-Bl wild-type mice were used in this experiment. Ten experimental animals included 4 females and 6 males. Seven control animals included five females and two males. All animals expressed cre under the control of the choline acetyltransferase promoter (B6;129S6-Chat$^{tm2(cre)Lowl}$/J breeding line obtained from Jackson Laboratory Stock 006410). Two control and one experimental animal (all female) also had a channelrhodopsin transgene (B6;129S-Gt(ROSA)26Sor$^{tm32(CAG-COP4*H134R/EYFP)Hze}$/J Jackson Laboratory stock 012569), which was inert and neutral to this experiment. At the time of stroke, each group ranged in age from 12-38 weeks old. Homecages were kept in a temperature and humidity maintained facility on a reverse light cycle to assure that behavioral testing would occur during normal waking hours. All mice had food and water available to them ad-libitum in their home cage and also in their reach chambers if subjected to long sessions. All procedures involving these mice were approved by the UT Southwestern Institutional Animal Care and Use Committee.

Automated Reach Training

All animals were trained in the automated reach chamber using the same procedure described in Chapter 1. After animals reached a stable baseline performance, they underwent injections of either DREADD of GFP virus (details below). After recovery, animals were allowed to reach a stable performance in baseline before receiving photothrombotic strokes.

Cylinder

Animals were tested for laterality of paw use using the cylinder test before stroke, at day 3 after stroke, and at week 7 after recovery. Animals were placed in the cylinder and remained until at least 10 rears were performed. High-speed video of the reach session was then used to score paw interactions with the cylinder using the same criteria as Shallert et al. [41].

Injection

Injection procedures were identical to those in Chapter 5, with the following exceptions. Mice were injected with a cre-dependent DREADD virus (AAV8-hSyn-DIO-hM3D(Gq)-mCherry, obtained thanks to the lab of Brian Roth) or a GFP virus (AAV8-CMV-GFP), both obtained from UNC Vector Core. The DREADD virus contained a double-

floxed antisense copy of the hM3D Gq receptor under control of the human synapsing promoter that will be flipped to sense and expressed in the presence of cre. The experimenter was blind to the identity of the virus during injection. The coordinates of injection was 1.7mm lateral to bregma, 4.7mm below the surface of dura, and .2, .3 and .4 mm posterior to bregma (3 injections per side, 6 total). Each injection consisted of 900nl of virus (approximate titer, 4×10^{12}).

Stroke

Stroke was induced surgically according to the same procedure as in Chapter 2.

Recovery

After stroke, animals were allowed to recover for at least 2 days with wet food available. On day 3 and day 7, they were allowed to try approximately 50 to 100 pulls in the reach chamber to assess post-stroke functional deficit. Starting on day 14 and every 7 days until week 6, animals were injected in the morning with 10mg/kg cloazapine-n-oxide (CNO) suspended in 1.2% DMSO and saline. Approximately 1 hour later, they were placed in the reach chamber and allowed to pull approximately 150-200 times. For approximately the first 50 pulls, the criteria for success was 20g as in baseline and post-stroke testing. If the animal's reinforcement rate for those 50 pulls was approximately 25% or less, the animal was changed to an adaptive criterion where the force requirement for reinforcement was 2g for the first 15

pulls, then equal to the rolling median of the past 15 pulls. If an animal could not reach the target pull number within ~6 or 7 hours from the time of injection, they were placed back in their homecage and allowed to try again on the following day. After the 6-week recovery, on week 7, animals performed a final reach session without a CNO injection to assess post-stroke recovery.

Sacrifice, perfusion and histology

After their final behavioral tests were complete, animals were injected subcutaneously one last time with 10mg/kg CNO. One hour later, they were placed in their normal reach chambers with the reach handle absent. Peanut oil deliveries occurred randomly every 5 to 25 seconds for 15 minutes. After the 15 minutes were up, each animal was transferred to a new homecage with various enrichment items: five marbles, one half of a plastic mouse tube, and peanut butter spots located around the cage and on objects. Mice had access to water in this new cage. They remained in this cage for 1.5 hours until they were sacrificed with an overdose of isothesia. Each mouse was then perfused and sectioned using the same procedure as described in Chapter 5.

Tissue samples from each animal was mounted on two slides for staining, with the first section of each slide starting at approximately 1.36mm anterior to bregma and every 12th subsequent section (360µm spacing) for a total of 12 sections. Sections were allowed to adhere, and rehydrated with PBS. Sections were then incubated in .3% triton-x in PBS for 2 hours at room temperature, then .1% triton-x and 10% bovine serum albumin for another 2

hours. The two slides for each animal were used to confirm viral infection and to probe for c-fos activation.

Slides for evaluating viral infection were rinsed thoroughly with PBS and incubated overnight for two nights in the dark at 4 degrees C in PBS with 10% BSA and 1:100 goat anti-choline acetyltransferase (ChAT) antibody (Millipore catalog #AB144P). On the second day, slices were rinsed thoroughly with PBS and washed with PBS for 10 minutes. Sections were then incubated in PBS with 1:500 donkey anti-goat alexa488 conjugated secondary antibody at room temperature for 2 hours. Slices were then washed with PBS for 10 minutes three times. Slides were coverslipped with Flouromount-G (Southern Biotech) and stored in the dark at 4 degrees until visualization.

The slides for visualizing c-fos expression were rinsed thoroughly with PBS and incubated overnight for two nights in the dark at 4 degrees C in PBS with 10% BSA, 1:100 goat anti-choline acetyltransferase (ChAT) antibody (Millipore catalog #AB144P) and 1:100 rabbit anti c-fos antibody (Abcam ab209794). On the second day, slices were rinsed thoroughly with PBS and washed with PBS for 10 minutes. Sections were then incubated in PBS with 1:100 donkey anti-goat alexa488 conjugated secondary antibody for experimental animals and 1:100 Donkey anti-goat cy3 conjugated secondary antibody for control animals at room temperature for 2 hours. Different secondary antibodies were used to avoid overlap with the spectrum of the endogenous flourophores of the virus. Slides were then washed again and incubated for one hour in 10% Natural Goat Serum (NGS) in PBS, then for 2 hours in 1:500 Goat anti-rabbit biotinylated secondary antibody in 10% NGS. One half hour before the end of this 2-hour incubation, a Vectastain Elite ABC Kit (Vector Laboratories) was used

to prepare a working solution of ABC reagent. This solution was vortexed for the majority of the final 30 minutes of the secondary antibody incubation. Slides were then washed again with PBS and incubated in the ABC reagent for 30 minutes. They were then washed in PBS several times and washed facedown on a slow agitator for 30 minutes in a large volume of PBS. Slides were then incubated in Tyramide Signal Amplification Plus Working Solution with Cy5 labelled tyramide (TSA plus fluorescence kit, Perkin Elmer) for 12 minutes, rinsed again in PBS for 3x10minutes and coverslipped with Vectashield. Slides were allowed to dry in the dark at 4 degrees overnight until visualization.

Histological visualization and analysis

To confirm viral infection, slides were scanned at 40x with an Zeiss Axioscan Z-1 slide scanner set for Cy3 and GFP or Alexa488 and m-Cherry filters. Images were visualized to confirm the expected flourophore expression.

To measure c-fos fluorescence in cholinergic cells, slides were scanned with a Zeiss LSM780 Inverted Confocal Microscope to detect Cy5 and Alexa488 or Cy3. Both NBs of each section were visualized entirely at 25X magnification. Using ImageJ freeware, scans were then separated by color and the ChAT-stained channel despeckled and thresholded so that the soma of the stained cells were clearly delineated. The analyze particles feature with a lower limit of 100μm^2 was used to make an outline of these cells, and that outline was overlayed on the Cy5 image. The mean grey value of the c-fos signal within each of these

cell outlines was measured and normalized by subtracting the signal from the average of three on-tissue areas on the same slide that clearly included no positive staining.

To establish a control that would verify that the c-fos staining was working properly, wild-type animals were injected with high levels of sodium chloride as in Sharp et al. [172] and sacrificed 90 minutes later. Other animals were sacrificed with no injection. This procedure causes excitation of cells of the paraventricular nucleus that modulate salinity homeostasis [172]. For these animals, staining was conducted identically as with DREADD experimental and control animals except that the step of incubation with ChAT antibody was conducted with no antibody in the blocking solution. After visualization, cells from the paraventricular nucleus that were visibly c-fos positive were identified and c-fos staining in their soma quantified in the same manner as ChAT cells in the other groups.

Once c-fos expression analysis was complete, slides were scanned with a Hammamatsu Nanozoomer slide scanner using the TxRed filter cube. Autoflourescence on this tissue showed a clear delineation between healthy and dead/missing tissue near the infarct. For the first slice of each slide and every other slice up to slice 12 (a space of 720 μm), the healthy tissue of the stroke- ipsilateral and contralateral hemispheres were outlined and their area determined using the NDPview software associated with the Nanozoomer using the same procedure as with cresyl violet stains in Chapter 5. The area of the stroke hemisphere was subtracted from that of the healthy hemisphere and the result multiplied by the 720 space between samples. These volumes were added together to obtain an indirect measure of approximate stroke volume between 1.36mm anterior and 2.96mm posterior to bregma.

Behavior Data Analysis

To confirm post-stroke deficit, a t-test was performed for each group on success rate in the reach task on day 7 after stroke vs. the average of the final 3 sessions of baseline (Figure 5). The deficit was calculated for each animal in each measurement as the difference between baseline average and the worst performance of either day 3 or day 7. To test for differences in recovery, the proportion of the post-stroke deficit recovered on week 7 was compared between the groups using an unpaired t-test.

RESULTS

Induction of stroke, deficit, and verification of infection

Photothrombotic stroke was successfully induced in all animals, and all but one animal showed functional deficits after stroke (Figure 6). The animal without functional deficits is not included in recovery data or analysis. Two additional DREADD-injected animals showed no m-cherry expression in their NB; these animals are also not included in recovery data or analysis.

Recovery

In the automated reach task, the performance of both DREADD-injected and control animals increased in variability between weeks 2 and 6, which were the weeks that they were injected with CNO. It is possible that though the CNO should have no biological impact outside of DREADD receptor activation, in fact some subtle effect may have influenced behavioral performance during this period [171]. Both groups still experienced similar amounts of weekly practice and reinforcement (Figure 10) however their behavior during this time is not shown here since it is so disordered. On week 7, with no CNO injection, performance on the automated reach task was biologically but not statistically different between the groups (Figure 7).

After stroke, group data indicates a small and nonsignificant decrease in right paw use as assessed by the cylinder test. After recovery, neither group increased their right paw use, and groups were not different in cylinder tests measurements at any point (Figure 8).

Post-experimental confirmation of activation

Based on pre-experimental tests, it was expected that animals in the control group would have a consistently lower level of c-fos staining in their NB cholinergic cells compared to the DREADD infected group. Animals in the DREADD group with c-fos staining that did not significantly differ from control animals were to be eliminated from the group. However, unexpectedly we discovered that as many animals in the control group

showed elevated c-fos fluorescence in NB ChAT-positive cells as did in the experimental group, so no animals were eliminated based on c-fos fluorescence. It is possible that both groups simply reached a similar range of basal activation because of other variables affecting NB activity. No matter the group, though, animals with higher c-fos staining in NB cholinergic cells tended to recover better (Figure 9)

Post-hoc analysis of the relationship between recovery and variables of interest

Groups were counterbalanced intentionally for reinforcement and practice between the groups and the differences that did occur in these variables did not predict differences in recovery (Figure 10). The two animals in the control group that showed both unexpectedly good recovery and unexpectedly high c-fos staining were different from others in either group in that they were trained for significantly longer (Figure 10, middle). For all animals except these two in either group, no apparent correlation between recovery and length of training is discernable, however such a correlation is seen in the control group with these animals added (Figure 10, bottom).

DISCUSSION

Results of this experiment indicate that augmenting NB cholinergic activation may cause an increase in stroke recovery (Figure 7). The group sizes were not adequate to assess a statistical difference between groups ($p=0.38$, $n=15$). This is an ongoing project and

additional animals are under study. While there was no statistically significant difference between groups in recovery, the separation of means was biologically significant. The variability within groups that contributed to lack of statistical significance may have been due to the variation that the DREADDs had in their activation of cholinergic neurons. C-fos staining indicates that the virus may have failed to increase excitability in target cells for some animals. It is also possible that the receptor was indeed increasing excitability in a similar fashion across subjects, but that other variables (perhaps related to pre-training or the stroke itself or other influences) also modulated excitability of cholinergic cells in both experimental and control groups. In the control group, there were only two animals that recovered well after stroke, and these two animals also happened to have high activation of NB cholinergic neurons. Animals in the DREADDs group that did not recover well often also had lower fos staining in the NB; in fact NB staining and recovery were correlated (Figure 9).

In addition to having high levels of c-fos staining in NB cholinergic neurons, the two animals who recovered well in the control group spent longer in training and baseline than other animals. One intriguing but entirely speculative explanation for these results might be that pre-training in this task may make the behavior more easily recovered afterward by making the NB more responsive to the stimuli relevant to the task. Such a speculation could form the basis for further study.

More animals are required to determine if the trend seen in this data are nonrandom and replicable. If so, and if more animals do not change the average recovery of the DREADDs group, then it may be important to note that the improvement seen did not constitute a total

groupwide recovery. This means that some measurement range remains to explore the question of if similar increases in activation of other modulatory systems might combine to cause a larger effect. Given the complexity of modulator interactions in the brain, which I will expound upon in the next chapter, it would not be surprising if combined stimulation had an additive or even nonlinear effect on both the extent of the difference and the variation within groups.

FIGURES

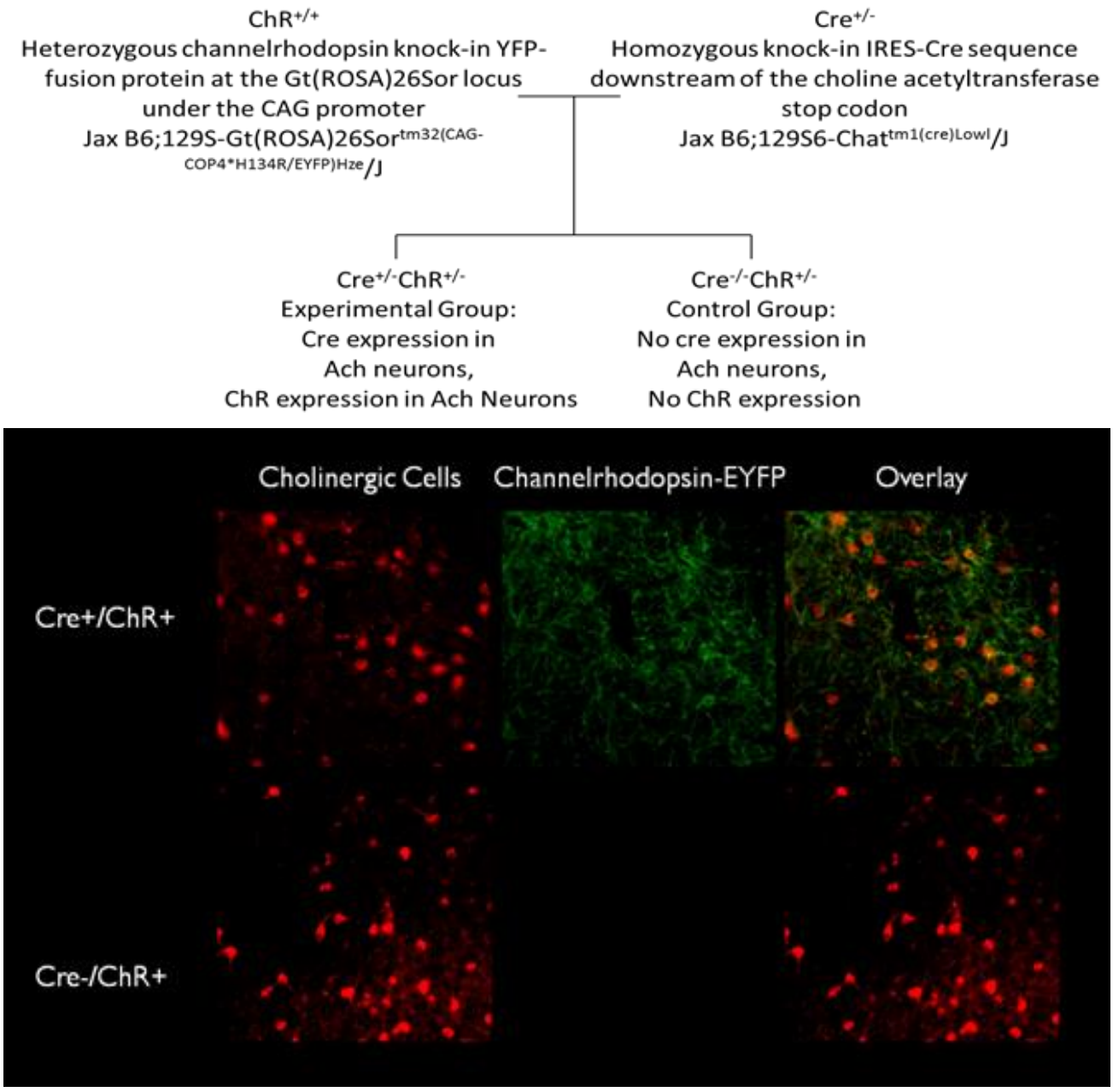


Figure 1
Breeding scheme (top) showing the production of animals with channelrhodopsin expressed in cholinergic cells and control littermates. Both groups express cre. Immunohistochemical staining for cholinergic cells (anti-choline acetyltransferase, red) co-localized with cell bodies visible via the EYFP transgene as expected only in one genotype (below).

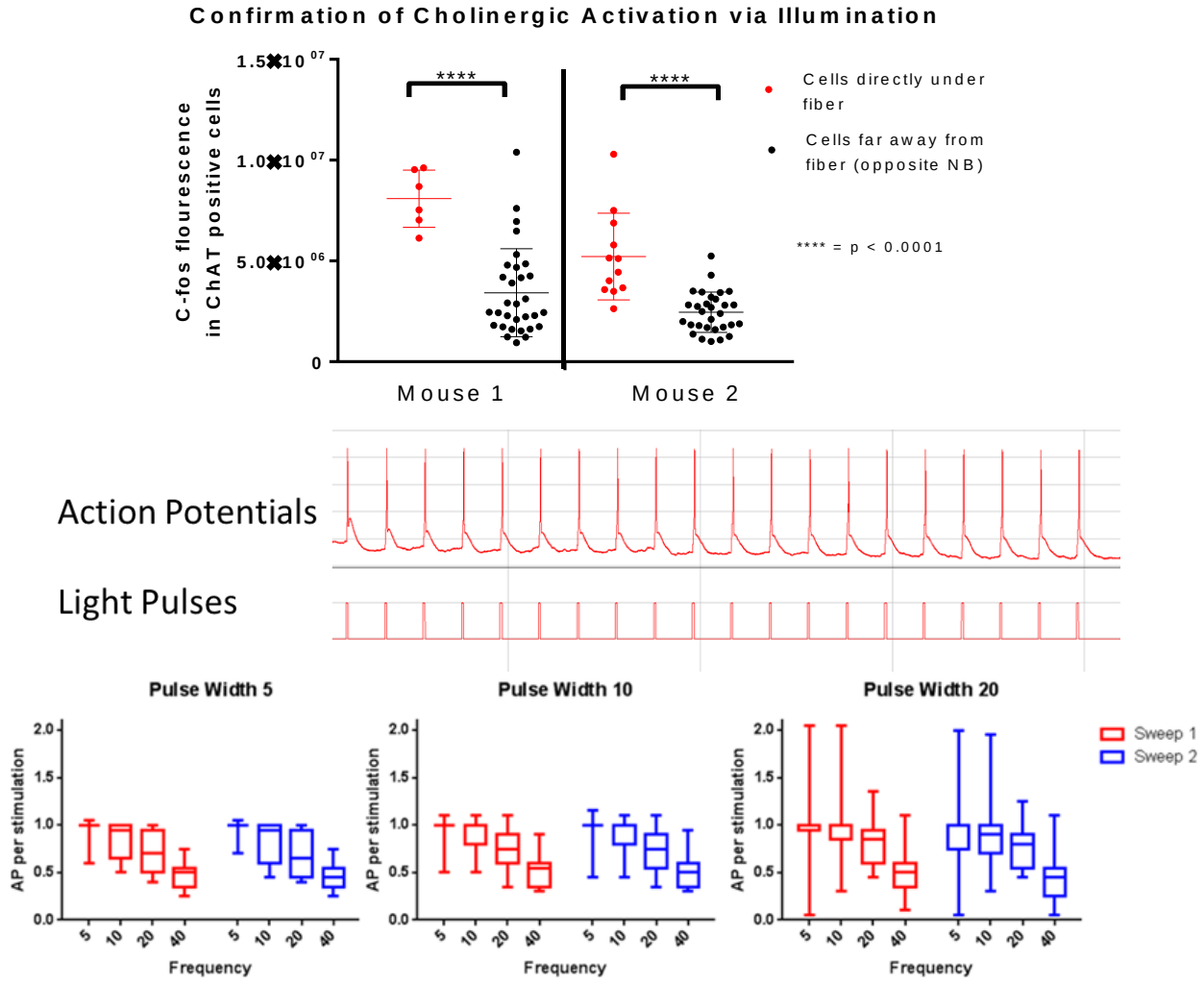


Figure 2

C-fos staining confirmed that cholinergic cells under the fiber were more active than those in the opposite NB (top). Recording from a single eYFP-positive cell while exposing it to light pulses shows clear control of action potentials using light (above). Slice recordings from eYFP-positive NB neurons (n=11) from multiple animals (below), exposing each cell to two trains of 20 pulses at each combination of pulse widths 5, 10, and 20ms and frequencies 5, 10, 20, and 40 Hz.

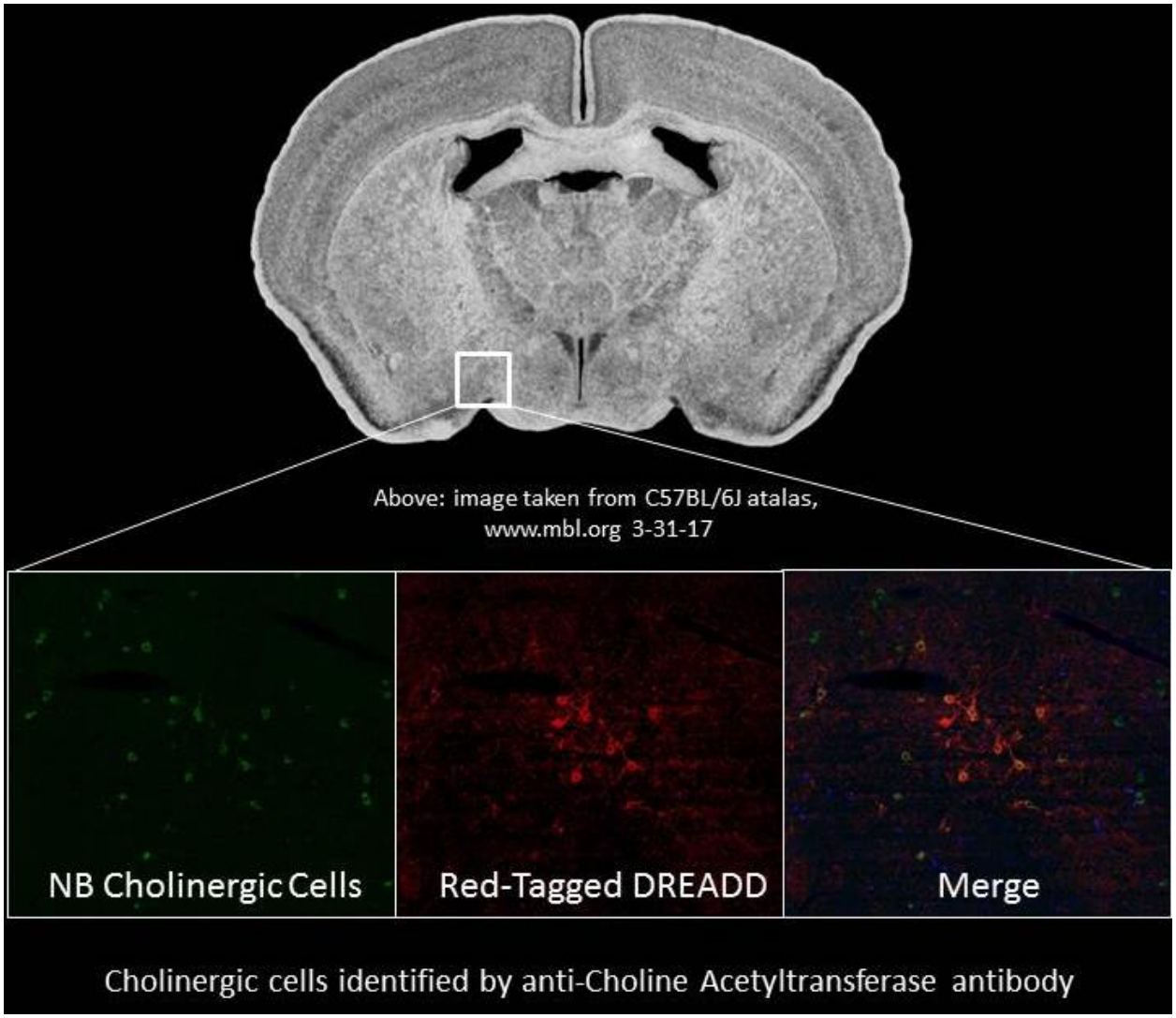
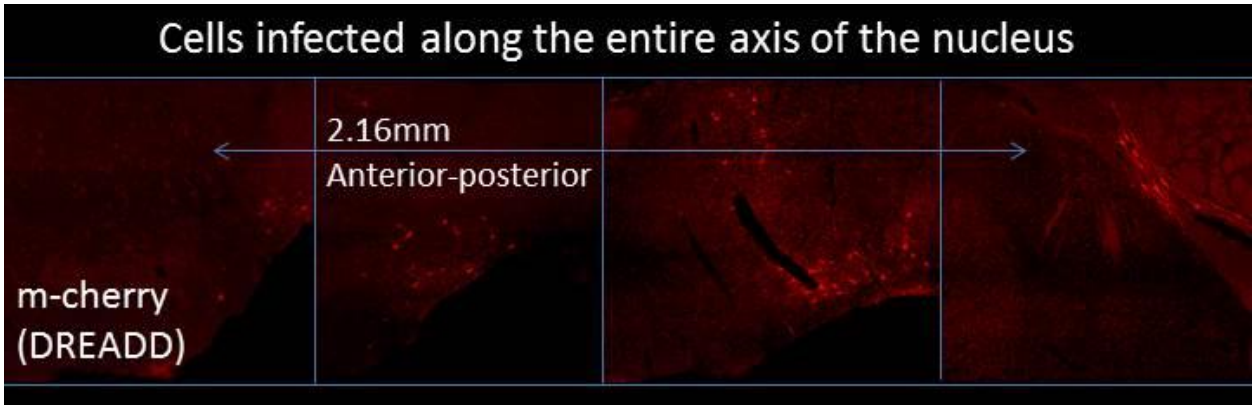


Figure 3
Injection of DREADD virus into nucleus basalis resulted in identification of m-cherry DREADD tag one week post-injection (red). Red tag co-localized with cholinergic cells identified via immunohistochemical stain for choline acetyltransferase (green).



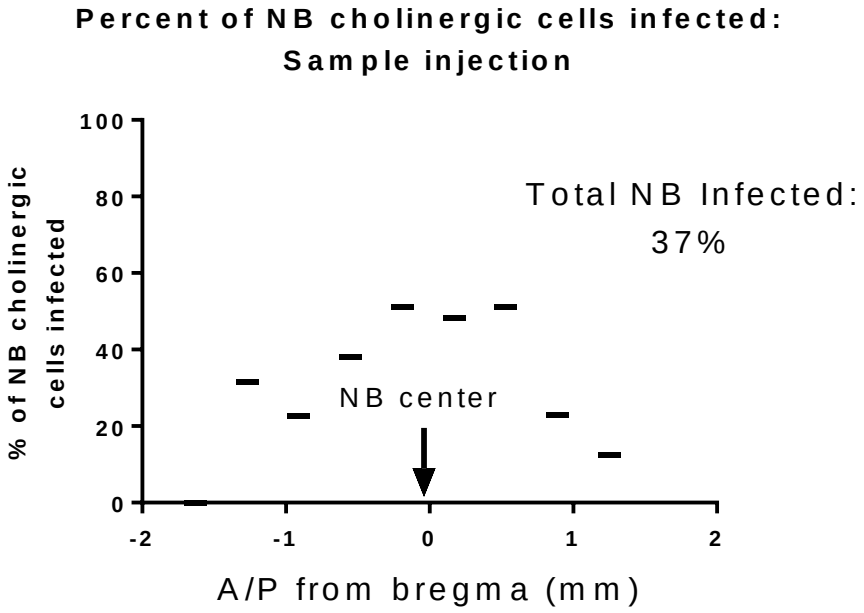
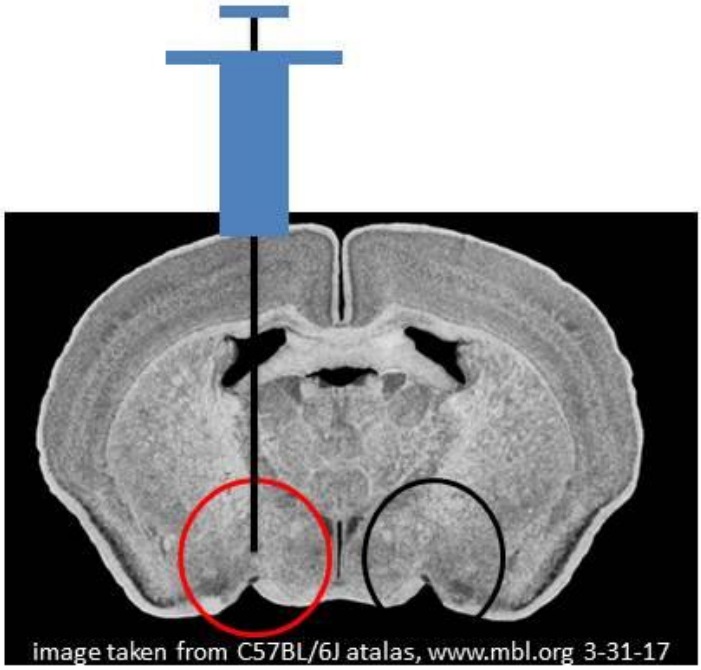


Figure 4
Above: Three separate injections of virus along the anterior-posterior axis of the nucleus resulted in expression throughout the nucleus. Below: Percentage of NB cholinergic cells in sample coronal sections of an injected animal.



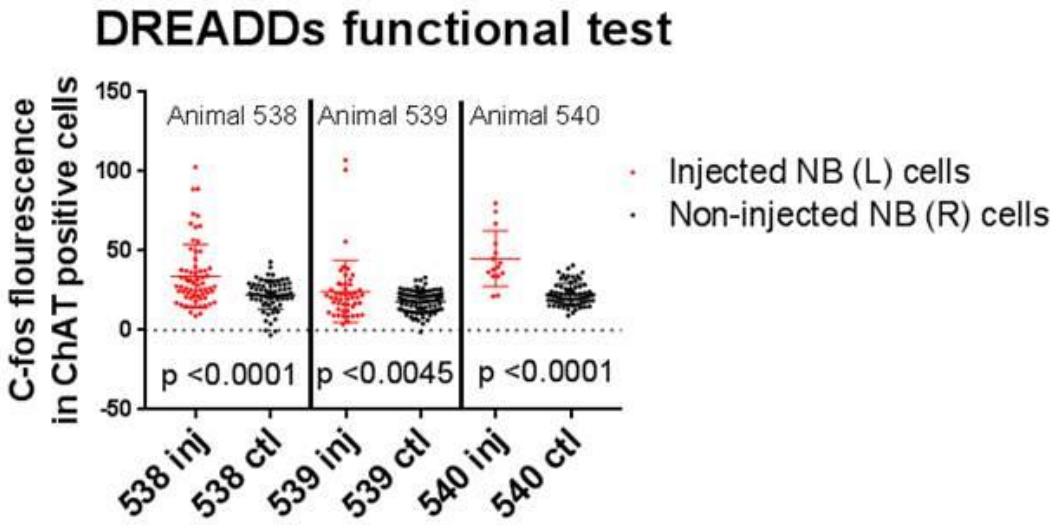


Figure 5
Animals injected unilaterally with DREADD virus consistently showed greater intensity c-fos staining in NB cholinergic cells on the injected side compared to cholinergic cells in opposite NB.

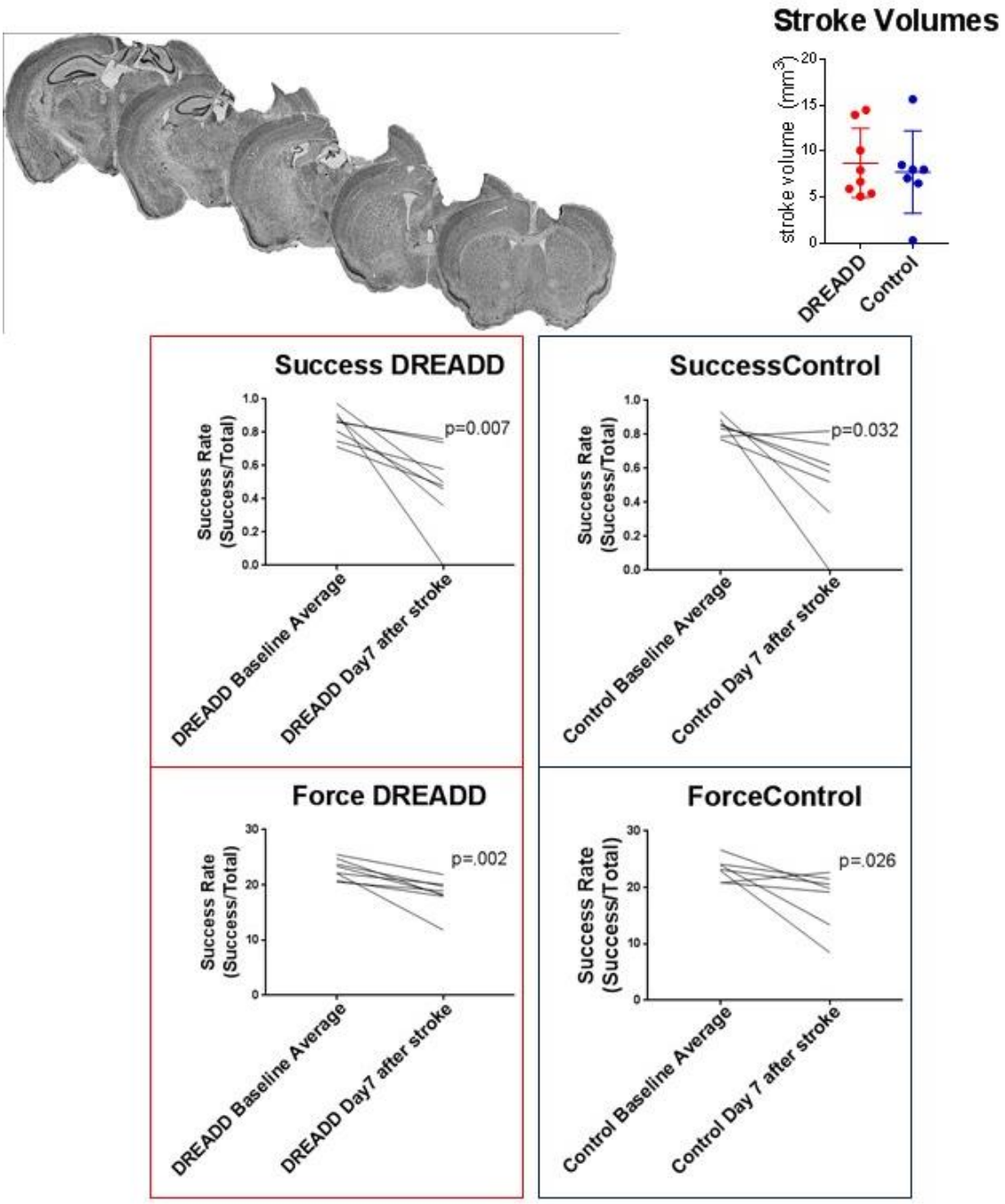


Figure 6

Photothrombotic stoke was successfully induced in both groups, causing a significant functional impairment 7 days after stroke in both primary outcome measures. Above, left: sample cresyl violet stain of coronal sections from an animal with phothtrombotic stroke induction. Above, right: infarct volumes from both groups. Below: functional deficits induced by stroke.

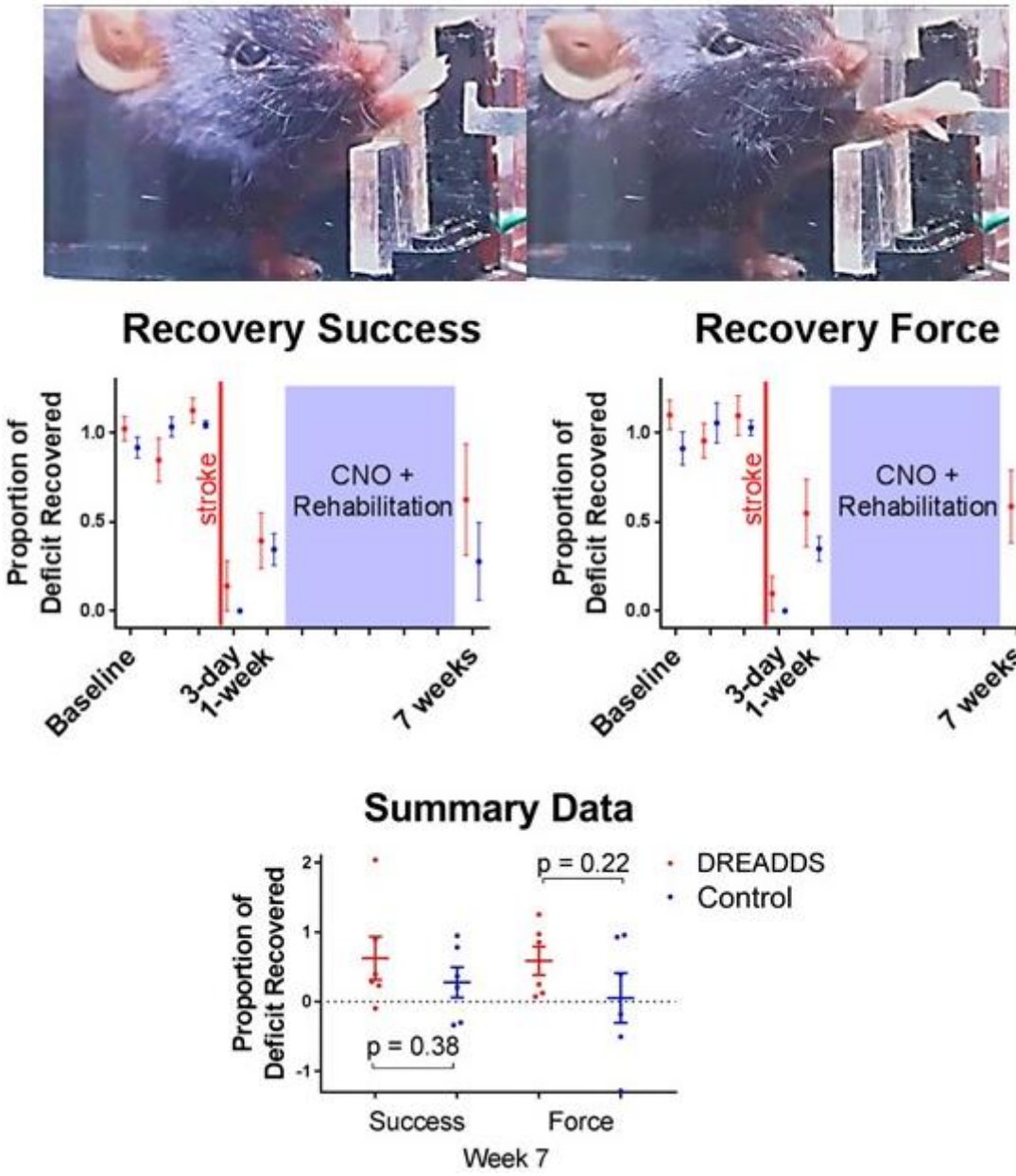


Figure 7

Post-stroke recovery for animals in the automated reach task. Above: pictures indicate the reach-grasp motion involved in the task. Middle and bottom: proportion of total deficit (the difference between baseline average and the worst performance of either 3 days or 7 days) recovered at 7 weeks showed a biologically significant difference in means with high individual variance and no statistical significance.

Figure 8
Post-stroke recovery for animals in the cylinder task. Left: an animal rearing in a cylinder. Right: after stroke, a non-significant shift in paw use away from the affected (right) limb did not change on average after recovery.

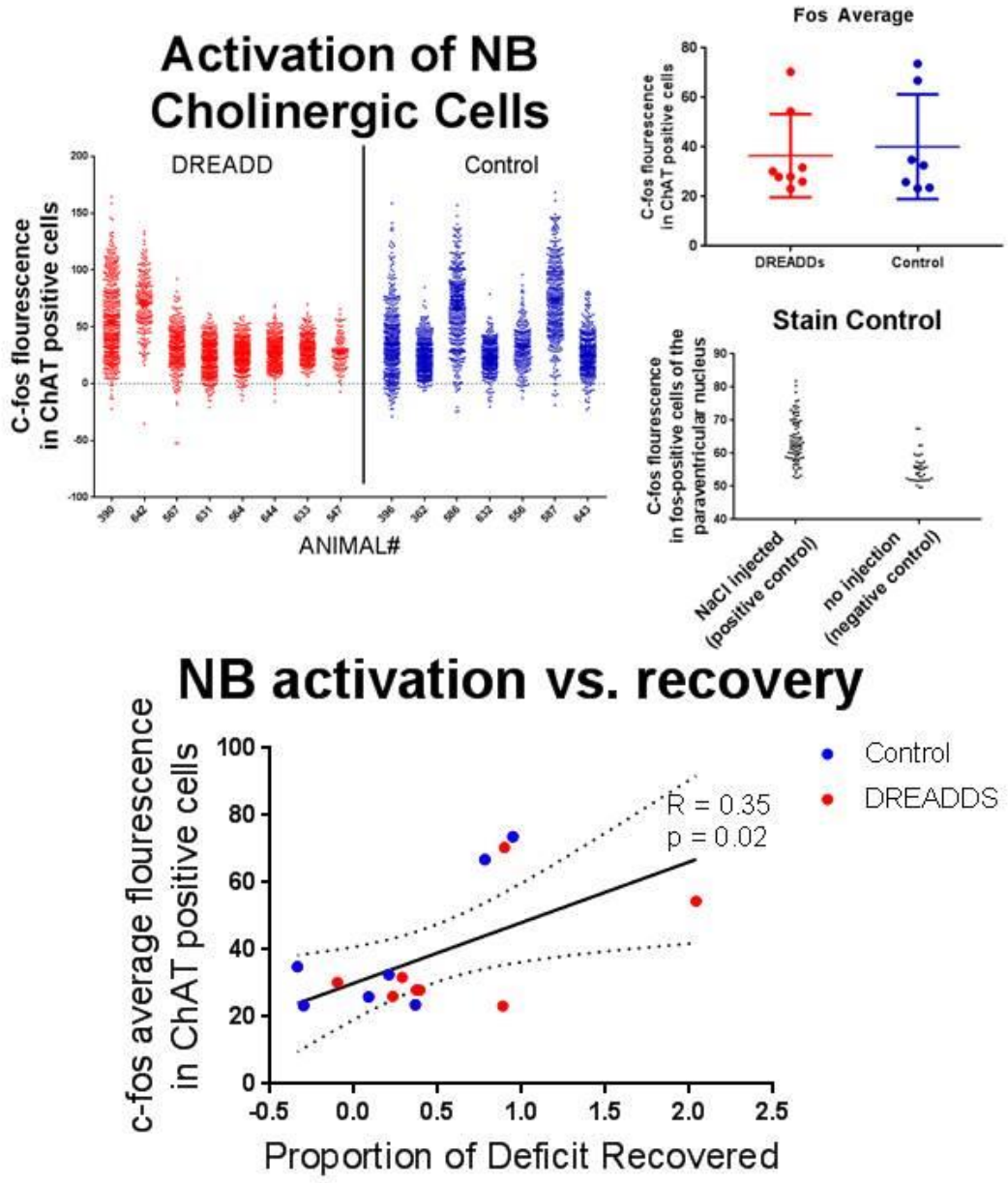


Figure 9
Above, left: c-fos staining from each quantified cell from the NB of each animal show clear differences in activation, but not group-consistent differences as expected. Above right: no difference existed between the groups in c-fos staining on average. The stain itself was valid; increase in c-fos staining was detected in paraventricular nucleus (the location of salinity homeostasis-modulating cells) in response to NaCL injection. Below: NB activation was related to recovery.

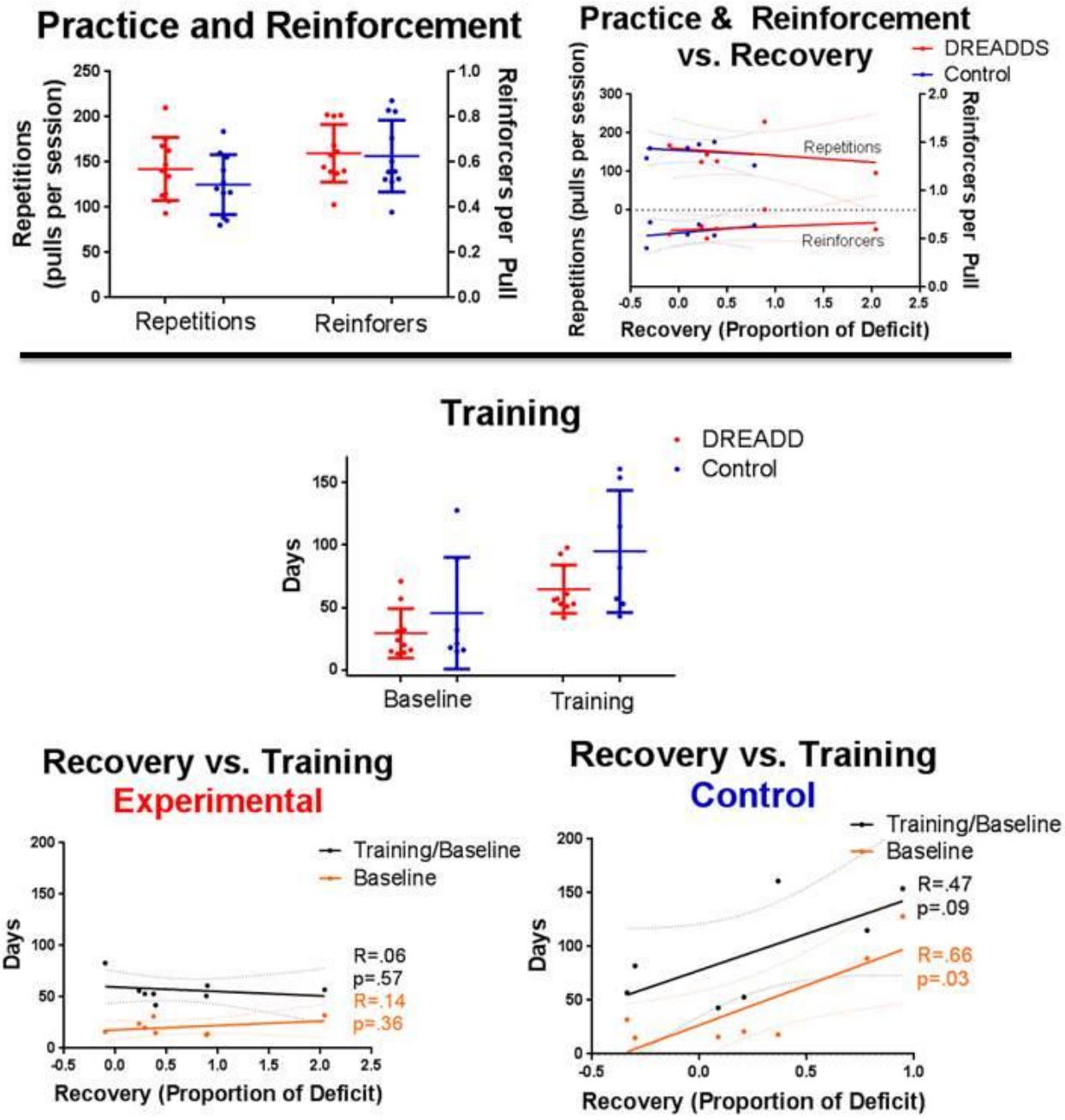


Figure 10
Above, left: no differences between the groups existed in practice or reinforcement rates. Above, right: variation in neither practice nor reinforcement rate was related to recovery. Middle: an inadvertent bias in pre-stroke training came about with the inclusion of two animals in the control group that were trained for significantly longer than any of the others

in either group. Below: in the experimental group no relationship existed between training time and recovery, but the inclusion of two overtrained animals in the control group created a correlation that might indicate a ~100day overtraining threshold for post-stroke effects.

CHAPTER SEVEN
Expanded Discussion and Future Directions

FURTHER CONSIDERATIONS IN THE DEVELOPMENT OF BEHAVIORAL ASSESSMENTS

Natural behavior might be screened in search of a better assessment

Our automated system represents an important advance in functional behavioral assessment after cortical stroke, but a few clues indicate that it can be further improved. The task, like many other explicitly trained reaching tasks, is less affected by cortical stroke when trained for a long duration before stroke, while human forelimb deficits follow no such pattern. As mentioned in Chapter 1, functional assessments might possibly be improved by finding forelimb tasks that do not need to be acquired via explicit training from the experimenter, but instead are "overtrained" in the natural course of the animal's development, and which still experience long-lasting deficit in response to cortical stroke. Even reach-and-grasp food tasks may not qualify under this category; these tasks also require training for stable performance before injury and induce cortical plasticity when trained [103] typical of a newly acquired motor pattern.

It may be possible to screen widely for species-specific forelimb behavior that naturally differs in a long-term time course after stroke: behavior folded into the subtle environmental contingencies of the mouse's everyday environment. If a way can be found to gather large volumes of data about forepaw motion patterns in the home cage, such patterns could be

monitored before and after cortical stroke to see what patterns change over extended timeframes. Such an approach carries three natural advantages. First, naturally learned behaviors will not involve explicit training investment since they will be acquired during the course of development. Second, the widely incorporated nature of the behaviors will align the degree of habitual performance with human case and provide more human-like post-stroke extra-rehabilitative practice opportunities in everyday life. Third, since the requirements of the screen would be longevity of perturbation, the original gold standard of our models will be already achieved. One technology currently available that could help in performing such a behavioral screen is motion capture technology. With the use of multiple cameras in the homecage and big data analysis methods, optical information about movement alone can be gathered and analyzed, and particular focus can be paid to forelimbs using visible markers (i.e. similar to Jhuang et al. [173]). Consistent patterns of motion can be extracted and screened for changes after cortical ischemic stroke. Depending on what is discovered, new methods for prompting and measuring the behavior may then be developed.

Automating the search for such behaviors could expand the search for a stroke-specific task beyond the perceptual biases of even the most experienced human observers. Humans, like mice, are animals subject to species-specific stimulus salience. Unless the behavior of other animals reliably predicts a threat or an established human-relevant resource, we are less likely to respond to it [174, 175]. This is likely a reciprocal phenomenon; it is possible that mice may not easily notice that a human has trouble buttoning a shirt, although they likely read our body language better than we can when it comes to predictors of picking them up. In short, much of what mice do that may be affected by injury may escape our radar since if it

has no historical importance to us, it also lacks salience. Using technological filters to get around our own perceptual biases constitutes a path toward finding naturally perturbed mouse forelimb behavior for future stroke model development.

Models might be improved by simulating behavioral context and dynamics

Implicit within stroke research and within the larger field of neuroscience lies an understanding of the significance of pre- and post-stroke environmental variables on brain injury [53, 176]. Yet while direct teaching and learning may be recognized as an important factors to investigate in modelling recovery, the particular structure of non-explicit behavioral functions are less frequently considered as potentially meaningful contextual variables in rodent models. To develop treatments that augment post-stroke learning with predictive validity may require more nuanced modelling of the complex dynamics that underlie that learning, which are often absent in rodent models of functional recovery. This constitutes yet another opportunity to improve the modelling of functional injury in stroke; if the pre- and post-stroke behavioral dynamics experienced by recovering humans are better recapitulated in the rodent, it may improve the validity and translation of new pharmacological, stimulation nor cell-based interventions. Below I will discuss a few points of behavioral dynamics that are rarely considered in rodent modelling of stroke recovery which may prove useful in developing more nuanced modelling.

Neither reinforcement nor learning are necessarily adaptive or desirable

As discussed in previous chapters, reinforcement can produce maladaptive behavior as well as adaptive; it need only be contingent on some other form of behavior than that desired. For example, the response cost of everyday tasks can be increased for a myriad of reasons after stroke including loss of muscle coordination, spasticity, or weakness. This might produce a negative reinforcement contingency on using other limbs for everyday tasks, which would in turn decrease opportunities for exercise and rehabilitative feedback on the affected limb. This would cause further atrophy and intensify the difficulty of using the limb, leading to a cycle of further functional loss. This point is acknowledged in research on constraint-induced movement therapy. Yet even if conditions are constrained so that the affected limb must be used, the changed condition of that limb's control circuits may place the preponderance of positive feedback on a form of motion that limits potential recovery (i.e. compensatory motions). For example, inappropriate muscular co-activation is common in stroke, which may make it difficult to lift a cup to the lips without inappropriate movements in the shoulder muscles. The form of motion that maximizes positive reinforcement (produces motion in the planned vector or brings the cup to the lips) and negative reinforcement (does not carry the strain of decoupling the co-activation throughout a complex motion) may involve compensatory torso or neck contortion that cancels out the shoulder motion rather than eliminating it. This set of motions and others like it may be heavily reinforced due to success in everyday tasks, and as compensation becomes more practiced the negative reinforcement placed on its use would increase. This increase in

compensatory skill may lead to an ultimate limitation on recovery for tasks that absolutely require independent muscle activation but do not carry strong, everyday feedback.

Ubiquitous natural feedback assures that learning will almost always occur

Feedback occurs constantly; it is not possible to consider a model in which no learning occurs after stroke, even when the experimenter does not explicitly design a reward-based rehabilitative scenario. Mice may be housed in a poorly or richly enriched environment, or may be in a rehabilitative activity or not, but this does not change the quantity of learning; rather, it changes the kind of learning that occurs. In a badly designed environment, an animal learns to stay still or to compensate or to simply abandon former activities in favor of others. In a facilitative environment, learning still occurs but can be directed toward better and recovered motions or greater levels of activity. The difference is not the existence of post-stroke learning; the difference is in content.

Natural feedback is dynamic

Even when the environment remains nominally identical, it may not functionally be so, either from individual to individual or in the same individual from time to time. For example, the presentation of consistent physical stimuli does not necessarily make stimulation consistent in the behavioral sense; an animal's history with particular stimuli will affect the ability of those stimuli to function in a learning paradigm. In the phenomenon of blocking, for example, if a neutral stimulus such as a tone is paired with an unconditioned stimulus such as food, the tone will not come to elicit responses appropriate to the food as predicted

by classical conditioning if another stimulus such as a light has already been conditioned and occurs in temporal proximity to the tone [177]. Differential conditioning can also occur based on temporal variation in the behavior itself, which may differ from animal to animal [178]. These simple principles can have ripple effects across operant behavior; stimuli in a behavioral chamber that take on conditioned reinforcing properties for one animal may be blocked for another, or different animals may respond to different stimulus features as conditioned reinforcement. In another example, when an animal learns a conditional discrimination using a fading paradigm that does not allow for the occurrence of prolific errors, the animal will not show a classic extinction burst as is typically predicted from the cessation of reinforcement and will adapt less readily to reinforcement perturbations [179, 180]. Small differences in the sequence of learning or in the timing of schedule extension of a task may also affect its response to perturbation [181]. After stroke, therefore, animals that were trained more efficiently and with fewer errors may be more sensitive to extinction encountered after injury and may produce less extinction-induced variation, which in turn may make them less successful at recovery. Even reinforcement is relative; under the right conditions, the opportunity to perform a motor behavior may actually reinforce eating rather than the other way around [182] and such dynamics may create a context and history-specific reinforcer efficacy [183]. Since these dynamics are specific to individual and to environment, the feedback provided in the task at hand relative to the power of feedback provided by competing activities may or may not produce consistent learning opportunities for different animals [184]. The relativity of reinforcement can be extreme. For example, an animal may actually respond to shock as a reinforcer [185] possibly because of constraints and avoidance

histories interacting with new contingencies [186] or because of reinforcement-predicting shocks [187] among other potential reasons. Similarly, the complex conditions and contrast-induced punishments of brain damage may produce some strange reinforcers too.

All this may or may not confound research on stroke recovery; when stringent methods are used such as pseudorandomization based on pre-stroke learning patterns and other factors, it is less likely that these things will matter. However, these factors may inject variation into behavioral assays that make it more difficult to see important signals. Perhaps more importantly, such factors may be relevant in the context of human injury. For example, we tend to use food deprivation to increase the relative efficacy of food deliveries for rodent subjects in stroke research. This effectively removes or blunts the chances that competing responses will occur that are reinforced with non-food consequences. This may not matter at all to our question at hand, however it also may. If the efficacy of a treatment depends on blunting alternate sources of reinforcement (i.e. if the treatment increases pain involved with practice movements in a way that would punish the motions of anyone with an alternative reinforcer available), it may be difficult to translate such a treatment from a hungry rat to a human. It may be independently important to determine not only if the treatment increases the efficacy of learning but also if it interferes with the efficacy of feedback that is not artificially boosted.

Injury may cause further feedback dynamics

Motor and sensory injury itself may alter the feedback functions of an environment. To illustrate using an easy example, cochlear damage alters the feedback functions of the audible

environment not because the sounds are different but because those same sounds affect auditory neurons in a critically different way. In some people, the sudden loss of downstream activity based on a certain frequency may cause the expansion of cortical areas sensitive to that frequency, such that tinnitus results [188]. This tends to happen in frequencies with which there is some experience; again, the results (function) of changed feedback depends on the history of the animal. In this case a sudden lack of stimulus previously highly related to many aspects of the environment and behavior may trigger maladaptive plasticity. Examples in stroke may be more complex but similar in kind. For example, an animal with an injury-induced perturbation in reaching may not receive feedback in the same way they did when learning the task originally. For example, in a phenomenon called reinforcement contrast, a diminished rate or magnitude of reward can cause a punishing effect and allocate behavior toward alternate responses even when the probability of reinforcement on the alternate remains unchanged [189-191], and this effect may be different between deprived food reinforcement or appetitive extra calories like sucrose [192]. Therefore rare food delivery for an animal previously reinforced at high rates may actually lead to diminished attempts and greater competition by alternative responses and contingencies. In other words, a stimulus that reinforced before may not do so any longer because of the contrast with previous rates of delivery with concomitant “effort”. As another example, stroke may destroy critical conditioned reinforcement functions in the environment and their re-learning or replacement may be blocked by pre-stroke learning histories. During the original learning process, proprioceptive, auditory, olfactory and other predictors of success are available to take on conditioned reinforcing properties; the exact feel of an accurate pull, the smell of a partially

open valve, the way the lip of the chamber pushes against the side of the arm during the right motion sequence may all become critical to performance, and these critical points may be different from animal to animal. After stroke, some of these predictors may be less available either due to sensory loss or to differences in movement execution. Without such signals in place, reinforcement may rest only on the final outcome and not on indications of proximity to success, leading to worse recovery in animals whose important cues happen to be disrupted (as would be expected if proprioception entered into motion-relevant conditioned signaling, its loss perturbs skilled motor behavior [193]). Other available cues that could have been newly conditioned in a new paradigm may be initially blocked because of the animal's previous exposure, rendering the learning environment more challenging.

Implications of behavioral dynamics for basic research

Again, these considerations may or may not be important to incorporate into a model. If such dynamics are consistent in the human case, their careful modelling may be important to some questions. If they are not consistent in the human case, testing under multiple conditions may be important for validity. They will be most useful to animal modelling if and when they become well characterized in humans. Many of the issues I mention (such as reinforcement efficacy, often described as motivation) are addressed to some degree by well-designed rehabilitative interventions, and the model need only imitate those interventions. However, rehabilitation is itself an autonomous field of basic research, and if that field advances at an ideal rate, its imitation will also be a moving target. A much simpler approach is simply to choose modelling conditions with these principles in mind and make sure that

such conditions could plausibly be reconstituted in the clinic and take into consideration unchangeable histories of the stroke patient. In other words, rather than trying to control for every possibility, it may be prudent simply to assure that no major contradiction exists in the model. Another important point is to communicate the behavioral conditions of the experiment and describe any behavioral dynamics were tracked or known, noting that they form a context apart from which treatment may not work in the same way. Currently, these considerations do not frequently enter into consideration at all in basic research, limiting the capability of the basic scientist to communicate the best conditions for success in translation.

My previous suggestion to further develop a behavioral model toward species-specific, not explicitly trained forelimb function also carries with it advantages in terms of these considerations. As I summarized previously, "the widely incorporated nature of the behaviors will align the degree of habitual performance with human case and provide more human-like post-stroke extra-rehabilitative practice opportunities in everyday life". This may be an initial step, though certainly not the only possible step, towards improving behavioral models in terms of behavioral context and dynamics.

FUTURE DIRECTIONS IN NEUROMODULATION

The isolation of the cholinergic system in this paper serves the analytical approach of the work; determining the effect of its manipulation in isolation is vital to interpreting the effects of multiple modulator interactions such as caused by VNS. However, this work makes no assumptions that the effects of acetylcholine should automatically be best understood as

autonomous. While the transmitter systems have all been linked to learning and memory in many different tasks, they do not appear to be mere repetitions of one another or redundant systems. In different tests, they have differential impacts on learning. For example, acetylcholine, dopamine, norepinephrine and serotonin have all proven vital for learning in the morris water maze, but the latter three have only a weak effect on learning in the radial maze, all but norepinephrine intensely affects passive avoidance, and all have higher effects on spontaneous alteration of choices in a T maze [194]. Thus, behavioral data do not indicate that these systems are redundant; it is more likely that they are playing complimentary roles. This can be seen in the sub-behavioral level in the effects of modulators on plasticity and STDP. The modulators named above have all been shown to be important for particular instances of cortical plasticity [81], however some forms of plasticity may require more than one modulator [139]. Modulators also have unequal effects on plasticity induction in particular regions, and those effects are interactive [154]. For example, in visual cortical neurons LTP is gated by noepinepherine and LTD by acetylcholine in isolation. Both are required for bi-directional STDP [152]. The role of one modulator does not always even recapitulate itself in different regions; for example dopamine is required for LTP gating in the striatum and amygdala but not in the hippocampus [154].

The non-redundancy of modulators does not mean that they have no common mechanisms; in fact they do influence similar signaling pathways through similar receptor types. For example, multiple modulators are coupled to Gs and Gq11 receptors. Gs receptors gate LTP in visual cortex while Gq11 gate LTD; each suppress the opposite process in isolation and both are required for bi-directional plasticity [195]. Thus, the independence of

modulatory roles cannot derive from complete nonoverlap of process (see also [196]). However, each modulator is associated with a slightly varied pattern of projection, a variety of receptors with different expression levels and geometry, and varied regional contexts in target wiring [81, 154, 196]. Thus, a great deal of differential impact between the systems is possible via both overlapping and non-overlapping mechanisms. The variable that seems to bind each system into a unit is not an autonomous mechanism of impact on plasticity or behavior but the coordinated activity of its cell cluster, which happens to connect with lawful consistencies on the level of behavior.

Analytic approaches to studying the effects of single components in isolation may seem less relevant when systems are found to have nonlinear combinatorial effects. In such a case, those forms of reductionistic thinking are discouraged that assume that understanding a whole will come from assembling knowledge derived from the study of each part. Neuromodulator interactions indicate that these systems in sum constitute such an unpredictable whole, to say nothing of the behavioral principles that lie on an even higher level of analysis. Yet this only discourages potential contributions of the autonomous study of subsystems in an academic manner. Setting boundary conditions on phenomena of interest at higher levels of analysis can narrow the range of relevant points to consider in lower ones, which can be done by making testing conditions physiologically valid (where possible), working in vivo as much as possible, and modelling behavioral context. In this way it is possible to limit study to boundary conditions that make the study of the whole as well as the connections between levels of analysis meaningful [197], and this study is a step in that direction. We have examined one component part, the cholinergic system, in a behaviorally

and physiologically valid context, coupled with training feedback that should naturally utilize other modulatory systems in a pattern with which our cholinergic augmentation can interact. Such a cross-level validated (and therefore narrower) form of subcomponent analysis could ultimately inform us about interactions between neuromodulators and the behavior of the whole by providing a highly contextualized baseline against which other such studies can be compared. For this reason, future study should build on this work not only by producing multiple lines of evidence about the role of the cholinergic system (i.e. using optogenetic approaches) but also by repeating experimentation with modifications of other modulators and with various modulators in combination. It is my hope that this work inspires a line of investigation in that direction.

APPENDIX A
A Supplemental Research Project on the Effects of Stroke on the Cholinergic System

During the course of my doctoral studies, I supervised two undergraduate honors theses, the most recent of which was completed by Dene Betz and provides preliminary data for a line of research complimentary to the investigation of the role of the NB cholinergic system in stroke recovery. Some clues indicate that stroke itself may perturb the cholinergic basal forebrain system, as described in previous sections. This may come about via network effects from damage to regions targeted by the NB or may be direct effects of ischemic injury affecting regions of the brain where ascending cholinergic pathways are located. In any case, the characterization of post-stroke effects on the cholinergic system can put a potentially important context onto the results of NB manipulation; if augmentation of NB cholinergic signals increase stroke recovery in the context of a fully intact NB system, then augmenting a damaged system may even produce greater clinical benefit. If augmentation of signals from a damaged system increase stroke recovery, those results may not hold for a fully intact system.

ABBREVIATED METHODS

For full methods of this project, see Dene Betz's undergraduate thesis [198]. Abbreviated methods are provided here in order to enable discussion.

In case forelimb motor learning constituted important context for changes in the cholinergic system after damage to forelimb cortical areas, and since this project did not involve explicit forelimb rehabilitation imposed by the experimenter, Dene exposed animals before and after strokes in the homecage to activities that provided the opportunity for forelimb use such as marbles to bury or small tubes from which to scoop out peanut butter. Photothrombotic strokes on C57/Bl6 mice were induced as described in previous chapters. For sham strokes, saline injections were substituted for Rose Bengal. Animals were sacrificed 3 days, 2 weeks and 4 weeks after stroke. The mice utilized were the same mice from Chapter 6 that expressed channelrhodopsin in their cholinergic cells. Importantly, that channelrhodopsin was expressed throughout processes as well as in the soma, allowing for the visualization via the attached EYFP tag of ascending cholinergic projections. Animals were perfused as described in previous chapters and sagittal sections visualized throughout each brain using two-photon serial tomography (TissueCyte 1000, TissueVision). Dene located sample sections using anatomical markers with which she quantified cell numbers in the nucleus basalis, quantified fluorescence of axons at a midpoint of one of the ascending pathways, and quantified fluorescence of axons and axon terminals in peri-infarct cortex (fluorescence values obtained by subtracting mean value on Image J from non-tagged background tissue). All procedures were blinded and regions of interest for fluorescent quantification or cell counting were determined using anatomical markers in non-fluorescent channels to avoid bias. Image-J fluorescence was chosen over more rigorous methods such as axon quantification because of its greater ease and the preliminary, probing nature of the project.

RESULTS

Results indicate a potential increase in mid-pathway axon fluorescence in the hemisphere ipsilateral to the stroke (Figure 1). Results do not indicate any potential differences in other measures, but the variation in this sparse sample indicate that this may be due to a high degree of noise in the measurement, which may be mitigated by more rigorous quantification methods.

DISCUSSION

These pilot data indicate that it may be fruitful to look more closely into potential changes in the cholinergic system induced by cortical stroke. If ascending pathways indeed change in fluorescence intensity with more subjects added, this may indicate either a rearrangement of axons that results in increased density in the region, an increase in fluorescent labelling in existing axons, an increase in axon diameter, or an increase in axon number. In the latter case, which is among the most probable, it may be that loss of target neurons induces axonal outgrowth toward damaged areas. If this is the case and if peri-infarct cortex shows no concomitant increase in axon terminals as indicated by this pilot, it may mean that new axons terminate elsewhere.

This pilot data addresses the question of whether the structure of the cholinergic system may be affected by loss of target neurons. The question of whether it may be

affected due to spatial overlap of ischemic damage with ascending pathways in humans could also be addressed by repeating the experiment with a subcortical stroke along the pathway in question. Together, these two experiments could elucidate both the existence of and the potential causes of post-stroke changes in the cholinergic system.

FIGURE

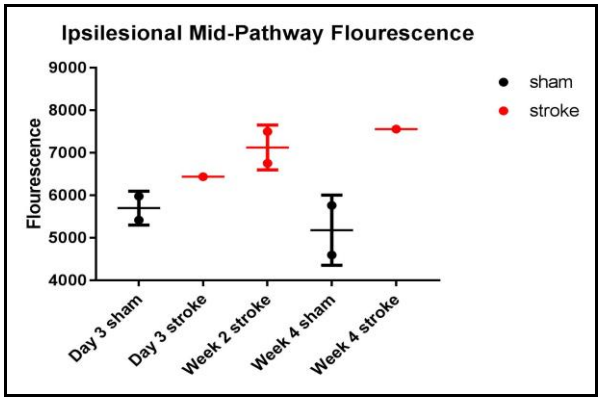


Figure 1
Pilot data showing a potential increase in the fluorescence of tagged cholinergic projections in the hemisphere ipsilateral to cortical ischemic injury (reprinted with permission from Betz [198]).

APPENDIX B
General Acknowledgements

These studies were financially supported by a National Institute of Health training grant (T32 NS069562 Cellular Biophysics of the Neuron Training Program), the Patrick and Beatrice Haggerty Foundation, the Beatrice Menne Haggerty Center for Research on Brain Injury and Repair in Stroke, the Texas Institute for Brain Injury and Repair, and through UTD by the National Institute of Health research project grants R01NS085167 and R44NS086344. Special thanks to Dr. Julian Meeks and Marina Maksimova for providing the electrophysiology data in Chapter 6 and for teaching me about the process of slice recording. Special thanks to Dr. Eric Meyers, Dr. Andrew Sloan, Dr. Michael Kilgard and Dr. Robert Rennaker for your constant support and collaboration with these projects. Special thanks to Dene Betz and Hanan Muzemil for all their hard work in the Goldberg lab, particularly for Hanan's support of Chapter 3 and Dene's support of Chapter 6. Thanks to Xiangmei Kong for foundational support for all of the work reported here. Thanks to all members of the Neurorepair Lab including the Dr. Mark Goldberg Lab and the Dr. Ann Stowe lab for their constant encouragement. Thanks to my committee and others at UT Southwestern and UT Dallas for their support and advice including Dr. Julian Meeks, Dr. Kim Huber, Dr. Robert Rennaker, Dr. Michael Kilgard, Dr. Peter Tsai, Dr. Denise Ramirez, Dr. Kate Luby-Phelps, Abhijit Bugde, Dr. Todd Roberts, Dr. Irene Bowen, Dr. James Bibb, Dr. Robert Greene and others who have been so gracious and helpful to me along the way.

BIBLIOGRAPHY

1. Dirnagl U: **Bench to Bedside: The Quest for Quality in Experimental Stroke Research**. *Journal of Cerebral Blood Flow & Metabolism* 2006, **26**(12):1465-1478.

2. Dirnagl U, Iadecola C, Moskowitz MA: **Pathobiology of ischaemic stroke: an integrated view**. *Trends in Neurosciences* 1999, **22**(9):391-397.

3. Zhang ZG, Chopp M: **Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic**. *The Lancet Neurology* 2009, **8**(5):491-500.

4. Philip M, Benatar M, Fisher M, Savitz SI: **Methodological quality of animal studies of neuroprotective agents currently in phase II/III acute ischemic stroke trials**. *Stroke* 2009, **40**(2):577-581.

5. Hoyte L, Kaur J, Buchan AM: **Lost in translation: taking neuroprotection from animal models to clinical trials**. *Experimental Neurology* 2004, **188**(2):200-204.

6. Douglas Kondziolka, Gary K. Steinberg, Lawrence Wechsler, Carolyn C. Meltzer, Elaine Elder, James Gebel, Sharon DeCesare, Tudor Jovin, Ross Zafonte, Jonathan Lebowitz *et al*: **Neurotransplantation for patients with subcortical motor stroke: a Phase 2 randomized trial**. *Journal of Neurosurgery* 2005, **103**(1):38-45.

7. Ringelstein EB, Thijs V, Norrving B, Chamorro A, Aichner F, Grond M, Saver J, Laage R, Schneider A, Rathgeb F: **Granulocyte Colony–Stimulating Factor in Patients With Acute Ischemic Stroke**. *Stroke* 2013:STROKEAHA. 113.001531.

8. Di Cesare F, Mancuso J, Woodward P, Bednar MM, Loudon PT, Group ASS: **Phosphodiesterase-5 Inhibitor PF-03049423 Effect on Stroke Recovery: A Double-Blind, Placebo-Controlled Randomized Clinical Trial**. *Journal of Stroke and Cerebrovascular Diseases* 2016, **25**(3):642-649.

9. The ISTcg: **The benefits and harms of intravenous thrombolysis with recombinant tissue plasminogen activator within 6 h of acute ischaemic stroke (the third international stroke trial [IST-3]): a randomised controlled trial**. *The Lancet*, **379**(9834):2352-2363.

10. Chollet F, Tardy J, Albucher J-F, Thalamas C, Berard E, Lamy C, Bejot Y, Deltour S, Jaillard A, Niclot P *et al*: **Fluoxetine for motor recovery after acute ischaemic stroke (FLAME): a randomised placebo-controlled trial**. *The Lancet Neurology* 2011, **10**(2):123-130.

11. Vetencourt JFM, Sale A, Viegi A, Baroncelli L, De Pasquale R, Oleary OF, Castrén E, Maffei L: **The antidepressant fluoxetine restores plasticity in the adult visual cortex**. *Science* 2008, **320**(5874):385-388.

12. Dawson J, Pierce D, Dixit A, Kimberley TJ, Robertson M, Tarver B, Hilmi O, McLean J, Forbes K, Kilgard MP *et al*: **Safety, Feasibility, and Efficacy of Vagus Nerve Stimulation Paired With Upper-Limb Rehabilitation After Ischemic Stroke**. *Stroke; a Journal of Cerebral Circulation* 2016, **47**(1):143-150.

13. **Recommendations for Standards Regarding Preclinical Neuroprotective and Restorative Drug Development**. *Stroke* 1999, **30**(12):2752-2758.

14. Lapchak PA, Zhang JH, Noble-Haeusslein LJ: **RIGOR Guidelines: Escalating STAIR and STEPS for Effective Translational Research**. *Translational Stroke Research* 2013, **4**(3):279-285.

15. Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent TA, Savitz SI, Lo EH: **Update of the stroke therapy academic industry roundtable preclinical recommendations**. *Stroke* 2009, **40**(6):2244-2250.

16. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG: **Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research**. *PLoS biology* 2010, **8**(6):e1000412.

17. Atkinson CS, Press GA, Lyden P, Katz B: **The ferret as an animal model in cerebrovascular research**. *Stroke* 1989, **20**(8):1085-1088.

18. Mestas J, Hughes CC: **Of mice and not men: differences between mouse and human immunology**. *The Journal of Immunology* 2004, **172**(5):2731-2738.

19. Davis LE, King MK, Schultz JL: **Fundamentals of neurologic disease**: Demos Medical Publishing; 2005.

20. Witte OW: **Photochemical and Endothelin Models of Focal Brain Ischemia**. In: *Rodent Models of Stroke.* edn. Edited by Dirnagl U. Totowa, NJ: Humana Press; 2010: 71-83.

21. Sozmen EG, Hinman JD, Carmichael ST: **Models that matter: white matter stroke models**. *Neurotherapeutics* 2012, **9**(2):349-358.

22. Engel O, Kolodziej S, Dirnagl U, Prinz V: **Modeling stroke in mice-middle cerebral artery occlusion with the filament model**. *JoVE (Journal of Visualized Experiments)* 2011(47):e2423-e2423.

23. Orset C, Haelewyn B, Vivien K, Vivien D, Young AR: **Rodent Models of Thromboembolic Stroke**. In: *Rodent Models of Stroke.* edn. Edited by Dirnagl U. Totowa, NJ: Humana Press; 2010: 55-70.

24. **Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS): bridging basic and clinical science for cellular and neurogenic factor therapy in treating stroke**. *Stroke* 2009, **40**(2):510-515.

25. Hepp-Reymond M-C, Wiesendanger M: **Unilateral pyramidotomy in monkeys: Effect on force and speed of a conditioned precision grip**. *Brain Research* 1972, **36**(1):117-131.

26. Wenzelburger R, Kopper F, Frenzel A, Stolze H, Klebe S, Brossmann A, Kuhtz-Buschbeck J, Golge M, Illert M, Deuschl G: **Hand coordination following capsular stroke**. *Brain : a journal of neurology* 2005, **128**(Pt 1):64-74.

27. Seitz RJ, Donnan GA: **Recovery potential after acute stroke**. *Frontiers in neurology* 2015, **6**.

28. Carter AR, Astafiev SV, Lang CE, Connor LT, Rengachary J, Strube MJ, Pope DL, Shulman GL, Corbetta M: **Resting interhemispheric functional magnetic resonance imaging connectivity predicts performance after stroke**. *Annals of neurology* 2010, **67**(3):365-375.

29. Corbetta M: **Functional connectivity and neurological recovery**. *Developmental psychobiology* 2012, **54**(3):239-253.

30. Biernaskie J, Corbett D: **Enriched rehabilitative training promotes improved forelimb motor function and enhanced dendritic growth after focal ischemic injury**. *Journal of Neuroscience* 2001, **21**(14):5272-5280.

31. Bonifer NM, Anderson KM, Arciniegas DB: **Constraint-induced movement therapy after stroke: efficacy for patients with minimal upper-extremity motor ability**. *Archives of physical medicine and rehabilitation* 2005, **86**(9):1867-1873.

32. Treger I, Aidinof L, Lehrer H, Kalichman L: **Modified constraint-induced movement therapy improved upper limb function in subacute poststroke patients: a small-scale clinical trial**. *Topics in stroke rehabilitation* 2012, **19**(4):287-293.

33. Hömberg V: **Neurological Rehabilitation: Chapter 14. Neurorehabilitation approaches to facilitate motor recovery**, vol. 110: Elsevier Inc. Chapters; 2013.

34. Mernoff ST, Lo AC: **Novel stroke rehabilitation interventions**. *Medicine and Health Rhode Island* 2011, **94**(12):360.

35. Kim Y-H, You SH, Ko M-H, Park J-W, Lee KH, Jang SH, Yoo W-K, Hallett M: **Repetitive transcranial magnetic stimulation–induced corticomotor excitability and associated motor skill acquisition in chronic stroke**. *Stroke* 2006, **37**(6):1471-1476.

36. Takeuchi N, Tada T, Toshima M, Chuma T, Matsuo Y, Ikoma K: **Inhibition of the unaffected motor cortex by 1 Hz repetitive transcranial magnetic stimulation enhances motor performance and training effect of the paretic hand in patients with chronic stroke**. *Journal of Rehabilitation Medicine* 2008, **40**(4):298-303.

37. Slater D, Burnham K, Cook T, Golden S: **Middle Cerebral Artery Stroke**. In: *Physical Medicine and Rehabilitation* Edited by Kishner S, vol. 2017. Medscape: Medscape; 2017.

38. Gor-García-Fogeda MD, Molina-Rueda F, Cuesta-Gómez A, Carratalá-Tejada M, Alguacil-Diego IM, Miangolarra-Page JC: **Scales to assess gross motor function in stroke patients: a systematic review**. *Archives of physical medicine and rehabilitation* 2014, **95**(6):1174-1183.

39. Platz T, Pinkowski C, van Wijck F, Kim I-H, Di Bella P, Johnson G: **Reliability and validity of arm function assessment with standardized guidelines for the Fugl-Meyer Test, Action Research Arm Test and Box and Block Test: a multicentre study**. *Clinical Rehabilitation* 2005, **19**(4):404-411.

40. Staines R, McIlroy W, Brooks D: **Functional impairments following stroke: implications for rehabilitation**. *Curr Issues Cardiac Rehab Prevent* 2009.

41. Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST: **CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury**. *Neuropharmacology* 2000, **39**(5):777-787.

42. Jones B, Roberts D: **The quantitative measurement of motor inco-ordination in naive mice using an accelerating rotarod**. *Journal of Pharmacy and Pharmacology* 1968, **20**(4):302-304.

43. Metz GA, Whishaw IQ: **The ladder rung walking task: a scoring system and its practical application**. *JoVE (Journal of Visualized Experiments)* 2009(28):e1204-e1204.

44. Shanina E, Schallert T, Witte O, Redecker C: **Behavioral recovery from unilateral photothrombotic infarcts of the forelimb sensorimotor cortex in rats: role of the contralateral cortex**. *Neuroscience* 2006, **139**(4):1495-1506.

45. Li H, Zhang N, Lin H-Y, Yu Y, Cai Q-Y, Ma L, Ding S: **Histological, cellular and behavioral assessments of stroke outcomes after photothrombosis-induced ischemia in adult mice**. *BMC neuroscience* 2014, **15**(1):58.

46. Schweizer TA, Macdonald RL: **The behavioral consequences of stroke**: Springer; 2014.

47. Meyers E, Sindhurakar A, Hays S, Sloan A, Carmel J, Kilgard M, Rennaker R: **A novel automated method for isolating and quantifying supination performance in a rat model of ischemic stroke**. *Neuromodulation* 2014, **17**(5):e82.

48. Balkaya M, Endres M: **Behavioral Testing in Mouse Models of Stroke**. In: *Rodent Models of Stroke.* edn. Edited by Dirnagl U. Totowa, NJ: Humana Press; 2010: 179-197.

49. Moon S-K, Alaverdashvili M, Cross AR, Whishaw IQ: **Both compensation and recovery of skilled reaching following small photothrombotic stroke to motor cortex in the rat**. *Experimental neurology* 2009, **218**(1):145-153.

50. Knieling M, Metz G, Antonow-Schlorke I, Witte O: **Enriched environment promotes efficiency of compensatory movements after cerebral ischemia in rats**. *Neuroscience* 2009, **163**(3):759-769.

51. Meyers E, Sindhurakar A, Choi R, Solorzano R, Martinez T, Sloan A, Carmel J, Kilgard MP, Rennaker RL, Hays S: **The supination assessment task: An automated method for quantifying forelimb rotational function in rats**. *Journal of neuroscience methods* 2016, **266**:11-20.

52. Hays SA, Khodaparast N, Sloan AM, Hulsey DR, Pantoja M, Ruiz AD, Kilgard MP, Rennaker Ii RL: **The isometric pull task: A novel automated method for quantifying forelimb force generation in rats**. *Journal of Neuroscience Methods* 2013, **212**(2):329-337.

53. Schulkin J: **Preoperative events: their effects on behavior following brain damage**: Psychology Press; 2014.

54. Langhorne P, Coupar F, Pollock A: **Motor recovery after stroke: a systematic review**. *Lancet Neurol* 2009, **8**(8):741-754.

55. Klein A, Sacrey LA, Whishaw IQ, Dunnett SB: **The use of rodent skilled reaching as a translational model for investigating brain damage and disease**. *Neuroscience and biobehavioral reviews* 2012, **36**(3):1030-1042.

56. Lai S, Panarese A, Spalletti C, Alia C, Ghionzoli A, Caleo M, Micera S: **Quantitative kinematic characterization of reaching impairments in mice after a stroke**. *Neurorehabilitation and neural repair* 2015, **29**(4):382-392.

57. Clarkson AN, Lopez-Valdes HE, Overman JJ, Charles AC, Brennan KC, Thomas Carmichael S: **Multimodal examination of structural and functional remapping in the mouse photothrombotic stroke model**. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* 2013, **33**(5):716-723.

58. Lee JK, Park MS, Kim YS, Moon KS, Joo SP, Kim TS, Kim JH, Kim SH: **Photochemically induced cerebral ischemia in a mouse model**. *Surgical neurology* 2007, **67**(6):620-625; discussion 625.

59. Hays SA, Khodaparast N, Sloan AM, Hulsey DR, Pantoja M, Ruiz AD, Kilgard MP, Rennaker RL, 2nd: **The isometric pull task: a novel automated method for quantifying forelimb force generation in rats**. *Journal of neuroscience methods* 2013, **212**(2):329-337.

60. Wong CC, Ramanathan DS, Gulati T, Won SJ, Ganguly K: **An automated behavioral box to assess forelimb function in rats**. *Journal of neuroscience methods* 2015, **246**:30-37.

61. Becker AM, Meyers E, Sloan A, Rennaker R, Kilgard M, Goldberg MP: **An automated task for the training and assessment of distal forelimb function in a mouse model of ischemic stroke**. *Journal of neuroscience methods* 2016, **258**:16-23.

62. Lemon RN: **Descending pathways in motor control**. *Annu Rev Neurosci* 2008, **31**:195-218.

63. Nudo RJ, Wise BM, SiFuentes F, Milliken GW: **Neural substrates for the effects of rehabilitative training on motor recovery after ischemic infarct**. *Science* 1996, **272**(5269):1791.

64. Sun J, Ke Z, Yip SP, Hu X-l, Zheng X-x, Tong K-y: **Gradually increased training intensity benefits rehabilitation outcome after stroke by BDNF upregulation and stress suppression**. *BioMed research international* 2014, **2014**.

65. Pruitt DT, Schmid AN, Danaphongse TT, Flanagan KE, Morrison RA, Kilgard MP, Rennaker RL, Hays SA: **Forelimb training drives transient map reorganization in ipsilateral motor cortex**. *Behavioural Brain Research* 2016, **313**:10-16.

66. Wang L, Conner JM, Nagahara AH, Tuszynski MH: **Rehabilitation drives enhancement of neuronal structure in functionally relevant neuronal subsets**. *Proceedings of the National Academy of Sciences* 2016, **113**(10):2750-2755.

67. Brown CE, Li P, Boyd JD, Delaney KR, Murphy TH: **Extensive turnover of dendritic spines and vascular remodeling in cortical tissues recovering from stroke**. *Journal of Neuroscience* 2007, **27**(15):4101-4109.

68. Conner JM, Chiba AA, Tuszynski MH: **The basal forebrain cholinergic system is essential for cortical plasticity and functional recovery following brain injury**. *Neuron* 2005, **46**(2):173-179.

69. Pruitt D, Danaphongse T, Schmid A, Morrison R, Kilgard M, Rennaker II RL, Hays S: **Traumatic brain injury occludes training-dependent cortical reorganization in the contralesional hemisphere**. *Journal of Neurotrauma* 2017(ja).

70. Biernaskie J, Chernenko G, Corbett D: **Efficacy of rehabilitative experience declines with time after focal ischemic brain injury**. *Journal of Neuroscience* 2004, **24**(5):1245-1254.

71. Berretta A, Tzeng Y-C, Clarkson AN: **Post-stroke recovery: the role of activity-dependent release of brain-derived neurotrophic factor**. *Expert review of neurotherapeutics* 2014, **14**(11):1335-1344.

72. Sawaki L, Butler AJ, Leng X, Wassenaar PA, Mohammad YM, Blanton S, Sathian K, Nichols-Larsen DS, Wolf SL, Good DC: **Constraint-induced movement therapy results in increased motor map area in subjects 3 to 9 months after stroke**. *Neurorehabilitation and neural repair* 2008, **22**(5):505-513.

73. Daniels CML, Ayers KL, Finley AM, Culver JP, Goldberg MP: **Axon sprouting in adult mouse spinal cord after motor cortex stroke**. *Neuroscience letters* 2009, **450**(2):191-195.

74. Dancause N, Barbay S, Frost SB, Plautz EJ, Chen D, Zoubina EV, Stowe AM, Nudo RJ: **Extensive cortical rewiring after brain injury**. *Journal of Neuroscience* 2005, **25**(44):10167-10179.

75. Jones TA, Chu CJ, Grande LA, Gregory AD: **Motor skills training enhances lesion-induced structural plasticity in the motor cortex of adult rats**. *Journal of Neuroscience* 1999, **19**(22):10153-10163.

76. Taub E, Uswatte G, Mark V, Morris D: **The learl1ed nonuse phenomenon: implications for rehabilitation**. *Eura Medicophys* 2006, **42**:241-255.

77. Allred RP, Cappellini CH, Jones TA: **The "good" limb makes the "bad" limb worse: Experience-dependent interhemispheric disruption of functional outcome after cortical infarcts in rats**. *Behavioral neuroscience* 2010, **124**(1):124.

78. Allred RP, Jones TA: **Maladaptive effects of learning with the less-affected forelimb after focal cortical infarcts in rats**. *Experimental neurology* 2008, **210**(1):172-181.

79. Mang CS, Campbell KL, Ross CJ, Boyd LA: **Promoting neuroplasticity for motor rehabilitation after stroke: considering the effects of aerobic exercise and genetic variation on brain-derived neurotrophic factor**. *Physical therapy* 2013, **93**(12):1707.

80. Zhang R, Wang Y, Zhang L, Zhang Z, Tsang W, Lu M, Zhang L, Chopp M: **Sildenafil (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats**. *Stroke* 2002, **33**(11):2675-2680.

81. Gu Q: **Neuromodulatory transmitter systems in the cortex and their role in cortical plasticity**. *Neuroscience* 2002, **111**(4):815-835.

82. Porter BA, Khodaparast N, Fayyaz T, Cheung RJ, Ahmed SS, Vrana WA, Rennaker RL, Kilgard MP: **Repeatedly pairing vagus nerve stimulation with a movement reorganizes primary motor cortex**. *Cerebral Cortex* 2012, **22**(10):2365-2374.

83. Khodaparast N, Hays SA, Sloan AM, Hulsey DR, Ruiz A, Pantoja M, Rennaker Ii RL, Kilgard MP: **Vagus nerve stimulation during rehabilitative training improves forelimb strength following ischemic stroke**. *Neurobiology of Disease* 2013, **60**:80-88.

84. Khodaparast N, Hays SA, Sloan AM, Fayyaz T, Hulsey DR, Robert L. Rennaker I, Kilgard MP: **Vagus Nerve Stimulation Delivered During Motor Rehabilitation Improves Recovery in a Rat Model of Stroke**. *Neurorehabilitation and Neural Repair* 2014, **28**(7):698-706.

85. Hays SA, Ruiz A, Bethea T, Khodaparast N, Carmel JB, Rennaker RL, Kilgard MP: **Vagus nerve stimulation during rehabilitative training enhances recovery of forelimb function after ischemic stroke in aged rats**. *Neurobiology of aging* 2016, **43**:111-118.

86. Pruitt DT, Schmid AN, Kim LJ, Abe CM, Trieu JL, Choua C, Hays SA, Kilgard MP, Rennaker RL: **Vagus nerve stimulation delivered with motor training enhances recovery of function after traumatic brain injury**. *Journal of neurotrauma* 2016, **33**(9):871-879.

87. Hulsey DR, Hays SA, Khodaparast N, Ruiz A, Das P, Rennaker RL, Kilgard MP: **Reorganization of motor cortex by vagus nerve stimulation requires cholinergic innervation**. *Brain stimulation* 2016, **9**(2):174-181.

88. Howland RH: **Vagus nerve stimulation**. *Current behavioral neuroscience reports* 2014, **1**(2):64-73.

89. Woolf NJ: **Cholinergic systems in mammalian brain and spinal cord**. *Progress in neurobiology* 1991, **37**(6):475-524.

90. Zaborszky L, van den Pol A, Gyengesi E: **The basal forebrain cholinergic projection system in mice**. *The mouse nervous system* 2012, **28**:684-718.

91. Rye D, Wainer B, Mesulam M-M, Mufson E, Saper C: **Cortical projections arising from the basal forebrain: a study of cholinergic and noncholinergic components employing combined retrograde tracing and immunohistochemical localization of choline acetyltransferase**. *Neuroscience* 1984, **13**(3):627-643.

92. McKinney M, Coyle JT, Hedreen JC: **Topographic analysis of the innervation of the rat neocortex and hippocampus by the basal forebrain cholinergic system**. *Journal of Comparative Neurology* 1983, **217**(1):103-121.

93. Freund TF, Gulyas A: **GABAergic interneurons containing calbindin D28K or somatostatin are major targets of GABAergic basal forebrain afferents in the rat neocortex**. *Journal of Comparative Neurology* 1991, **314**(1):187-199.

94. Gritti I, Mainville L, Mancia M, Jones BE: **GABAergic and other noncholinergic basal forebrain neurons, together with cholinergic neurons, project to the mesocortex and isocortex in the rat**. *The Journal of comparative neurology* 1997, **383**(2):163-177.

95. Freund TF, Meskenaite V: **gamma-Aminobutyric acid-containing basal forebrain neurons innervate inhibitory interneurons in the neocortex**. *Proceedings of the National Academy of Sciences* 1992, **89**(2):738-742.

96. Chernyshev B, Panasyuk YA, Semikopnaya I, Timofeeva N: **Activity of neurons in the basal magnocellular nucleus during performance of an operant task**. *Neuroscience and behavioral physiology* 2004, **34**(9):907-918.

97. Hangya B, Ranade SP, Lorenc M, Kepecs A: **Central cholinergic neurons are rapidly recruited by reinforcement feedback**. *Cell* 2015, **162**(5):1155-1168.

98. Richardson RT, DeLong MR: **Electrophysiological studies of the functions of the nucleus basalis in primates**. In: *The basal forebrain.* edn.: Springer; 1991: 233-252.

99. Richardson RT, DeLong MR: **Functional implications of tonic and phasic activity changes in nucleus basalis neurons**. In: *Activation to acquisition.* edn.: Springer; 1991: 135-166.

100. Cybulska-Klosowicz A, Zakrzewska R, Kossut M: **Brain activation patterns during classical conditioning with appetitive or aversive UCS**. *Behavioural brain research* 2009, **204**(1):102-111.

101. Masuda R, Fukuda M, Ono T, Endo S: **Neuronal responses at the sight of objects in monkey basal forebrain subregions during operant visual tasks**. *Neurobiology of learning and memory* 1997, **67**(3):181-196.

102. Reed A, Riley J, Carraway R, Carrasco A, Perez C, Jakkamsetti V, Kilgard MP: **Cortical map plasticity improves learning but is not necessary for improved performance**. *Neuron* 2011, **70**(1):121-131.

103. Kleim JA, Barbay S, Nudo RJ: **Functional reorganization of the rat motor cortex following motor skill learning**. *Journal of neurophysiology* 1998, **80**(6):3321-3325.

104. Adkins DL, Boychuk J, Remple MS, Kleim JA: **Motor training induces experience-specific patterns of plasticity across motor cortex and spinal cord**. *Journal of applied physiology* 2006, **101**(6):1776-1782.

105. Polley DB, Steinberg EE, Merzenich MM: **Perceptual learning directs auditory cortical map reorganization through top-down influences**. *Journal of Neuroscience* 2006, **26**(18):4970-4982.

106. Biane JS, Takashima Y, Scanziani M, Conner JM, Tuszynski MH: **Thalamocortical projections onto behaviorally relevant neurons exhibit plasticity during adult motor learning**. *Neuron* 2016, **89**(6):1173-1179.

107. Takeuchi N, Izumi S-I: **Maladaptive plasticity for motor recovery after stroke: mechanisms and approaches**. *Neural plasticity* 2012, **2012**.

108. Calautti C, Leroy F, Guincestre J-Y, Marie R-M, Baron J-C: **Sequential activation brain mapping after subcortical stroke: changes in hemispheric balance and recovery**. *Neuroreport* 2001, **12**(18):3883-3886.

109. Cramer SC, Bastings EP: **Mapping clinically relevant plasticity after stroke**. *Neuropharmacology* 2000, **39**(5):842-851.

110. Ramanathan D, Conner JM, Tuszynski MH: **A form of motor cortical plasticity that correlates with recovery of function after brain injury**. *Proceedings of the National Academy of Sciences* 2006, **103**(30):11370-11375.

111. Puckett AC, Pandya PK, Moucha R, Dai W, Kilgard MP: **Plasticity in the rat posterior auditory field following nucleus basalis stimulation**. *Journal of neurophysiology* 2007, **98**(1):253-265.

112. Kilgard MP, Merzenich MM: **Cortical map reorganization enabled by nucleus basalis activity**. *Science* 1998, **279**(5357):1714-1718.

113. Kilgard MP, Pandya PK, Vazquez J, Gehi A, Schreiner CE, Merzenich MM: **Sensory input directs spatial and temporal plasticity in primary auditory cortex**. *Journal of neurophysiology* 2001, **86**(1):326-338.

114. Chen N, Sugihara H, Sharma J, Perea G, Petravicz J, Le C, Sur M: **Nucleus basalis-enabled stimulus-specific plasticity in the visual cortex is mediated by astrocytes**. *Proceedings of the National Academy of Sciences* 2012, **109**(41):E2832-E2841.

115. Chubykin AA, Roach EB, Bear MF, Shuler MGH: **A cholinergic mechanism for reward timing within primary visual cortex**. *Neuron* 2013, **77**(4):723-735.

116. Meintzschel F, Ziemann U: **Modification of practice-dependent plasticity in human motor cortex by neuromodulators**. *Cerebral Cortex* 2006, **16**(8):1106-1115.

117. Torres E, Perry T, Blokland A, Wilkinson LS, Wiley R, Lappi D, Dunnett SB: **Behavioural, histochemical and biochemical consequences of selective immunolesions in discrete regions of the basal forebrain cholinergic system**. *Neuroscience* 1994, **63**(1):95-122.

118. Nag N, Baxter MG, Berger-Sweeney JE: **Efficacy of a murine-p75-saporin immunotoxin for selective lesions of basal forebrain cholinergic neurons in mice**. *Neuroscience letters* 2009, **452**(3):247-251.

119. Hamlin AS, Windels F, Boskovic Z, Sah P, Coulson EJ: **Lesions of the basal forebrain cholinergic system in mice disrupt idiothetic navigation**. *PloS one* 2013, **8**(1):e53472.

120. Moreau PH, Cosquer B, Jeltsch H, Cassel JC, Mathis C: **Neuroanatomical and behavioral effects of a novel version of the cholinergic immunotoxin mu p75-saporin in mice**. *Hippocampus* 2008, **18**(6):610-622.

121. Berger-Sweeney J, Stearns NA, Murg SL, Floerke-Nashner LR, Lappi DA, Baxter MG: **Selective immunolesions of cholinergic neurons in mice: effects on neuroanatomy, neurochemistry, and behavior**. *Journal of Neuroscience* 2001, **21**(20):8164-8173.

122. Nilsson O, Leanza G, Rosenblad C, Lappi D, Wiley R, Björklund A: **Spatial learning impairments in rats with selective immunolesion of the forebrain cholinergic system**. *Neuroreport* 1992, **3**(11):1005-1008.

123. Croxson PL, Browning PG, Gaffan D, Baxter MG: **Acetylcholine facilitates recovery of episodic memory after brain damage**. *Journal of Neuroscience* 2012, **32**(40):13787-13795.

124. Fine A, Hoyle C, Maclean C, Levatte T, Baker H, Ridley R: **Learning impairments following injection of a selective cholinergic immunotoxin, ME20. 4 IgG-saporin, into the basal nucleus of Meynert in monkeys**. *Neuroscience* 1997, **81**(2):331-343.

125. Conner J, Kulczycki M, Tuszynski M: **Unique contributions of distinct cholinergic projections to motor cortical plasticity and learning**. *Cerebral Cortex* 2010, **20**(11):2739-2748.

126. Ramanathan D, Tuszynski MH, Conner JM: **The basal forebrain cholinergic system is required specifically for behaviorally mediated cortical map plasticity**. *Journal of Neuroscience* 2009, **29**(18):5992-6000.

127. Arnold HM, Fadel J, Sarter M, Bruno JP: **Amphetamine-stimulated cortical acetylcholine release: role of the basal forebrain**. *Brain research* 2001, **894**(1):74-87.

128. Martinsson L, Hårdemark HG, Eksborg S: **Amphetamines for improving recovery after stroke**. *The Cochrane Library* 2007.

129. Garcia-Colunga J, Awad J, Miledi R: **Blockage of muscle and neuronal nicotinic acetylcholine receptors by fluoxetine (Prozac)**. *Proceedings of the National Academy of Sciences* 1997, **94**(5):2041-2044.

130. Zorowitz RD: **Road to recovery: drugs used in stroke rehabilitation**. *Expert review of neurotherapeutics* 2004, **4**(2):219-231.

131. Nadeau SE, Behrman AL, Davis SE, Reid K: **Donepezil as an adjuvant to constraint-induced therapy for upper-limb dysfunction after stroke: an exploratory randomized clinical trial**. *Journal of rehabilitation research and development* 2004, **41**(4):525.

132. Berthier ML, Hinojosa J, del Carmen Martín M, Fernández I: **Open-label study of donepezil in chronic poststroke aphasia**. *Neurology* 2003, **60**(7):1218-1219.

133. Berthier M, Green C, Higueras C, Fernandez I, Hinojosa J, Martin M: **A randomized, placebo-controlled study of donepezil in poststroke aphasia**. *Neurology* 2006, **67**(9):1687-1689.

134. Berthier ML, Green C: **Donepezil improves speed and accuracy of information processing in chronic post-stroke aphasia**. In: *Neurology: 2007*: LIPPINCOTT WILLIAMS & WILKINS 530 WALNUT ST, PHILADELPHIA, PA 19106-3621 USA; 2007: A10-A10.

135. Hasselmo ME: **The role of acetylcholine in learning and memory**. *Current opinion in neurobiology* 2006, **16**(6):710-715.

136. Gold PE: **Acetylcholine modulation of neural systems involved in learning and memory**. *Neurobiology of learning and memory* 2003, **80**(3):194-210.

137. Rasmusson D: **The role of acetylcholine in cortical synaptic plasticity**. *Behavioural brain research* 2000, **115**(2):205-218.

138. Fink CG, Murphy GG, Zochowski M, Booth V: **A dynamical role for acetylcholine in synaptic renormalization**. *PLoS Comput Biol* 2013, **9**(3):e1002939.

139. Bear MF, Singer W: **Modulation of visual cortical plasticity by acetylcholine and noradrenaline**. 1986.

140. Fanselow EE, Richardson KA, Connors BW: **Selective, state-dependent activation of somatostatin-expressing inhibitory interneurons in mouse neocortex**. *Journal of Neurophysiology* 2008, **100**(5):2640-2652.

141. Delmas P, Brown DA: **Pathways modulating neural KCNQ/M (Kv7) potassium channels**. *Nature Reviews Neuroscience* 2005, **6**(11):850-862.

142. Lambe EK, Picciotto MR, Aghajanian GK: **Nicotine induces glutamate release from thalamocortical terminals in prefrontal cortex**. *Neuropsychopharmacology* 2003, **28**(2):216-225.

143. Kruglikov I, Rudy B: **Perisomatic GABA release and thalamocortical integration onto neocortical excitatory cells are regulated by neuromodulators**. *Neuron* 2008, **58**(6):911-924.

144. Oldford E, Castro-Alamancos M: **Input-specific effects of acetylcholine on sensory and intracortical evoked responses in the "barrel cortex" in vivo**. *Neuroscience* 2003, **117**(3):769-778.

145. Yang Y, Ge W, Chen Y, Zhang Z, Shen W, Wu C, Poo M, Duan S: **Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine**. *Proceedings of the National Academy of Sciences* 2003, **100**(25):15194-15199.

146. Henneberger C, Papouin T, Oliet SH, Rusakov DA: **Long-term potentiation depends on release of D-serine from astrocytes**. *Nature* 2010, **463**(7278):232-236.

147. De Pittà M, Brunel N, Volterra A: **Astrocytes: orchestrating synaptic plasticity?** *Neuroscience* 2016, **323**:43-61.

148. Agulhon C, Fiacco TA, McCarthy KD: **Hippocampal short-and long-term plasticity are not modulated by astrocyte Ca2+ signaling**. *Science* 2010, **327**(5970):1250-1254.

149. Chen N, Sugihara H, Sharma J, Perea G, Petravicz J, Le C, Sur M: **Nucleus basalis-enabled stimulus-specific plasticity in the visual cortex is mediated by astrocytes**. *Proceedings of the National Academy of Sciences* 2012, **109**(41):E2832–E2841.

150. Takata N, Mishima T, Hisatsune C, Nagai T, Ebisui E, Mikoshiba K, Hirase H: **Astrocyte calcium signaling transforms cholinergic modulation to cortical plasticity in vivo**. *Journal of Neuroscience* 2011, **31**(49):18155-18165.

151. Navarrete M, Perea G, de Sevilla DF, Gómez-Gonzalo M, Núñez A, Martín ED, Araque A: **Astrocytes Mediate In Vivo Cholinergic-Induced Synaptic Plasticity**. *PLoS biology* 2012, **10**(2):e1001259.

152. Seol GH, Ziburkus J, Huang S, Song L, Kim IT, Takamiya K, Huganir RL, Lee H-K, Kirkwood A: **Neuromodulators control the polarity of spike-timing-dependent synaptic plasticity**. *Neuron* 2007, **55**(6):919-929.

153. Couey JJ, Meredith RM, Spijker S, Poorthuis RB, Smit AB, Brussaard AB, Mansvelder HD: **Distributed network actions by nicotine increase the threshold for spike-timing-dependent plasticity in prefrontal cortex**. *Neuron* 2007, **54**(1):73-87.

154. Pawlak V, Wickens JR, Kirkwood A, Kerr JN: **Timing is not everything: neuromodulation opens the STDP gate**. *Spike-timing dependent plasticity* 2010:138.

155. Salgado H, Köhr G, Trevino M: **Noradrenergic 'tone'determines dichotomous control of cortical spike-timing-dependent plasticity**. *Scientific reports* 2012, **2**:417.

156. Yang K, Dani JA: **Dopamine D1 and D5 receptors modulate spike timing-dependent plasticity at medial perforant path to dentate granule cell synapses**. *Journal of Neuroscience* 2014, **34**(48):15888-15897.

157. Meunier CNJ, Callebert J, Cancela J-M, Fossier P: **Effect of dopaminergic D1 receptors on plasticity is dependent of serotoninergic 5-HT1A receptors in L5-pyramidal neurons of the prefrontal cortex**. *PloS one* 2015, **10**(3):e0120286.

158. Selden NR, Gitelman DR, Salamon-Murayama N, Parrish TB, Mesulam M-M: **Trajectories of cholinergic pathways within the cerebral hemispheres of the human brain**. *Brain : a journal of neurology* 1998, **121**(12):2249-2257.

159. Pineiro R, Pendlebury S, Smith S, Flitney D, Blamire A, Styles P, Matthews P: **Relating MRI changes to motor deficit after ischemic stroke by segmentation of functional motor pathways**. *Stroke* 2000, **31**(3):672-679.

160. Kataoka K, Hayakawa T, Kuroda R, Yuguchi T, Yamada K: **Cholinergic deafferentation after focal cerebral infarct in rats**. *Stroke* 1991, **22**(10):1291-1296.

161. Manns ID, Alonso A, Jones BE: **Discharge properties of juxtacellularly labeled and immunohistochemically identified cholinergic basal forebrain neurons recorded in association with the electroencephalogram in anesthetized rats**. *Journal of neuroscience* 2000, **20**(4):1505-1518.

162. Wenk GL: **The nucleus basalis magnocellularis cholinergic system: one hundred years of progress**. *Neurobiology of learning and memory* 1997, **67**(2):85-95.

163. Brown MT, Tan KR, O'Connor EC, Nikonenko I, Muller D, Lüscher C: **Ventral tegmental area GABA projections pause accumbal cholinergic interneurons to enhance associative learning**. *Nature* 2012, **492**(7429):452-456.

164. Kitabatake Y, Hikida T, Watanabe D, Pastan I, Nakanishi S: **Impairment of reward-related learning by cholinergic cell ablation in the striatum**. *Proceedings of the National Academy of Sciences* 2003, **100**(13):7965-7970.

165. Steriade M, Datta S, Pare D, Oakson G, Dossi RC: **Neuronal activities in brain-stem cholinergic nuclei related to tonic activation processes in thalamocortical systems**. *Journal of Neuroscience* 1990, **10**(8):2541-2559.

166. Fuller PM, Saper CB, Lu J: **The pontine REM switch: past and present**. *The Journal of physiology* 2007, **584**(3):735-741.

167. Steriade M: **Acetylcholine systems and rhythmic activities during the waking–sleep cycle**. *Progress in brain research* 2004, **145**:179-196.

168. Yeomans JS, Mathur A, Tampakeras M: **Rewarding brain stimulation: role of tegmental cholinergic neurons that activate dopamine neurons**. *Behavioral neuroscience* 1993, **107**(6):1077.

169. Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K: **Millisecond-timescale, genetically targeted optical control of neural activity**. *Nature neuroscience* 2005, **8**(9):1263-1268.

170. Roth BL: **DREADDs for neuroscientists**. *Neuron* 2016, **89**(4):683-694.

171. MacLaren DA, Browne RW, Shaw JK, Krishnan Radhakrishnan S, Khare P, España RA, Clark SD: **Clozapine-n-oxide administration produces behavioral effects in Long-Evans rats - implications for designing DREADD experiments**. *eneuro* 2016.

172. Sharp FR, Sagar S, Hicks K, Lowenstein D, Hisanaga K: **c-fos mRNA, Fos, and Fos-related antigen induction by hypertonic saline and stress**. *Journal of Neuroscience* 1991, **11**(8):2321-2331.

173. Jhuang H, Garrote E, Yu X, Khilnani V, Poggio T, Steele AD, Serre T: **Automated home-cage behavioural phenotyping of mice**. *Nature communications* 2010, **1**:68.

174. Roelfsema PR, van Ooyen A, Watanabe T: **Perceptual learning rules based on reinforcers and attention**. *Trends in cognitive sciences* 2010, **14**(2):64-71.

175. Cassaday HJ, Moran PM: **Latent inhibition and other salience modulation effects: same neural substrates**. *Latent Inhibition: Cognition, Neuroscience, and Applications to Schizophrenia* 2010:342-371.

176. Subramanian SK, Massie CL, Malcolm MP, Levin MF: **Does provision of extrinsic feedback result in improved motor learning in the upper limb poststroke? A systematic review of the evidence**. *Neurorehabilitation and neural repair* 2010, **24**(2):113-124.

177. Vom Saal W, Jenkins HM: **Blocking the development of stimulus control**. *Learning and Motivation* 1970, **1**(1):52-64.

178. Donahoe JW, Vegas R: **Pavlovian conditioning: the CS-UR relation**. *Journal of Experimental Psychology: Animal Behavior Processes* 2004, **30**(1):17.

179. Prather DC: **Trial-and-error versus errorless learning: Training, transfer, and stress**. *The American journal of psychology* 1971:377-386.

180. Terrace H: **Extinction of a discriminative operant following discrimination learning with and without errors**. *Journal of the Experimental Analysis of Behavior* 1969, **12**(4):571-582.

181. Kincaid SL: **Effects of Gradual and Rapid Variable-Interval Schedule Thinning on Concurrent Schedule Performance in Pigeons**: West Virginia University; 2013.

182. Premack D: **Reversibility of the reinforcement relation**. *Science* 1962, **136**(3512):255-257.

183. Klatt KP, Morris EK: **The Premack principle, response deprivation, and establishing operations**. *The Behavior Analyst* 2001, **24**(2):173.

184. Williams BA: **The blocking of reinforcement control**. *Journal of the experimental analysis of behavior* 1975, **24**(2):215-225.

185. Sidman M: **Normal sources of pathological behavior**. *Science* 1960.

186. Sidman M: **BY-PRODUCTS OF AVERSIVE CONTROL**. *Journal of the Experimental Analysis of Behavior* 1958, **1**(3):265-280.

187. Holz WC, Azrin NH: **Discriminative properties of punishment**. *Journal of the Experimental Analysis of Behavior* 1961, **4**(3):225-232.

188. Eggermont JJ: **Hearing loss, hyperacusis, or tinnitus: what is modeled in animal research?** *Hearing research* 2013, **295**:140-149.

189. Williams BA: **Contrast, signaled reinforcement, and the relative law of effect**. *The American Journal of Psychology* 1980:617-629.

190. Capaldi ED, Sheffer JD: **Contrast and reinforcement in consumption**. *Learning and Motivation* 1992, **23**(1):63-79.

191. Williams BA: **Behavioral contrast and reinforcement value**. *Learning & behavior* 1991, **19**(4):337-344.

192. Papini MR: **Diversity of adjustments to reward downshifts in vertebrates**. *International Journal of Comparative Psychology* 2014, **27**(3).

193. Gordon J, Ghilardi MF, Ghez C: **Impairments of reaching movements in patients without proprioception. I. Spatial errors**. *Journal of neurophysiology* 1995, **73**(1):347-360.

194. Myhrer T: **Neurotransmitter systems involved in learning and memory in the rat: a meta-analysis based on studies of four behavioral tasks**. *Brain Research Reviews* 2003, **41**(2):268-287.

195. Huang S, Treviño M, He K, Ardiles A, de Pasquale R, Guo Y, Palacios A, Huganir R, Kirkwood A: **Pull-push neuromodulation of LTP and LTD enables bidirectional experience-induced synaptic scaling in visual cortex**. *Neuron* 2012, **73**(3):497-510.

196. Briand LA, Gritton H, Howe WM, Young DA, Sarter M: **Modulators in concert for cognition: modulator interactions in the prefrontal cortex**. *Progress in neurobiology* 2007, **83**(2):69-91.

197. Bechtel W, Hamilton A: **Reduction, integration, and the unity of science: Natural, behavioral, and social sciences and the humanities**. *General philosophy of science: Focal issues* 2007:377-430.

198. Betz D: **Imaging and Quantifying the Response of Cholinergic Projections from the Nucleus Basalis of the Cortex after Cortical Stroke**. *Undergraduate Honors Thesis.* University of Texas, Dallas; 2017.