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Acute inactivation of the contralesional hemisphere for longer durations improves recovery after cortical injury

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Résumé

Au cours des dernières années, des méthodes non-invasives de stimulations permettant de moduler l'excitabilité des neurones suivant des lésions du système nerveux central ont été développées. Ces méthodes sont maintenant couramment utilisées pour étudier l'effet de l'inhibition du cortex contralésionnel sur la récupération motrice à la suite d'un accident vasculocérébral (AVC). Bien que plusieurs de ces études rapportent des résultats prometteurs, les paramètres permettant une récupération optimale demeurent encore inconnus.
Chez les patients victimes d'un AVC, il est difficile de débuter les traitements rapidement et d'initier l'inhibition dans les heures suivant la lésion. L'impact de ce délai est toujours inconnu. De plus, aucune étude n'a jusqu'à maintenant évalué l'effet de la durée de l'inhibition sur la récupération du membre parétique. Dans le laboratoire du Dr Numa Dancause, nous avons utilisé un modèle bien établi de lésion ischémique chez le rat pour explorer ces questions. Nos objectifs étaient d'évaluer 1) si une inactivation de l'hémisphère contralésionnel initiée dans les heures qui suivent la lésion peut favoriser la récupération et 2) l'effet de la durée de l'inactivation sur la récupération du membre parétique.

Suite à une lésion dans le cortex moteur induite par injections d'un vasoconstricteur, nous avons inactivé l'hémisphère contralésionnel à l'aide d'une pompe osmotique assurant l'infusion continue d'un agoniste du GABA (Muscimol). Dans différents groupes expérimentaux, nous avons inactivé l'hémisphère contralésionnel pour une durée de 3, 7 et 14 jours suivant la lésion. Dans un autre groupe, le Muscimol a été infusé pour 14 jours mais à un débit moindre de façon à pouvoir étudier le lien entre la fonction du membre non-parétique et la récupération du membre parétique. Les données comportementales de ces groupes ont été comparées à celles d'animaux ayant récupéré de façon spontanée d'une lésion similaire.

Nos résultats indiquent que l'augmentation de la durée de l'inactivation (de 3 à 14 jours) accélère la récupération du membre parétique. De plus, les deux groupes ayant reçu une inactivation d'une durée de 14 jours ont montré une plus grande récupération fonctionnelle que le groupe n'ayant pas reçu d'inactivation de l'hémisphère contralésionnel, le groupe contrôle. Nos résultats suggèrent donc que l'inactivation de l'hémisphère contralésionnel initiée dans les heures suivant la lésion favorise la récupération du membre parétique.

La durée d'inhibition la plus efficace (14 jours) dans notre modèle animal est beaucoup plus longues que celles utilisées jusqu'à maintenant chez l'homme. Bien qu'il soit difficile d'extrapoler la durée idéale à utiliser chez les patients à partir de nos données, nos résultats suggèrent que des traitements de plus longue durée pourraient être bénéfiques.

Finalement, un message clair ressort de nos études sur la récupération fonctionnelle après un AVC: dans le développement de traitements basés sur l'inhibition de l'hémisphère contralésionnel, la durée de l'inactivation est un facteur clef à considérer.

Mots-clés: accident vasculocérébral, AVC, avant-bras, contralésionnel; cortex; inhibition; inactivation; lésion; main; rat; récupération motrice

Abstract

With the introduction of non-invasive brain stimulation methods aimed at modulating the excitability of cortical areas after stroke, many groups are intensively investigating the effects of inhibition of the contralesional hemisphere on functional recovery. Although the reported results of these studies are very promising, limitations of enrolling acute stroke patients as well as technical difficult of establishing continuous inhibition protocols have left several open ended questions regarding the treatment parameters and patient selection. For example, the efficacy of inhibition treatment in acute setting after stroke and the effect of treatment duration are two questions that are virtually unexplored.

Therefore, in the laboratory of Prof. Numa Dancause, we took advantage of a well established rodent model of cortical ischemic lesion to gain direct and objective insight about the importance of contralesional inactivation on motor recovery of the paretic limb. Using an Endothelin-1 rodent model of ischemic cortical lesion, we pharmacologically inactivated the contralesional hemisphere with a GABA agonist (Muscimol). By doing so we were interested in the effect of early treatment when contralesional inactivation is initiated rapidly after the lesion.

Early after induction of cortical ischemic lesion, the contralesional hemisphere was inactivated with continuous infusion of the Muscimol for 3, 7 or 14 days in three different groups of animals. In a fourth group, Muscimol was infused at slower rate for 14 days to provide additional insights on the relation between the effects of inactivation on the non-paretic forelimb behavior and the recovery of the paretic forelimb. We included a group of animals with spontaneous recovery that received no inactivation after lesion.

Our results indicated that increasing inactivation duration (from 3 to 14 days) accelerated the recovery of grasping function. Both groups with 14 days of inactivation had similar recovery profiles and performed better than animals that spontaneously recovered. In fact, the duration of inactivation, not the intensity, correlated with the better functional outcomes.

Our results support early contralesional inactivation to improve recovery of the paretic forelimb after cortical lesion. Moreover, based on our results, the duration of inactivation is the most important factor to correlate with the functional outcomes. Therefore, by providing precise temporal and behavioral evidence, our results provide a window of opportunity for the researchers in which the current gap in our understanding of the clinical efficacy of contralesional inhibition in acute phase after stroke can be approached with more confidence.

Keywords: contralesional; cortex; forelimb; hand; inhibition; inactivation; lesion; stroke; rat; recovery

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To the Teachers

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Throughout my graduate studies I have been blessed to be around remarkable individuals who helped me in various aspects of my development. Clearly my acknowledgement must start with my advisor and mentor, Prof. Numa Dancause who was brave enough to take a chance on me two and a half years ago. I will forever be influenced by his excellent guidance. Numa has shown me how to be a compassionate and successful mentor while establishing and maintaining a top-level research laboratory. He is, and will continue to be, my role model as I progress in my clinical-academic career.

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CHAPTER I.

General introduction

Stroke is the leading cause of disability worldwide (Go et al., 2013). Morbidity and mortality with acute stroke is substantial (Hartmann et al., 2001) however in long term, some of the initial deficits of the survived patients are subject to various degrees of recovery (Langhorne et al., 2009). Depending on the underlying cause of the stroke, up to sixty percent of stroke patients will still suffer from significant functional deficits a year after stroke incidence (Petty et al., 2000). With 700,000 cases per year in north America, this signifies that one person dies from stroke every three minutes and two are left with various degrees of permanent disability (Feigin et al., 2009).

The recent improvements in the management of acute stroke are reflected in the substantial increase of stroke survivors, many of whom show persistent neurological deficits in terms of paresis, aphasia, apraxia or neglect (Langhorne et al., 2009). Therefore, investigating the reparative events in the brain after stroke, neuroprotective therapies and developing strategies to augment the efficacy of rehabilitative techniques is the priority of stroke research.

In the last few years, several approaches to increase adaptive plasticity and recovery after stroke have been proposed and are currently being tested. In particular, many groups are intensively investigating the effects of inhibition of the contralesional hemisphere on functional recovery (Hummel and Cohen, 2006). The rationale behind the treatment strategy used in most studies is the concept of post-stroke interhemispheric imbalance (Liepert et al., 2000; Nowak et al., 2009). According to this hypothesis, increased activity in the contralesional hemisphere after stroke exerts an augmented inhibitory influence on the ipsilesional hemisphere. By doing so, it

interferes with function and adaptive plasticity in the ipsilesional hemisphere and hence the recovery of the paretic arm. Therefore, one proposed method of reducing interhemispheric imbalance is to inhibit the contralesional hemisphere.

Interhemispheric inhibition in health and after stroke

In normal conditions, both hemispheres are engaged in a mutual transcallosal interhemispheric inhibition which contributes to the functional coupling of the two hemispheres at the onset and during performance of voluntary movements (Jeeves et al., 1988; Ferbert et al., 1992; Gerloff et al., 1998; Di Lazzaro et al., 1999; Grefkes et al., 2008). It has been shown that when one hemisphere is inhibited in healthy subjects with low frequency transcranial stimulation methods, the excitability of contralateral hemisphere is augmented (Plewnia et al., 2003; Schambra et al., 2003; Kim et al., 2004), the performance of the ipsilateral hand is increased (Kobayashi et al., 2004; Dafotakis et al., 2008) and the interhemispheric inhibition from contralateral hemisphere to the inhibited hemisphere is enhanced significantly (Lang et al., 2004).

The transcallosal imbalance after stroke follows a similar principle. A substantial body of animal and human studies has documented increased excitability of contralesional sensorimotor brain areas after stroke (Liepert et al., 2000; Marshall et al., 2000; Abo et al., 2001; Manganotti et al., 2002; Wittenberg et al., 2007) because of the decreased inhibitory transcallosal inputs from ipsilesional to the contralesional (intact) hemisphere. The disinhibited contralesional cortex (Traversa et al., 1998; Liepert et al., 2000; Cicinelli et al., 2003; Rossini et al., 2003) exerts further inhibitory transcallosal inputs into the ipsilesional hemisphere (Murase et al., 2004), which possibly contributes to the loss of function in addition to that of the damage to corticospinal fibers (Nowak et al., 2009). The magnitude of increased excitability in

contralesional hemisphere (Liepert et al., 2000; Manganotti et al., 2002, 2008; Nardone and Tezzon, 2002a; Shimizu et al., 2002) and the interhemispheric inhibition from contralesional to ipsilesional hemisphere (Murase et al., 2004; Duque et al., 2005) is found to be proportional to the motor impairment in stroke patients.

Possible mechanism of hyperexcitability of contralesional hemisphere after stroke

The increased neuronal activity of the contralesional hemisphere occurs in a very short time (< 1 hour) after cortical damage (Mohajerani et al., 2011). In this short time, the post-stroke rewiring and experience cannot not be cause of the enhanced contralesional activity. This effect is most probably a result of immediate circuit loss or de-afferentaion of glutamatergic transcallosal fibers from the ipsilesional to the contralesional hemisphere (Buchkremer-Ratzmann and Witte, 1997). In fact, if the lesion spares the cortical layer V (from which the main callosal output arises) there will be no subsequent contralesional hyperexcitability.

In the contralesional hemisphere of acute and subacute stroke patients with cortical and subcortical lesions, the balance of excitatory and inhibitory activity is shifted towards an increase of excitatory activity (a lower threshold for activation of excitatory interneurons) due to decreased potency of inhibitory circuits (Bütefisch et al., 2003a). In human stroke subjects, experimental paired pulse stimulation paradigms with non-invasive stimulus patterns to measure short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF) has been widely used to study the changes of the potency of inhibitory and excitatory circuits at the cortical level. SICI is a type of intracortical inhibition that can be studied by paired TMS. The protocol to measure SICI involves a sub-threshold conditioning stimulus followed by a supra-threshold test stimulus applied at the same cortical site with one coil (Kujirai et al., 1993). The test response is

typically inhibited at inter-stimulus intervals (ISI) of 1-6 ms. There is considerable evidence that SICI occurs within the cortex itself rather than in sub-cortical structures and reflects the potency of GABAergic inhibitory circuits (Kujirai et al., 1993; Chen et al., 1998; Di Lazzaro et al., 1998). ICF is tested using protocols similar to SICI with a sub-threshold conditioning stimulus followed by a supra-threshold test stimulus that is also applied at the same cortical site with one coil (Kujirai et al., 1993). Facilitation occurs at an ISI of 8-30 ms. Similar to SICI, ICF occurs at the cortical level rather than in subcortical structures (Ziemann et al., 1996b; Nakamura et al., 1997) and appears to be mediated by separate neuronal populations from SICI (Chen et al., 1998; Ashby et al., 1999; Strafella and Paus, 2001).

For example, Bütefisch and collaborators documented a marked hyperexcitability of contralesional hemisphere in terms of decreased SICI after paired-pulse stimulation of contralesional hemisphere (Bütefisch et al., 2003a). In this experiment, they used a paired pulse paradigm with a single TMS coil where conditioning stimulus (CS) of about 80% of motor threshold (MT) was delivered 2 ms before a test stimulus (TS) with intensity of 110% of MT at the cortical area that would elicit a maximal response in first dorsal interosseous muscle (FDI) of the non-paretic hand. The diminished SICI they found was attributed to GABA down regulation (Ziemann et al., 1996a, 1996b; Ashby et al., 1999). Moreover, Bütefisch and collaborators (2003a) demonstrated decreased threshold for excitation of excitatory circuits of the contralesional hemisphere. For the latter experiment, they used a paired pulse paradigm with a constant ISI of 2 ms, while the CS varied from 20% to 100 % of MT and TS was fixed at 110% of MT. They observed that in CL hemisphere of stroke patients, the excitation threshold was decreased to less than 50-60% of MT compared to normal subject where the threshold is about 70% of MT. So all in all, the new electrophysiological evidence in human stroke survivors has

demonstrated a hyperexcitability of CL hemisphere, which is linked to the down regulation of GABA.

Contralesional GABA-Receptor down regulation has been demonstrated by more direct molecular studies as well. Qü and collaborators, in a series of experiments using autoradiography, investigated the density of [^{3}H] muscimol binding sites to GABA-A receptors in ipsi- and contralateral hemisphere in two animal models of stroke 7 days after the lesion in mice (Qü et al., 1998b), and rats (Qü et al., 1998a). In both experiments, they observed a drastic decrease of the [^{3}H] muscimol binding in contralesional hemisphere. In the latter study, in addition to the autoradiographic evidence of [^{3}H] muscimol binding in contralesional hemisphere, they used electrophysiology on 400 µm rat brain slices 7 days after the lesion. In the cortex of contralesional hemisphere, they recorded field potentials from layer 2 and stimulated the layer 6 in a pattern that activates GABA mediated inhibitory circuits. They observed a reduction of GABA mediated inhibition in CL hemisphere that correlated with the diminished density of [^{3}H] muscimol binding (autoradiography) in these animals.

Similarly, Lee and Yamashita (Lee et al., 2011) showed a decrease in expression of $GABA_A$ R-α1 subunits in the contralesional hemisphere for at least two weeks after a traumatic brain injury (TBI) in the primary somatosensory cortex of mice. This study used real-time reverse-transcriptase PCR to look at the gene expression in terms of mRNA of various $GABA_A$-Receptor subunits. The results demonstrated a decreased expression of α1 and γ2 subunits in CL hemisphere as soon as 7 days after injury. Next, they investigated the temporal down-regulation of $GABA_A$ R-α1 protein in CL hemisphere by immunohistochemical staining: At 2 weeks after the injury the expression of $GABA_A$ R-α1 was decreased in all cortical layers and this effect lingered on in Layer II, III, and V till 4 weeks after the injury. In this study, there was a

correlation between the downregulation of GABA and upregulation of activity dependent gene (Alivin-1) in the CL hemisphere. Hence, the down-regulation of GABA could be associated with increased neuronal activity in CL hemisphere.

Contralesional inhibition after stroke

The precise role of lesion induced changes in the contralesional cortex in functional reorganization and recovery after stroke is yet to be determined, but at least in small cortical lesions, recent studies have suggested a detrimental role for increased excitability of homotopic contralesional cortex (Liepert et al., 2000; Neumann-Haefelin and Witte, 2000; Nardone and Tezzon, 2002b; Duque et al., 2005; Manganotti et al., 2008). In the current literature, this phenomenon has been widely attributed to abnormally increased interhemispheric inhibition from contralesional to ipsilesional primary motor cortex (Murase et al. 2004; Duque et al. 2005) after stroke.

Consistent with the model of interhemispheric inhibition, new lines of investigation have provided insight about the importance of decreasing the excitability of contralesional hemisphere in promoting recovery after stroke. Support for this hypothesis is provided by fMRI connectivity studies that demonstrated that improvements of motor function after low frequency repetitive transcranial magnetic stimulation (rTMS) over contralesional M1 is associated with a reduction of interhemispheric inhibition between the contralesional and ipsilesional primary motor cortices (Grefkes et al., 2010).

TMS and transcranial direct current stimulation (tDCS) methods have been the mainstay of the above-mentioned experiments to change the excitability of contralesional hemisphere. TMS is a well-established non-invasive method in brain research to interfere with and measure the

cortical activity (Dimyan and Cohen, 2010). A very short magnetic pulse introduced via the scalp induces an electric field in the underlying brain that activates the axons and/or neurons. When applied as repetitive rTMS in a train of several thousand pulses, the TMS can be used to change the excitability of cortical neurons for minutes to several hours depending on the stimulation frequency and duration (Rothwell, 1997; Di Lazzaro et al., 2004). The exact neurobiological mechanisms of the sustained effect of rTMS in humans in not clear yet, but it has been shown to resemble long term depression (Di Lazzaro et al., 2004).

In humans, reducing the excitability of primary motor cortex of the contralesional hemisphere by means of low-frequency (≤ 1Hz) rTMS is shown to improve the function of the paretic hand in sub-acute and chronic stages of stroke (Hummel and Cohen, 2006). Recent evidence suggest that the neuromodulatory interventions by means of low frequency rTMS are particularly more effective when applied on a regular basis (Conforto et al., 2011; Avenanti et al., 2012). For example, in a sham controlled study, Avenanti and collaborators found that inhibiting the contralesional hemisphere with daily sessions of rTMS in the span of 10 days resulted in a significant increase in excitability of ipsilesional M1(Avenanti et al., 2012). The intervention in Avenanti's study caused a significant decrease in interhemispheric inhibition to the ipsilesional cortex in terms of decreased ipsilesional silent period (Avanzino et al., 2007) meaning that the observed effect was mediated by transcallosal rather than corticospinal pathways (Boroojerdi et al., 1996; Meyer et al., 1998). The behavioral improvements after the aforementioned intervention, as indicated by grip force and dexterity, persisted for three months.

Cathodal (i.e. inhibitory) tDCS has also been widely used in proof-of-principle studies to reduce the excitability of contralesional hemisphere after stroke (Fregni et al., 2005; Hummel et al., 2005; Sparing et al., 2009). Despite the fundamental neuromodulatory difference between the

tDCS and rTMS, the functional outcomes following the contralesional hemisphere inhibition at least in mild to moderate hand impairment seems to be similar (Hummel and Cohen, 2006). In tDCS, a small transcranial current (1-2 mA) is applied via surface electrodes. Because of the electrical resistance of SCALP and cranium, this current is not sufficient enough to induce action potentials in cortical neurons. Thus, in contrast to TMS, it does not cause a muscle twitch when used. tDCS alters the level of intrinsic postsynaptic activity by causing a shift in the membrane potential. Depending on the direction of the electrical flow, a bi-directional shift in the membrane potential can be achieved. Hence tDCS can be used to increase (anodal stimulation) or decrease (cathodal stimulation) the excitability of neurons underneath the stimulation electrode (Nitsche and Paulus, 2000).

Zimmerman and collaborators recently showed that applying cathodal tDCS on the contralesional hemisphere significantly improves learning of a new finger sequence in stroke patients (Zimerman et al., 2012). By employing a paired pulse paradigm on the ipsilesional M1, they associated the improvements in the motor performance to decreased potency of inhibitory circuits in the ipsilesional M1. This implies that interhemispheric modulation of GABAergic circuits in ipsilesional hemisphere is responsible for the behavioral effect following contralesional inhibition. Likewise, similar to rTMS studies, the behavioral gain achieved by contralesional cathodal tDCS correlates with significant movement related fMRI activity in ipsilesional cortex in moderately impaired stroke patients (Stagg et al., 2012). In other words, the ipsilesional increase in the M1 BOLD signal following the contralesional inhibition is associated with better motor performance. This is perfectly in line with a very recent meta-analysis (Rehme et al., 2012) that has demonstrated that irrespective of the method of contralesional inhibition,

restoration of neuronal activity in ipsilesional primary motor cortex is the most critical factor that underlies motor recovery after stroke.

Limitations of human studies: major questions to be answered

The effect of lesion size

The studies reviewed above strongly suggest that non-invasive contralesional brain stimulation can become an important treatment approach in neurorehabilitation after stroke. This claim is supported by functional imaging studies that cast light on the pathophysiological disturbances in cortical networks after stroke. According to these studies, persistent overactivity of contralesional areas is often observed in patients with less successful recovery of function, a finding that is typically associated with severe corticospinal tract lesions (Ward et al., 2007; Schaechter et al., 2008).

Thus, the initial impairment and the lesion size seem to be a critical factor in treatment response after contralesional hemisphere inhibition. Nair and collaborators recently applied cathodal tDCS on the contralesional M1 for 5 days in a group of chronic stroke patients with severe impairments of upper extremity (Nair et al., 2011). The authors discovered that in severely paretic patients, tDCS was able to effectively reduce the contralesional overactivity, however the ipsilesional activity was increased only in a few patients and was not significantly different from that of sham stimulation. Similar negative findings have been attributed in part to ceiling effect (Malcolm et al., 2007) meaning that the amount of improvement achievable in severely impaired patients might be limited because of the extensive circuit loss.

In addition to the effect of lesion size, the previous studies have left open questions regarding the importance of inter-subject differences in stimulation sensitivity (Hamada et al.,

2012), lesion location and time after stroke (Hesse et al., 2011), and duration or number of treatment sessions necessary to achieve a sustained effect (Bestmann et al., 2004, 2010; Sarfeld et al., 2012). None of these possible parameters, however, has been effectively tested in a well controlled study (Khedr and Fetoh, 2010), which makes drawing a unanimous conclusion for proper patient selection virtually impossible.

The choice of inhibition: what cortical circuits are engaged?

A subset of studies is reporting no additional benefit over motor training with non-invasive brain stimulation methods other than low frequency rTMS or tDCS. For example, in two recent studies, Talelli and collaborators used continuous theta burst stimulation (cTBS) to inhibit the contralesional primary motor cortex of chronic stroke patients in single sessions (Talelli et al., 2007) or multiple sessions in combination with physical therapy for two weeks (Talelli et al., 2012). Compared to sham stimulation, patients demonstrated no additional benefit of contralesional inhibition in terms of grip force or skilled hand movements.

One possible mechanism for these contradictory results can be the difference of low frequency rTMS and cTBS in engaging the GABAergic interneurons in the stimulated cortex (Grefkes and Fink, 2012). Recent evidence from animal models suggest that cTBS and low frequency rTMS have selective effects on certain classes of cortical interneurons (Trippe et al., 2009). Low frequency rTMS induces a steady increase of the activity dependent proteins of the cortical inhibitory interneurons possibly leading to sustained but gradual increase of secretion and reuptake of GABA at the synaptic level, however, cTBS causes strong activation of inhibitory neurons leading to immediate but not sustained GABA release (Trippe et al., 2009; Benali et al., 2011). The latter animal evidence is supported by a recent study using magnetic

resonance spectroscopy that demonstrated robust but transient increase of cortical GABA content following cTBS in human motor cortex (Stagg et al., 2009).

As mentioned earlier in this chapter, down regulation of GABAergic system in the contralesional hemisphere seems to underlie the transcallosal imbalance after stroke. In this light, do the contradictory results from human studies simply reflect the differences of the various non-invasive inhibitory modalities in potentiating the GABAergic system?

The optimal time to deliver the intervention

With the introduction of non-invasive brain stimulation methods aimed at modulating the excitability of cortical areas after stroke, understanding the post-stroke adaptive and maladaptive events has become very critical to optimize the neuromodulatory interventions and proper patient selection. The evolution of activation in the sensory-motor cortex from early contralesional activity to late ipsilesional activity suggests that a dynamic bihemispheric reorganization of motor networks occurs during recovery from hemiparesis (Marshall et al., 2000). Therefore the immediate phase after stroke, when the transcallosal imbalance is at its maximal strength, seems to be a critical window for contralesional inhibition. Nonetheless, few studies have tested the effect of contralesional inhibition very early after stroke (Conforto et al., 2011), none of which has included more than one or two patients with less than one week after the ischemic attack. Instead, a rapidly growing number of studies using diverse protocols of inhibition of the contralesional cortex are implementing non invasive stimulation methods in subacute and chronic stages after cerebral ischemic attacks when the evidence of transcallosal imbalance gradually subside (Cramer, 2008).

While the human studies might have the limitations of enrolling acute stroke patients in proof of principle studies or implement comprehensive selection criteria, the well-established animal stroke models provide a window of opportunity in which the effect of contralesional inhibition can be investigated in a well controlled experimental environment. Using such a model, we used a pharmacological method of inactivation using a GABA agonist (Muscimol) to gain direct and objective insight about the importance of potentiating the GABA system in contralesional hemisphere on motor recovery of the paretic limb. In particular, we were interested in the effect of early treatment when contralesional inactivation is initiated rapidly after the lesion, the period during which increased activity in the contralesional hemisphere is at its highest.

CHAPTER II. Manuscript submission

The following manuscript is resubmitted to the journal of Experimental Neurology in November 2013.

Cover Page:

Acute inactivation of the contralesional hemisphere for longer durations improves recovery after cortical injury

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(The authors information is removed from the submission cover page according to the regulations of Canadian government to protect the personal information)

Running title: Acute contralesional inactivation after lesion

Summary:

A rapidly growing number of studies using inhibition of the contralesional hemisphere after stroke are reporting improvement in motor performance of the paretic hand. These studies have used different treatment onset time, duration and non-invasive methods of inhibition. Whereas these results are encouraging, several questions regarding the mechanisms of inhibition and the most effective treatment parameters are currently unanswered. In the present study, we used a rat model of cortical ischemic lesion to study the effects of GABA-mediated inactivation on motor recovery. In particular, we were interested in understanding better the effect of inactivation duration when it is initiated within hours following a cortical lesion. Cortical lesions were induced with endothelin-1 microinjections. The contralesional hemisphere was inactivated with continuous infusion of the GABA-A agonist Muscimol for 3, 7 or 14 days in three different groups of animals. In a fourth group, Muscimol was infused at slower rate for 14 days to provide additional insights on the relation between the effects of inactivation on the non-paretic forelimb behavior and the recovery of the paretic forelimb. In spontaneously recovered animals, the lesion caused a sustained bias to use the non-paretic forelimb and long-lasting grasping deficits with the paretic forelimb. Animals in which the contralesional hemisphere was inactivated for 3 and 7 days did not show such bias to use their non-paretic forelimb and inactivation of 14 days resulted in a bias to use the paretic forelimb. In contrast, infusion at a slower rate for 14 days did not caused this bias to use the paretic forelimb. Increasing inactivation duration also accelerated the recovery of grasping function. Both groups with 14 days of inactivation had similar recovery profiles and performed better than animals that spontaneously recovered. Whereas the plateau performance of the paretic forelimb correlated with the duration of contralesional inactivation, it was not correlated with the spontaneous use of the forelimbs or with grasping performance of the

non-paretic hand. Our results support that contralesional inactivation initiated within hours after a cortical lesion can improve recovery of the paretic forelimb. In our model, increasing the duration of the inactivation improved motor outcomes but the spontaneous use and motor performance of the non-paretic forelimb had no impact on recovery of the paretic forelimb.

Highlights:

- GABA-mediated inactivation of the contralesional hemisphere can improve recovery
- Inactivation initiated within hours after cortical lesions can improve recovery
- Inactivating the contralesional hemisphere for longer duration has better outcomes
- Inactivation duration correlates better with recovery than non-paretic limb behavior

Keywords: contralesional; cortex; forelimb; hand; inhibition; inactivation; lesion; stroke; rat; recovery

Introduction:

Several studies have shown that following a lesion, there an increase of cortical excitability in the contralesional hemisphere (Meyer et al., 1985; Mohajerani et al., 2011; Sakatani et al., 1990). The hyperexcitability in the contralesional cortex (Buchkremer-Ratzmann et al., 1996) is associated with a diminution of GABAergic inhibition (Witte and Stoll, 1997) and a reduction of GABA-A receptor binding (Lee et al., 2011; Qu et al., 1998), suggesting that it may be related to a decreased potency of the inhibitory GABAergic system. Longitudinal imaging studies in humans have shown that the contralesional activity is typically maximal early after the injury and progressively diminishes with time and recovery (Carey et al., 2006; Jaillard et al., 2005; Marshall et al., 2000).

In the last few years, several modulatory approaches using non-invasive stimulation techniques to favor adaptive plasticity and recovery after stroke have been proposed and are currently being tested. In particular, many groups are intensively investigating the effects of inhibition of the contralesional hemisphere on behavioral recovery (Hummel and Cohen, 2006). The rationale behind the treatment strategy used in most studies is based on the concept of interhemispheric imbalance (Liepert et al., 2000; Nowak et al., 2009). According to this hypothesis, hyperexcitability in the contralesional hemisphere results in an augmented inhibitory influence on the ipsilesional hemisphere. In this manner, the contralesional hemisphere would interfere with function and adaptive plasticity in the ipsilesional hemisphere and with recovery of the paretic arm. However, this hypothesis is far from being universally accepted.

While some studies using protocols of inhibition of the contralesional cortex with non-invasive stimulation techniques show improvement in motor performance of the paretic hand (Khedr et al., 2009; Nowak et al., 2008; Takeuchi et al., 2005), inhibition of contralesional areas

with atypically high activity in chronic stroke patients was also shown to interfere with performance of the paretic hand (Johansen-Berg et al., 2002; Lotze et al., 2006). To date, only a few studies have used multiple treatments sessions, many of them with different treatment duration and onset (Boggio et al., 2007; Fregni et al., 2006; Khedr et al., 2009). Therefore, the effect of inhibition duration on behavioral recovery is virtually unexplored. No study has yet tested the effect of contralesional inhibition initiated within hours following the lesion, when the interhemispheric imbalance should be maximal. Moreover, the mechanisms through which non-invasive stimulation methods can induce cortical inhibition and to what extent they act through GABA are not fully understood, leaving open the question if potentiating GABA-mediated inhibition of the contralesional hemisphere can improve recovery.

To provide some insight on these issues, we used a well-established method of inactivation consisting of continuous infusion of the GABA-A agonist Muscimol (Martin, 1991) in a rat model of ischemic cortical lesion. In particular, our objectives were to confirm that GABA-mediated inactivation and very early inactivation could favor recovery, and to study the effect of duration of contralesional inactivation on motor outcomes. These data increase our understanding of the basic interactions between inactivation of the contralesional hemisphere and recovery and may provide useful cues for the development of treatments based on contralesional inhibition after stroke.

Methods:

Animals

A total of 53 Sprague-Dawley rats (Charles River, QC, CA) of approximately 2 months of age and weighing 250-300 grams were included in the study. All animals were housed in solitary standard Plexiglas cages with reverse day-night cycle (7am-7pm). They were handled only

during the dark cycle. Upon their arrival at our facility, animals were familiarized with banana food pellets in the Montoya staircase test (Biernaskie and Corbett, 2001; Montoya et al., 1991) for 10 work-days (Figure 1). To incite reaching behavior in the Montoya test, food access was carefully monitored during the two weeks of familiarization. For each rat, the daily food minimum corresponded to 5% of its body weight. Rats had free access to 85% of their daily minimum in the home cage. They could obtain more food to surpass the 100% value in the Montoya staircase apparatus. On any given day, if the animal did not attain its daily minimum in the Montoya, additional food was supplied in its home cage to reach the daily minimum. Baseline data were collected at the end of the familiarization period. Spontaneous use of forelimbs in exploratory behavior was documented with the cylinder wall test (Schallert et al., 2000) and grasping function with the Montoya Staircase test (Montoya et al., 1991). Animals that performed above the inclusion criteria (see Montoya Staircase test below) were randomly assigned to an experimental group. During the post-lesion period, food was restricted for 12-14h prior to each behavioral testing session and animals were given free access to food after testing. The weight of the animals was recorded daily during the 2 weeks prior to the lesion and weekly after the lesion. If an animal lost more than 10% of its original body weight at any point during the experiment, it was excluded. Two animals from Group 14D were excluded from the study during the recovery period because of weight loss and seizures. Animals had ad libitum access to water at all times. Our experimental protocol followed the guidelines of the Canadian Council on Animal Care and was approved by the Comité de Déontologie de l'Expérimentation sur les Animaux of the Université de Montréal.

Measurement of Forelimb Asymmetry

To detect spontaneous asymmetrical use of forelimbs, rats were placed in a transparent cylinder of 19cm diameter and 33cm height for 3-30 minutes or until 60 touches to the cylinder wall was achieved (Schallert et al., 2000). Animals were videotaped from above using a high definition digital video camera (30 frames/second). The videos were analyzed frame-by-frame offline to count the use of paretic versus non-paretic limbs during vertical exploration of the cylinder wall. The forelimb asymmetry score was calculated using the following equation:

$$Asymmetry\ score = \frac{touches\ with\ paretic\ forelimb\ - touches\ with\ nonparetic\ forelimb}{total\ number\ of\ touches}$$

Montoya Staircase test: Grasping and retrieving performance

Rats were placed in a Plexiglas chamber (6-cm wide, 12-cm high and 30cm long) with a central platform (2.3-cm wide, 6-cm high and 19-cm long) that supports the weight, separating the right and left forelimbs (Biernaskie and Corbett, 2001; Montoya et al., 1991). A pair of staircases with seven steps on each side was loaded into the Plexiglas chamber on both sides of the central platform. Each step had a smooth well that can hold one to three standard 45mg banana flavored food pellets (Bioserve Inc., Frenchtown, NJ, USA). During the familiarization period, animals had a session of Montoya staircase in the morning and one in the afternoon, the two separated by 3 to 4 hours. In a session, the rats had 4 trials with each hand (8 trials per day in totals). Initially, for each trial, every well on one side of the staircase was filled with 3 food pellets and 15 minutes were given to retrieve the pellets. In the following days, the number of pellets in each well and the time provided was progressively tapered according to the performance of the rat. However, by the 8^{th} day, only one pellet per well and three minutes per trial were given to all rats. On the 9^{th} and 10^{th} days of the familiarization period, the performance in terms of the

number of eaten pellets was recorded and used to establish if the animal reached our inclusion criteria. To be included in the study, rats needed to eat 4 out of 7 pellets in 3 of the 4 trials on both days with one of the two arms. Based on these criteria, 9 animals were excluded from the study. The ischemic lesion was induced in the cortex contralateral to the arm with the best performance score.

Surgical Procedures

All surgical procedures were conducted aseptically. Anesthesia was induced with Ketamine (80mg/kg, intra-peritoneal) and maintained with isoflurane (~2% in 100% oxygen) delivered via a custom-made facial mask adapted to our stereotaxic frame. Pulse rate and oxygen saturation were monitored and documented during the surgery. A self-regulating heating blanket (Harvard Apparatus, Holliston, MA) was used to maintain body temperature during the surgery. A midline incision was made to expose the skull and neck muscles. A small incision was made between C0 and C1 to release cerebrospinal fluid of the cisterna magna and reduce intracranial pressure.

In order to confirm the location of the motor areas of 3-month-old Sprague Dawley rats, we conducted intracortical microstimulation (ICMS) mapping experiments in naïve animals (n=3) (Figure 1B). Following craniotomy and durectomy, anesthesia was switched to ketamine hydrochloride (~10mg/kg/10 minutes; intraperitoneal) for the collection of electrophysiological data. A glass coated tungsten microelectrode (~1 MΩ) was used for electrical stimulation applied at a depth of ~1600μm. Stimulation consisted of a 40ms train of 13 monophasic cathodal pulses of 200 μs delivered at 350 Hz from an electrically isolated, constant current stimulator (Dancause et al., 2006; Nudo et al., 1992; Nudo et al., 1996). Pulse trains were repeated at 1 Hz intervals; current was ≤100 μA. Microelectrode interpenetration distances were ~333μm.

Stimulations were done to identify the caudal and rostral forelimb areas (CFA and RFA). The two areas were typically separated with neck and vibrissa representations. Based on ICMS maps, we selected the stereotaxic coordinates for six injections of endothelin-1 (ET-1) as in Fang and collaborators (Fang et al., 2010) (Figure 1C). ICMS mapping was conducted in additional animals (n=3) at the end of the recovery period (day 60 after the lesion) and confirmed that the lesion destroyed large portion of the CFA and appeared to leave the RFA intact (Figure 1B).

To induce the cortical lesion, six 0.6-mm holes were drilled over the caudal forelimb area (CFA) based on stereotaxic coordinates (+1.5, +0.5, -0.5mm anteroposterior, +2.5, +3.5mm mediolateral to bregma; Figure 1B) (Fang et al., 2010). Using a 1µl Hamilton syringe, a microinjection of endothelin-1 (ET1) (EMD chemicals, CA, USA) was made in each hole at a depth of 1.5 mm in the cortex (0.33µl, 0.3 µg/µl, 3nl/s). Following each injection of endothelin-1 (ET-1), the holes were sealed with bone wax. After the lesion induction, an additional 0.6-mm hole was drilled over the center of the contralesional CFA (+0.5mm anterior and +3mm lateral to bregma). A cannula (0.36mm, brain infusion kit 1; Alzet, CA, USA) was implanted at the depth of 1.5mm below the cortical surface and secured in place with acrylic cement (Figure 1D). The cannula was connected to an osmotic pump filled with Muscimol (10mM, Tocris Bioscience, Bristol, UK), a non-toxic GABA-A agonist. Muscimol was chosen because it is a well-established and simple way to reliably inactivate neural tissue for sustained periods without causing damage (Martin, 1991; Martin et al., 1999; Reiter and Stryker, 1988). Neck muscles were then glued back with dermal adhesive and the skin sutured. After the surgery, animals received a regimen of pain, anti-inflammatory and antibiotics medication and their recovery was closely followed.

Experimental groups

In 3 different experimental groups (Table 1), the contralesional hemisphere was inactivated for 3, 7 or 14 days with infusion of Muscimol (10mM) at a rate of 0.5µl/hour (Groups 3D, 7D and 14D) beginning within 2 hours following the lesion. An additional group (Group $14D_{slow}$) also received 14 days of Muscimol infusion, but delivered at a slower rate (0.25µl/hour). Because no pump model could provide infusion of 3 days, the plastic tube between the pump and the cannula was cut and sealed in a minor procedure 72 hours after the lesion in Group 3D.

In control animals (Group $Lesion_{no\ pump}$), to reproduce the mechanical damage caused by the cannula, a stainless steel rod of the same diameter as the cannula was lowered at a depth of 1.5mm and immobilized in acrylic. In additional control rats (n=3), a pump infusing saline (0.5µl/hour) for 14 days was implanted after the cortical lesion. No statistical difference of performance on the Montoya staircase tests was found between the two control groups (One way ANOVA paretic hand $F=0.10$; $p=0.76$; non-paretic hand $F=0.23$ $p=0.64$). The absence of behavioral effects from saline infusion is in line with previous publications using comparable infusion methods in the motor cortex (Martin et al., 2000). On post-lesion day 56, the animal was killed, the brain fixed, cryoprotected and cut coronally for anatomical reconstruction and estimation of the lesion size (MicroBrightField, VT, USA).

Reversible inactivation using Muscimol infusion of long duration

The effects of chronic brain inactivation using GABA-A agonist Muscimol on neural activity and behavior have been carefully documented (Hata and Stryker, 1994; Martin, 1991; Martin and Ghez, 1999; Reiter and Stryker, 1988). In kittens, recording of neural activity in the visual cortex following infusion with osmotic pumps for 8 to 11 days with parameters similar to the ones we

used (0.5µl/h of 10Mm) showed that inactivation extended over a radius of 1 to 3.5mm (Reiter and Stryker, 1988). To confirm that our injection protocol resulted in comparable spread, we filled the osmotic pump with Fluorophore-conjugated Muscimol molecule (BODIPY® TMR-X conjugate; Molecular probes). We infused fluorescent Muscimol at 0.5µl/h for 3 days in one animal (Group 3D; Figure 2A), for 7 days (Group 7D; Figure 2B) in one animal and for 13 days (Group 14D; Figure 2C) in one animal. Whereas the pump model infuses for 14 days, we chose to perfuse on day 13 to insure the infusion was still at its maximum. Finally we infused fluorescent Muscimol at 0.25µl/hour in one animal for 13 days (Group $14D_{slow}$; Figure 2D). Following the infusion period, animals were perfused, the tissue processed and images acquired according to an established protocol using this product (Allen et al., 2008). The radius for each animal was an average of the maximal radius found on a coronal section and the AP radius calculated from the span of sections showing the fluorescence. We found that Groups 3D and 7D had identical diffusion radius (1.2mm). Group $14D_{slow}$ had the smallest (1.1mm) and Group 14D the largest radius (1.9mm) of Muscimol diffusion. However, these differences are small and their significance questionable considering the variability of the diffusion radius reported by others (Reiter and Stryker, 1988). Thus, analyses and conclusions in the present set of experiments are based on the behavioral effect of the inactivation on the non-paretic forelimb and not on the rate of infusion or hypothetical radius of inactivation.

To show that in our model, Muscimol effectively induced a reversible contralesional inactivation and did not cause permanent damage to the contralesional hemisphere, we have conducted a series of controls. First, we conducted ICMS mapping in the contralesional hemisphere at the end of the recovery period in one animal of Group $Lesion_{no\ pump}$, Group 7D and Group 14D (Figure 3). We found that we could evoke movements from the cortex immediately

surrounding the cannula using normal current intensity and could not differentiate the responses in the animals that received Muscimol from the $Lesion_{no\ pump}$ or from naïve animals. We also visually inspected the Nissl stained tissue around the cannula in all of our animals and found no differences between experimental groups (Figure 4). To further confirm this finding, we calculated the size of the lesion made by cannula in the contralesional hemisphere in Nissl-stained sections for Group 14D and $14D_{slow}$, the two groups with Muscimol infusion of the longest duration, and compared it to the lesions made by the metal rod in the Group $Lesion_{no\ pump}$. In the contralesional hemisphere, the cannula made a small mechanical lesion in all groups. On average for Groups $Lesion_{no\ pump}$, 14D and $14D_{slow}$, the lesion made by the cannula was $0.15mm^3$, corresponding to 1.6% of the volume of the ET-1 lesion. A one-way ANOVA between the three groups found no significant difference ($F=2.15$, $p=0.16$) and, combining Groups $14D+14D_{slow}$ and comparing the lesion size to the Group $Lesion_{no\ pump}$ also did not show any difference (t-test; equal variance not assumed: $F=0.88$, $p=0.40$).

Altogether, these data support that Muscimol transiently inactivated the contralesional hemisphere and did not result in any permanent damage in our experimental groups. Thus, whereas there is permanent damage to the contralesional hemisphere, it was a small mechanical damage caused by the insertion of the cannula that was of identical size across experimental groups and controlled for in our Groups $Lesion_{no\ pump}$.

Histology and anatomical reconstruction

At the end of the experiment, animals were given a lethal dose of sodium pentobarbital and were transcardially perfused with heparinized 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS. Brains were extracted and post-fixed in 4% paraformaldehyde.

The brains were cryoprotected in a solution of 20% sucrose and 2% dimethyl sulfoxide (DMSO) over-night followed by 20% sucrose for 48 hours and quickly frozen at -55°C utilizing methyl butane and stored at -80°C (Brocard et al., 2010). Coronal sections were cut with a cryostat (40µm thickness). One out of six sections were Nissl stained and used for analysis of the lesion size and location. The lesion size was calculated with a software (MicroBrightField, Colchester, VT, USA) using the following formula:

$$Lesion\ size\ (\%) = \frac{volume\ of\ contralesional\ cortex - volume\ of\ ipsilesional\ cortex}{volume\ of\ contralesional\ hemisphere} \times 100$$

Statistical Analysis of experimental results

All values are reported as mean ± SEM, unless specified, and significance was considered at $p < 0.05$. Lesion volume between groups of animals was compared with a one-way ANOVA. For the Cylinder and Montoya tests, two repeated measures ANOVAs were conducted using animal group, time and group x time as factors and Tukey-HSD for post-hoc testing. Pre-lesion baseline data and post-intervention recovery period data (day 21 to 56 post-lesion) were included in this analysis. Statistical analysis during the intervention period was impossible because behavioral data could not be collected in all animals (see Results).

Mixed modeling, adjusting for correlations between individual measurements over time, was conducted to identify the plateau performance of the paretic limb on the Montoya test. Regressions were used to investigate how different factors correlated with this plateau performance for each rat. The autoregressive correlation structure, which indicates that for each

individual, observations taken close in time tend to be more highly correlated than observations taken far apart in time, was assumed in the analysis. To avoid making unfounded assumptions on the shape of the effect of time, we treated time as a categorical variable in the analysis. Regression results report the Spearman's correlation coefficient and t-test on the slope of the distribution. Statistical significance for the t-test was adjusted according to the Bonferroni correction factor ($p < 0.017$).

Results:

Cortical lesions

The average ET-1 induced lesion volume was 6.8±2.5mm^3 (Mean ± standard deviation). As reported by others using a similar ET-1 lesion protocol (Fang et al., 2010), ischemic lesions destroyed all cortical layers and there was no significant difference of lesion size between experimental groups (F=0.206, P = 0.892) (Figure 5).

General behavior during the intervention period

During the infusion of Muscimol, rats were typically much less active. Some rats made no movement in the Montoya staircase tests (Figure 6). In Group 3D, some rats were still inactive at day 7 after the lesion, showing that the reversal of the effects of inactivation took more than four days in some cases. Group 7D and 14D were more severely affected. More than half of the animals in these groups were inactive in the Montoya staircase in the first week during infusion of Muscimol. The side effects of Muscimol became problematic with time for some animals of Group 14D, and two rats from this group had to be excluded (see Methods). Using a slower rate of infusion (Group 14D$_{slow}$) diminished the inactivity caused by infusion of Muscimol. In fact, the general behavior of animals from Group 14D$_{slow}$ was the least affected by Muscimol delivery, supporting that the rate of infusion had a greater influence on these deleterious effects than the duration of inactivation.

The effect of inactivation on grasping performance of the non-paretic forelimb

The animals receiving Muscimol showed signs of impairments of grasping performance with the non-paretic forelimb in the Montoya test, confirming the effectiveness of the pharmacological

agent. The analysis of the performance of the non-paretic forelimb in the Montoya test was done on the baseline and the post-intervention recovery period to includes all animals in each group. The ANOVA showed a significant effect of group ($F=9.237$, $P<0.001$), time ($F=49.244$, $P<0.001$), and time x group ($F=11.034$, $P<0.001$). The pre-lesion performance was similar between all groups. In Group $Lesion_{no\ pump}$, the ET-1 lesion and the mechanical damage caused by the metal rod did not decrease the performance of the non-paretic forelimb to a significant level (Figure 7). In Groups 3D and 7D, whereas inactivation affected grasping during the intervention period, performance was not statistically different from pre-lesion baseline or from Group $Lesion_{no\ pump}$ in the post-intervention recovery period. The effect of inactivation on the non-paretic forelimb function of animals in the Group 14D was strikingly different. Grasping performance was significantly lower than pre-lesion baseline and from Group $Lesion_{no\ pump}$ until the 35^{th} day after the lesion. Thus 14 days of inactivation resulted in behavioral impairments of the non-paretic forelimb that lasted more than 2 weeks after the end of infusion. Using a slower infusion rate for 14 days in Group $14D_{slow}$ did not affect the level of impairments on grasping performance caused by the inactivation. Together with results from Figure 6, these data show that a slower rate of infusion had less detrimental effects on the general state of the animals but still had a comparable impact on the function of the non-paretic forelimb.

The effect of inactivation on spontaneous use of forelimbs

Rats also reared less often during the infusion of Muscimol. This was particularly true for animals in Group 7D and 14D and less in Group 3D and 14Dslow (missing data points in Figure 8). We found that animals either did no touch the cylinder wall at all or when they did explore, we obtained a large number of touches. In only 5 rats, we obtained one data collection session

with less than 55 touches, but more than 22. We considered that more than 20 touches were sufficient to provide an average for that session and thus included that session in the group average. Four of these 5 sessions were during the infusion of Muscimol and thus, were not included in the statistical analyses. In all other data collection sessions (n=457), we obtained more than 55 touches. Thus, the laterality scores obtained for each rat, in any session, are robust.

Analysis of the spontaneous use of the forelimbs in the cylinder test was done for the post-intervention recovery period and includes all animals in each group. The ANOVA showed a significant effect of group ($F=32.549$, $P<0.001$) and group x time ($F=4.338$, $p<0.001$). After the cortical lesion, animals in Group $Lesion_{no\ pump}$ used their non-paretic forelimb more often throughout the post-intervention recovery period (Figure 8). Thus, the lesion created a persistent bias for the use of the non-paretic forelimb. Following inactivation of short duration (Group 3D), there was an initial bias toward the use of the non-paretic forelimb. However, the animals recovered symmetrical use of their forelimbs at day 28. Inactivation of longer duration caused an initial bias to use the paretic forelimb. Whereas animals in Groups 7D returned to symmetrical use of their forelimb by day 28, the bias for the paretic forearm persisted until day 35 in Group 14D. The laterality index of Group 14D was different from $Lesion_{no\ pump}$ group throughout recovery. The use of a slower infusion rate for 14 days in Group $14D_{slow}$ still resulted in a bias to use the paretic forelimb on day 21. However, the animals returned to a symmetrical forelimb use during spontaneous exploration on day 28 and their laterality index was not different from Group $Lesion_{no\ pump}$, much like for Groups 3D and 7D.

The effect of inactivation on post-intervention recovery of the paretic forelimb

In the Montoya staircase test, grasping performance of the paretic forelimb showed a significant effect of group ($F=6.016$, $P<0.001$), time ($F=49.528$, $P<0.001$) and time x group ($F=5.739$,

P<0.001). Pre-lesion performance was similar among all experimental groups (Figure 9). The Group $Lesion_{no\ pump}$ had significant deficits until the 56^{th} day after the lesion. Inactivation of the contralesional hemisphere initially worsened the paretic forelimb function during the intervention period but then improved it in the post-intervention recovery period. Groups 3D and 7D recovered faster from the lesion than Group $Lesion_{no\ pump}$. They reached pre-lesion performance at day 35 and 28, respectively, and Group 7D transiently performed better than Group $Lesion_{no\ pump}$ on day 28. Group 14D had significant deficits and poorer performance than other groups on day 21. However, by day 28 and for the rest of the post-intervention recovery period, these animals had no deficits and performed significantly better than Group $Lesion_{no\ pump}$. Grasping performance of Group $14D_{slow}$ was similar to other groups and better than Group 14D on day 21. This group performed better than Group $Lesion_{no\ pump}$ from day 28 to 49. Thus, the beneficial effects of inactivation on recovery of the paretic forelimb were mostly preserved when a slower infusion rate was used to inactivate the contralesional hemisphere for 14 days. These results suggest that the beneficial effect of contralesional inactivation depends mainly on the duration of the inactivation and not the rate of infusion.

The effect of post-intervention behavior and inactivation duration on final level of recovery

We found that plateau performance on the Montoya test for the paretic limb was reached at day 7 for Group $Lesion_{no\ pump}$, day 21 for Group 3D, day 28 for Group 7D and day 35 for Groups 14D and $14D_{slow}$. Thus, from day 35 to day 56, all groups had a stable level of performance. For each animal, we calculated the average Montoya score between days 35 to 56 to obtain a 'plateau performance score'. Three regressions were conducted to study factors potentially correlating with the plateau performance score.

First, we performed a regression between the asymmetry score on the Cylinder test at day 21 and the plateau performance score for each animal (Figure 10A). A positive correlation between these factors would suggest that increased spontaneous use of the paretic forelimb at the beginning of the post-intervention recovery period results in better recovery of the paretic forelimb. We found a weak correlation between the two factors ($R = 0.28$) and a non-significant slope of the distribution ($p = 0.13$).

Second, we performed a regression analysis between the Montoya score of non-paretic forelimb at day 21 and the plateau performance score of the paretic forelimb (Figure 10B). Here, a negative correlation between these factors suggests that poor performance of the non-paretic forelimb at the beginning of the post-intervention period results in better recovery of the paretic forelimb. We found a weak negative correlation between the two factors ($R = -0.12$) and a non-significant slope of the distribution ($p = 0.58$).

A third regression was conducted between the inactivation duration value (0, 3, 7, 14) and the plateau performance score to test how well the inactivation duration correlated with the level of recovery (Figure 10C). A positive correlation between these factors suggests that a longer duration of inactivation results in better recovery of the paretic forelimb. The correlation between the two factors was higher than other variables tested ($R = 0.52$) and the slope of the distribution was highly significant ($p = 0.0004$). Overall, the correlation analyses suggest that the level of recovery was weakly influenced by the spontaneous use of forelimbs or on grasping function of the non-paretic forelimb after inactivation of the contralesional hemisphere. In contrast, the duration of inactivation had a great impact.

Discussion:

We used a rat model of cortical lesions to study the basic interaction between GABA-mediated inactivation of the contralesional hemisphere and behavioral recovery. Inactivation was initiated rapidly following the lesion and was maintained for increasing durations in different groups of animals. During the Muscimol delivery period, inactivation resulted in general inactivity of the animals, impaired the use of the non-paretic forelimb and worsened the function of the paretic forelimb. In the post-intervention recovery period, the adverse effects of inactivation progressively reverted. Rats with inactivation of the contralesional hemisphere recovered their grasping skills faster than untreated animals and the time of recovery was shorter for animals with inactivation of longer duration. In comparison to $Lesion_{no\ pump}$ group the grasping function of the paretic forelimb showed a tendency to be greater following inactivation of longer durations. In rats with 14 days of inactivation, this trend reached significance four weeks after the lesion and remained significant for the rest of the experiment. Using a slower infusion rate to inhibit the contralesional hemisphere for 14 days diminished the deleterious effect on the general behavior during inactivation while still preserving most of the beneficial effects on chronic recovery. Final recovery scores were correlated to the duration of inactivation but not to the spontaneous use of the forelimbs or the function of the non-paretic forelimb.

In our model, we found that acute inactivation of the contralesional hemisphere can favor recovery of the paretic forelimb. Inactivation of longer duration results in more pronounced and sustained recovery of function. Final recovery is more affected by the duration of inactivation than the effects of inactivation on general behavior or on the non-paretic forelimb.

Spontaneous recovery following ET-1 lesions in the sensorimotor cortex of rats

Cortical microinjections of ET-1 are now a common approach to produce focal cortical lesions in rats (Windle et al., 2006). As shown by others using similar lesion protocols, we found relatively small variability in lesion size among animals, facilitating comparison of behavioral recovery between experimental groups (Fang et al., 2010).

In spontaneously recovering animals, the Group $Lesion_{no\ pump}$ in the present study, lesions caused typical motor deficits of the paretic forelimb. The Cylinder and Montoya tests both revealed initial deficits of the paretic forelimb that recovered in the first 3 weeks. The recovery then slowed to reach a plateau and animals had small but persistent long-term deficits (Biernaskie and Corbett, 2001).

For the non-paretic forelimb, we found no change of performance in the Montoya test after lesion. Together with Cylinder test results, our data confirm that the mechanical damage caused by implantation of the cannula did not affect the function of the non-paretic forelimb.

Effects of GABA-mediated inactivation on general behavioral inactivity

We inactivated the contralesional hemisphere with chronic infusion of the GABA-A agonist Muscimol. This method has carefully documented effects on neural activity and behavior. With the parameters of infusion we used to test the effect of duration on recovery (10mM, 0.5µl/hour) for 8 to 11 days, Muscimol was shown to inhibit activity within a radius of 1 to 3.5mm from the cannula (Reiter and Stryker, 1988). Using fluorescent Muscimol, we found that the radius of diffusion in our experiment had similar values, ranging from 1.1mm (Group $14D_{slow}$) to 1.9mm (Group 14D). In animals of Groups 3D, 7D and 14D, the Muscimol appeared to diffuse further below the cortex, affecting the callosum and the dorsal striatum (Figure 2). In Group $14D_{slow,}$ Muscimol appeared to be limited to the cortex. It is thus possible that the general inactivity,

which we found to be the lowest in Group $14D_{slow}$ is a result of a spread of Muscimol to these additional structures. However such conclusion must be taken with caution and warrants more data, specifically knowing the variability of Muscimol diffusion reported by others using similar techniques (Reiter and Stryker, 1988).

The level of general behavioral inactivity of the animal caused by Muscimol infusion after cortical lesion could have influence the level of recovery the paretic limb. However, using a slower infusion rate for 14 days (Group $14D_{slow}$) was less detrimental to the general behavioral activity than 3 days of inactivation using the faster infusion rate (Group 3D). As the recovery of the paretic limb was comparable in Groups 14D and $14D_{slow}$ (Figure 9), respectively the group with the most and least pronounced effects on general behavioral inactivity (Figure 6), it appears that this inactivity add little effect on the recovery of the paretic forelimb.

Effects of GABA-mediated inactivation function of the non-paretic forelimb

In kittens, after sustained inhibition that lasts for 30 days, there is no injury to the inhibited tissue and grasping function returns to normal within 2-3 days (Martin et al., 2000). In adult rats following cortical lesions, we found that similar parameters of Muscimol infusion resulted in more sustained behavioral deficits of the non-paretic forelimb. Following 14 days of inactivation, animals had significantly poorer grasping performance with the non-paretic forelimb 3 weeks after the end of Muscimol delivery. It is possible that the volume of inactivation in relation to small brain size of the rat caused these effects. An alternative explanation may be that the cortical lesion potentiated the effects of inactivation in the contralesional hemisphere. It is well known that lesions trigger multiple changes in the contralesional cortex (Buchkremer-Ratzmann

et al., 1996; Jones and Schallert, 1994), some of which can increase skill learning of the non-paretic arm (Bury and Jones, 2002). Acute inhibition of this hemisphere after a lesion could have a particularly stable impact on the non-paretic forelimb. In humans, it may prevent patients from developing useful compensatory strategies with the non-paretic forelimb, something that should be investigated further.

The sustained functional impact on non-paretic forelimb caused by the 14 days inactivation raises a fundamental question. Are functional gains of the paretic forelimb due to central effects of the inhibition, increased use and practice with the paretic forelimb or a reversal of learned-non use (Taub, 2000)? This issue cannot be completely addressed with the present data. However, our regression analyses show that the spontaneous use and grasping function of the non-paretic forelimb were poorly correlated to the recovery of the paretic forelimb. In contrast, duration of inactivation was highly correlated with final level of recovery, suggesting that inhibition duration is much more critical and thus that central mechanisms were more involved in the recovery of the paretic forelimb. Following lesions in the CFA, increased use and motor learning with the non-paretic forelimb can decrease the recovery of the paretic arm (Allred and Jones, 2008; Allred et al., 2005). The adverse effects of the non-paretic forelimb behavior on recovery are not seen in animals that sustain a bilateral CFA injury or a partial callosal transection (Allred et al., 2010). These data suggest that the negative effect of the non-paretic forelimb training is mediated through the contralesional cortex and its callosal interactions with the ipsilesional hemisphere. Similarly, it is possible that the inactivation in our model improved the non-paretic forelimb recovery by limiting the interhemispheric influence of the contralesional CFA on the ipsilesional hemisphere.

Finally, it is worth mentioning that the effects on general motor behaviors or on the non-paretic forelimb we found have not been reported in human studies using inhibition of the contralesional hemisphere after stroke. These results support that in our model, not only was the duration of inactivation longer, but inactivation was also more profound than what has been used so far in human protocols. These differences should be taken into consideration in the interpretation of the results. However, the improved recovery of paretic forelimb and the absence of long-term adverse effects in any of our experimental group, suggest that inhibition of longer duration and higher intensity could be beneficial.

The effects of inactivation duration on the recovery of grasping function of the paretic forelimb

In humans, hyperexcitability of the contralesional hemisphere has been shown in several studies and is generally attributed to GABA down regulation (Butefisch et al., 2003). In rodents, there is a rapid increase of somatosensory evoked potentials in the contralesional cortex (Sakatani et al., 1990). After a few days, the contralesional hyperexcitability (Buchkremer-Ratzmann et al., 1996) is associated with a reduction of GABA-A receptors (Qu et al., 1998). According to the interhemispheric imbalance hypothesis, the contralesional hyperexcitabilty is thought to contribute to ipsilesional diaschisis and to interfere with recovery of the paretic forelimb through an increase of interhemispheric inhibition (Liepert et al., 2000; Nowak et al., 2009).

In the present study, inactivation of neural activity in the contralesional hemisphere with a GABA-agonist initially resulted in a decrease of function of the paretic forelimb. Similar short-term detrimental effects to the paretic forelimb have been reported in mice after traumatic cortical injury (Lee et al., 2011). In our study, following recovery for a longer period, we found

that these detrimental effects are transient. All experimental groups showed that GABA-mediated inactivation of the contralesional hemisphere initiated rapidly after the lesion can improve the rate and/or extent of recovery of the paretic forelimb.

In the present experiments, we chose to initiate contralesional inactivation within hours following the lesion. Longitudinal imaging studies in humans show that the increased contralesional activity is at its highest early after the lesion and is progressively resorbed with time and recovery (Jaillard et al., 2005; Marshall et al., 2000). Accordingly, inhibition of the contralesional hemisphere early after lesion should be most beneficial to recovery. Only a few human studies have initiated treatment within the first week following stroke and their results indicate an improvement of recovery of the paretic forelimb (Khedr et al., 2009; Khedr et al., 2010). However, early interventions, such as forced-use of the paretic forelimb, can increase functional deficits (Bland et al., 2000), raising doubts on how early any intervention should be initiated after lesion. In our model, results suggest that initiating inactivation of the contralesional hemisphere within hours following the lesion has no long-term detrimental effect on recovery of the paretic forelimb, regardless of inactivation duration.

In humans, studies using a single inhibition treatment appear to have short-lasting effects (Mansur et al., 2005; Nowak et al., 2008; Takeuchi et al., 2005) in comparison to treatments using multiple sessions (Emara et al., 2010; Fregni et al., 2006). However, to date, there has been no systematic investigation of treatment duration on motor outcomes. The comparison of Groups 3D, 7D and 14D conveys a compelling message that increasing contralesional inactivation duration promotes recovery of the paretic forelimb. Of course, due to numerous differences such as the method of inhibition and limitations of our animal model, absolute numbers for treatment duration in stroke patients using non-invasive methods of inhibition cannot directly be inferred

from our data. However, it is interesting to note that the most beneficial treatment was of 14 days, a period that corresponds to the time during which most of the spontaneous recovery occurs in our model. In humans, this period of faster recovery is of approximately one month, independently of the initial level of impairments (Duncan and Lai, 1997). To date, treatment durations in human studies have all been much shorter than this critical period. Whereas our data do not resolve the issue of inhibition duration in humans after stroke, they do strongly indicate that duration is a crucial factor to consider in treatment design.

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Chapter III. General Discussion

Modeling human stroke in rats

More than 80% of human cerebrovascular accidents are ischemic in origin (Go et al., 2013). Majority of these accidents are focal in nature and induced by arterial occlusion in the territory of middle cerebral artery (Roger et al., 2012). Thus, animal models of stroke are developed to induce ischemia in the same arterial territory (Sicard and Fisher, 2009; Bacigaluppi et al., 2010). These models are used to investigate the mechanisms involved in the evolution of ischemic injury, which in turn can lead to development of strategies to minimize the ischemic damage or enhance functional recovery after stroke (Turner et al., 2011).

Rats are the most commonly used animals for modeling the stroke for several reasons: (1) the brain anatomy and blood supply relatively resembles the humans, (2) their small size enables the experimenters to use well established anatomical and physiological methods easily, (3) low cost, (4) genetic homogeneity within one strain, (5) well established and accurate behavioral tests, and (6) easier public and institutional acceptance compared to primate models (Kleim et al., 2007).

There are several rat models of stroke (Table 2). Each of these models encompasses particular elements of human stroke (Carmichael, 2005; Bacigaluppi et al., 2010). With careful consideration of lesion size, mechanism and purpose, the rat models can be used to investigate various targets of human neuroprotective therapies: inflammatory cascade, reperfusion injury, change of excitability of remote brain regions, and sensorimotor cortical reorganization (Murphy and Corbett, 2009)(Gerloff et al., 2006). Rodent models can be easily steered to investigate the cell death and repair after stroke with precise control on the temporal and spatial elements implicated in the stroke (Bacigaluppi et al., 2010).

The current rat models of stroke

Middle Cerebral Artery occlusion (MCAo) models

Given the high proportion of involvement of middle cerebral artery in clinical stroke, the MCAo models are commonly used methods of inducing ischemia in rats. These models involve permanent or temporary interruption of MCA blood supply most commonly by insertion of an intraluminal suture in the internal carotid artery (Ginsberg and Busto, 1989). This technique entails unilateral dissection of the animal's neck, ligation of distal part of the external carotid artery (ECA), and insertion of a 0-5 nylon suture into the internal carotid artery (ICA). The suture will eventually rest at the bifurcation of anterior and middle cerebral arteries and depending on the experimental design can be used to cause a permanent or temporary ischemia (Carmichael, 2005). Given the high complication rate of the suture MCAo including the unwanted ischemia in the ECA territory (Dittmar et al., 2003), failure rate and extensive cervical soft tissue damage, other methods of MCAo have been developed (Gerriets et al., 2004). Embolization with either embospheres (Gerriets et al., 2003) or throboemboli (Wang et al., 2001) are two methods that have been tried with some success. Embospheres are titanium oxide round microspheres with predetermined sizes that are locally injected into bloodstream to provide embolization at the level of arteries, arterioles or capillaries depending on the size of microspheres. In their clinical application, embospheres provide consistent and predictable results for effective embolization of uterine fibroids, hypervascular tumors or arteriovenous malformations. When delivered to the ICA of rats with a 0.5 mm nylon tubing, 0.4-0.5 mm embospheres provide a permanent occlusion of large to medium sized arteries in the territory of ICA. In thromboembolic lesion, a small amount (5µl) of pre-formed clot is injected into the ICA hoping to produce ischemia distal to the arteries blocked by the clot (Chen Xu et al., 2000).

Vasoconstriction by injection of ET1 around the MCA after visualization of the MCA (craniotomy) or by stereotaxic coordinates has been shown to be advantageous above the abovementioned endovascular techniques (Nikolova et al., 2009).

The lesion size, location, and the level of impairment after MCAo models are highly dependent on the method and duration of MCAo, as well as the rat strain (Duverger and MacKenzie, 1988). Irrespective of the method, all MCAo models will results is highly variable lesions (Sharkey and Butcher, 1995; Gerriets et al., 2004; Nikolova et al., 2009), that may or not involve frontal, parietal, temporal and occipital cortex, or various subcortical structures including the thalamus, hypothalamus and striatum (Kanemitsu et al., 2002). Most importantly, in contrast to primates, MCAo in rodents tend to spare the forelimb representation area in the sensorimotor cortex (Gharbawie et al., 2005; Windle et al., 2006). Thus, such models are not well suited to study motor recovery following injury to motor areas of the cortex in rodents.

Photothrombosis

Considering the disadvantages of MCAo models, simpler stroke lesion induction techniques are gaining popularity among stroke researchers as alternative models (Bacigaluppi et al., 2010). Photothrombosis is one of these alternative models. This model uses intravenous injection of photosensitive dye “Rose Bengal” and photo-oxidation by transcranial illumination of specific brain areas (Watson et al., 1985). Recently, laser photocoagulation of penetrating arteries after craniotomy and visualization of surface brain arteries has also been used with success to induce mini stroke lesions (Mohajerani et al., 2011). Either way, the photo-oxidation causes release of oxygen radicals inside the blood vessels and subsequently endothelial damage, which leads to activation of coagulation cascades. This establishes permanent vascular occlusion in the illuminated tissue.

The advantage of this model (in the transcranial illumination method) is ability to create very focal lesions with minimal surgical intervention (Carmichael, 2005). The biggest disadvantage of this model is that very little re-perfusion or ischemic penumbra is achieved by the non-physiological thrombotic insult. Moreover, the endothelial damage leads to significant extravasation and vasogenic (and/or cytotoxic) edema that is often the hallmark of traumatic brain injury rather than ischemic stroke in humans (Carmichael, 2005).

The endothelin-1 (ET-1) models

In the present study, we used an ET-1 model of ischemic stroke in rats that uses multiple microinjections of ET-1 in the rat cortex. Our objective of using this model was to meet the following criteria:

1- to mimic pathophysiological changes in focal human stroke (i.e. ischemia followed by re-perfusion)

2- to create reproducible trans-cortical lesions with precision in size

3- to effectively target the caudal forelimb area

4- to induce long-lasting, reproducible and measurable functional deficits enabling us to study the recovery of function

5- to enable monitoring of physiological parameters and analysis of brain tissue over a large sample size

6- feasibility of multiple interventions including lesion induction, cannula implantation and pump installation in one short surgery session with minimal anatomic distortion or surgical manipulation

ET-1 is a potent vasoconstrictor (Yanagisawa et al., 1988). When injected directly into the brain tissue, applied topically to the surface of the cortex or injected adjacent to large cerebral

arteries, ET-1 reduces blood flow to the level that causes ischemic injury (Windle et al., 2006). The reduction of blood flow is rapid but not instantaneous with no endothelial damage (Macrae et al., 1993), and is accompanied by reperfusion over several hours (Biernaskie et al., 2001). The perivascular injection of ET-1 is already discussed in the previous section on MCAo models. Although this technique provides many technical advantages over the traditional MCAo models, the resultant lesion location and variability is very similar to the traditional models and not suitable for the objectives of the present study.

Both the topical application and intracortical microinjections of ET-1 produce dose-dependent ischemic lesions with minimal vasogenic edema (Windle et al., 2006). They provide precision in size and injury location, and produce long lasting reaching and grasping deficits (Gilmour et al., 2004; Windle et al., 2006). The topical application of ET-1, however, has important differences from the ET1 intracortical microinjections that make it a less favorable model to answer our experimental questions. For example, consistent involvement of deep cortical layers has been very important for our experimental design. Because it has been already shown in rodent models that involvement of the layer V, is necessary to induce down-regulation of GABA receptors and hyper-excitability in the contralesional hemisphere (Buchkremer-Ratzmann and Witte, 1997). The representative cortical lesions induces by topical application of ET1 fail to include the deep cortical layers consistently (Windle et al., 2006; Jones et al., 2009). Moreover, the topical application of ET-1 entails performing a craniotomy, which in addition to increasing the time of the surgery would cause an additional functional deficit making the quantification of deficit and recovery of hand function a constant experimental challenge (Cole et al., 2011).

Cresyl violet histology for infarct quantification in our study, as well as the behavioral testing revealed that by using ET-1 microinjections we were able to satisfy our lesion induction criteria. The histological analysis of coronal brain sections revealed that we were able to achieve consistent lesion size among experimental groups (figure 5). The lesions involved all of the cortical layers and consisted of a necrotic core surrounded by glia scar (Franco et al., 2012). The optimal placement of ET-1 microinjections to target the CFA and to produce sensorimotor deficits is already explored and validated (Rouiller et al., 1993; Windle et al., 2006; Fang et al., 2010). On the rostro-caudal plane, our lesions extended on average from bregma AP: +2.2 mm to begma AP: -0.8 mm (Paxinos and Watson, 2005) which is similar to the validated CFA lesion span in the previous studies. There was no significant different between the groups regarding the starting and ending coordinates of the lesions on histology (data not presented). In summary, ET-1 microinjections allowed us to achieve reproducible lesions in terms of size and location, which enabled us to compare the post stroke functional deficit and recovery between the experimental groups.

In spontaneously recovering animals, the $Lesion_{no\ pump}$ group in the present study, lesions caused typical motor deficits of the paretic forelimb (Gilmour et al., 2004; Windle et al., 2006). The Cylinder and Montoya tests both revealed profound initial deficits of the paretic forelimb that rapidly recovered in the first 3 weeks reaching a plateau of persistent long-term deficits validating the model for investigating the long term recovery of function (Livingston-Thomas et al., 2013).

Reversible contralesional inactivation with GABA

In the present study, we used a well-established model of stroke aiming to investigate whether continuous GABA-mediated pharmacological inactivation of the contralesional hemisphere in the acute phase of stroke would benefit the functional recovery. Our goal was not to propose a treatment modality. It was to test the implication of post-stroke transcallosal imbalance in a well-controlled experimental environment to provide insight about the current gaps in the literature. In particular, our objectives were to confirm that GABA-mediated inactivation and very early inactivation could favor recovery, and to study the interaction between duration and volume of contralesional inhibition and motor outcomes. This information increases our understanding of the basic interactions between inhibition of the contralesional hemisphere and recovery and may provide useful cues for the development of treatments based on contralesional inhibition after stroke.

We used chronic infusions of 10mM Muscimol to inhibit the contralesional hemisphere. We chose Muscimol because its pharmacological profile is shown to be a precise reflection of GABA receptor stimulation (Beaumont et al., 1978; Andén et al., 1979) and thus for a long time, Muscimol has been an excellent candidate for studying the effects of GABA potentiation (Naik et al., 1976). Moreover, this natural alkaloid is structurally similar to GABA (Krogsgaard-Larsen and Johnston, 1978) and has selective affinity and specificity for GABA-A receptor sites (Beaumont et al., 1978) with a weak agonist effect on GABA-B receptors (Krogsgaard-Larsen et al., 1994). Muscimol binds to GABA-A receptors to directly regulate gating of the chloride ion channel, which subsequently leads to hyper-polarization (Macdonald and Olsen, 1994). Given the ubiquitous distribution of GABA-A receptors (Chu et al., 1990), Muscimol can be used to

inhibit the neuronal activity virtually in all regions of central nervous system while sparing the conduction of fibers-of-passage (Majchrzak and Di Scala, 2000).

Muscimol induced inactivation has a rapid onset and lasts several hours depending on the dose (Martin and Ghez, 1993, 1999). In our experimental animals, the effects of inactivation appeared overall six hours after the placement of the osmotic mini pump which is the time needed for the pumps to reach a steady state of function (personal communication with the manufacturer). During the infusion of Muscimol, rats were typically less active in terms of exploratory behavior and grooming. They walked in a circular pattern of motion indicating significant weakness in the forelimb contralateral to the inhibition site (Brailowsky et al., 1988). Although we did not use a measure to document the locomotor slowness of these animals (Di Scala et al., 1990), the pattern of inactivity of our animals in the cylinder and Montoya staircase tests (Figure 6) provides clear clues about the effect of unilateral inactivation on the general state of the experimental animals. Our observation indicated that in the animals that did not show a complete inactivity in the cylinder test, the activity was slow. For example, acquiring 60 exploratory touches in the cylinder test typically requires less than three minutes, whereas during the inactivation (if the animals was not inactive) this time increased from three to forty-five minutes.

The decreased activity of our experimental animals that received Muscimol at the rate of 0.5 µl/hr might be a reflection of reduced synaptic activity in the interconnected areas adjacent to the core of inactivation in addition to the inactivation of CFA alone. In a series of experiments, Martin and collaborators provided a comprehensive analysis of spread of Muscimol induced inactivation (Martin, 1991; Martin and Ghez, 1993, 1999). By using autoradiographic measurement of [^{14}C] glucose uptake following cortical injection of Muscimol (1µg/mL) in rats,

they demonstrated that the effects of focal inactivation extended beyond the region of drug spread (Martin, 1991). They revealed a small area (1mm) of strong hypometabolism in the injection area, surrounded by a much larger area of milder hypometabolism. This hypometabolic area exceeded the spread of the Muscimol that was evaluated with [^{3}H] Muscimol injection, indicating that the extension of inactivation might have been due to reduced synaptic activity in interconnected neurons. Martin and collaborators demonstrated similar results with chronic infusion of Muscimol. With chronic cortical infusion of 10mM Muscimol at the rate of 0.5 µl/hr for two weeks (same as our 14D group) they demonstrated a 6.5–8 mm radius of reduced activity in cortex of kittens surrounding a 3 mm core of inactivity at the injection site (Martin and Lee, 1999). If these results could be applied to our rat model, the locomotor deficits in our animals, could have been the result of decreased neuronal activity in the medial frontal cortex; where chronic infusions of GABA is previously shown to produce similar behavioral effects (Di Scala et al., 1990).

As mentioned above, when animals were not completely inactive in the cylinder during the inactivation, the cylinder test data could be collected albeit in a longer time span (30-45 minutes) rather than a typical cylinder session (3 minutes). The cylinder test in these animals showed that inactivation caused a change of laterality of hand usage from decreased non-paretic limb usage in the 3D group to a complete reversal of forelimb usage in the 7D and 14D groups. It is already shown that chronic unilateral GABAergic inactivation for 3 to 14 days causes evident weakness of the contralateral forelimb (Brailowsky et al., 1988); therefore, the subsequent non-reliance on the non-paretic (inhibited) forelimb for rearing in the cylinder test seems to reflect the efficacy of inactivation in our animals. In the Montoya staircase, the inactivation caused reversible functional deficit in the non-paretic limb. All inactivation treatment groups in our study

demonstrated very low functional scores in Montoya staircase during the infusion of Muscimol denoting the effectiveness of cortical inactivation in reducing the grasping and retrieving performance of the non-paretic forelimb.

The absence of decreased activity in our 14Dslow group that received inactivation at a slower rate (0.25 µl/hr), despite the measurable deficits of hand function as well as transient reversal of laterality index in the cylinder test, demonstrates that in this group reduction of Muscimol flow rate caused a more focal but effective inactivation that involved the forearm representation area without probable involvement of the adjacent cortical areas. In fact, our preliminary experiments to measure the spread of Muscimol confirms that in the 14Dslow group the spread of Muscimol is confined to the forelimb representation area and the adjacent areas may not be involved (Figure 10).

The decreased activity of all of our inactivation groups subsided immediately after the inactivation period demonstrating the reversibility of Muscimol inactivation (Majchrzak and Di Scala, 2000). However, the reversal of the grasping deficit and retrieval performance of the non-paretic limb differed depending on the duration of inactivation (Figure 7). After three days of inactivation (Group 3D) the grasping and retrieving performance of the non-paretic limb returned back to normal in less than a week. For the 7D group we observed a significant deficit one week after the inactivation had stopped. In both groups with 14 days of inactivaition (14D and 14Dslow) the deficit of the non-paretic limb continued for three weeks beyond the period of inactivation.

Our critical analysis of the behavioral data after chronic infusions of Muscimol, suggest that depending on the duration of inactivation, some affects may not be immediately reversible. To our knowledge, little attention has been given to possible long lasting effects of GABA

potentiation. Hence, it is not possible to differentiate if the delayed reversal of grasping performance is due to central effects of unilateral GABA potentiation or merely a delayed reversal of learned non-use of the non-paretic limb (Taub, 2000). The reversibility is particularly important when animals are tested again. In this respect, scarce empirical evidence demonstrating the course of neuronal changes after chronic cortical inactivation is available. Chronic cortical infusions of Muscimol (Martin and Ghez, 1999; Lee et al., 2011) or GABA (Meneses et al., 1993) using mini-osmotic pumps are shown previously to cause no histological damage compared to saline infusions at the cortical injection site, therefore cortical damage due to chronic infusion of Muscimol cannot explain the delayed reversal of grasping and retrieving performance. Our histological examination of the infusion site of Muscimol did not show any difference between the inactivation groups and our $Lesion_{no\ pump}$ animals in terms of damage to neural tissue or formation of scar tissue around the injection site. Therefore, similar to Martin and collaborators we have observed no tissue damage related to chronic microinfusions of Muscimol.

The effects of contralesional inactivation on the grasping function of the paretic forelimb

Recent developments in stroke research highlight the importance of post-stroke increase of neuronal excitability in contralesional hemisphere that is linked to GABA down regulation (Bütefisch et al., 2003b). The increased excitability of contralesional hemisphere after stroke (Liepert et al., 2000; Manganotti et al., 2002, 2008; Nardone and Tezzon, 2002a; Shimizu et al., 2002) and the subsequent interhemispheric inhibition from contralesional to ipsilesional hemisphere (Murase et al., 2004; Duque et al., 2005) are shown to interfere with the recovery of stroke patients.

In rodents, unilateral cortical damage leads to a rapid increase of contralesional neuronal excitability (Abo et al., 2001; Mohajerani et al., 2011) and decreased intracortical inhibition (Buchkremer-Ratzmann and Witte, 1997), which is due to the reduction of GABA-A receptors (Qü et al., 1998a) and decreased production of GABA-A receptor subunits (Lee et al., 2011). According to longitudinal studies, the hyperexcitability of contralesional hemisphere is maximal shortly after stroke and gradually subsides over a period of several months (Marshall et al., 2000). Hence, in the present study, we chose to initiate the GABA-A potentiation of contralesional hemisphere within hours following the cortical ischemic lesion. Hypothetically, inhibition of the contralesional hemisphere early after lesion could be most beneficial to recovery. However, only a few human studies have aimed to implement inhibition treatment early following stroke in which very few patients within one week of stroke are included (Conforto et al., 2011).

In our experiments, infusions of Muscimol in the contralesional hemisphere initially resulted in general hypoactivity of animals and a decrease of function of the paretic forelimb. Similar short-term detrimental effects to the paretic forelimb have been reported after chronic contralesional infusion of Muscimol in mice with cortical lesions (Lee et al., 2011). In our study, following recovery for a longer period, we found that these detrimental effects are transient. All experimental groups showed that GABA mediated inactivation of the contralesional hemisphere initiated rapidly after the lesion can improve the extent of recovery of the paretic forelimb.

As discussed earlier, our experimental animals demonstrated a decreased functional reliance on their non-paretic limb during the inactivation period for rearing in the cylinder and grasping/retrieval in the Montoya staircase. In light of the better functional recovery of these animals in the post-intervention recovery period, it was essential for us to determine if the non-

reliance on the non-paretic limb (versus the effect of inhibition per se) was the main driver for the better functional outcomes. In other words, we wanted to examine if the better outcomes were related to non-reliance on the non-paretic limb as is observed in the studies using the constraint-induced therapy. Our correlation analysis (Figure 10) provides evidence that in our experimental animals the decrease of spontaneous use of non-paretic forelimb, as well as the functional deficit of non-paretic forelimb in Montoya test only weakly influenced the recovery. However, the duration of inactivation had a significant impact on the better functional outcomes of the groups with inactivation treatment.

Conclusions

In humans, studies using a single inhibition treatment appear to have promising but short-lasting effects on the level of spasticity and performance in fine motor tasks (Mansur et al., 2005; Takeuchi et al., 2005; Nowak et al., 2008). Same neuromodulatory interventions, when applied on a regular basis, seem to be more effective and yield longer-lasting effects on the recovery of paretic limb (Fregni et al., 2006; Emara et al., 2010; Conforto et al., 2011; Avenanti et al., 2012). To date, there has been no systematic investigation of treatment duration on motor outcomes, and our study is the first trying to address this question in a well-controlled laboratory environment. In fact the comparison of Groups 3D, 7D and 14D in our study demonstrates that increasing the duration of contralesional inactivation promotes recovery of the paretic forelimb in a time dependent manner. Whereas, providing absolute figures for duration (or the number) of treatment sessions in stroke patients is out of scope of the present study, our results strongly indicate that duration of treatment is a factor that should be accounted for when comparing the efficacy of contralesional inhibition in stroke subjects.

It is important to note that in our study, the most beneficial effects were observed when the inactivation treatment targeted the contralesional hemisphere throughout the first 14 days after the ischemic lesion: a period corresponding to the time during which most of the spontaneous recovery occurs in our model. Moreover, shorter durations of inactivation resulted in less prominent but still sustained effects on the recovery of function. In humans, independent of the initial level of impairment, the period of maximal recovery is approximately one month (Duncan PW and Lai SM, 1997). Hence, the acute phase after the stroke seems to be a golden interval of intervention for contralesional inhibition that should be considered while enrolling stroke patients in clinical trials. The current patient care protocols in acute setting after stroke (e.g.

thrombolysis or endovascular interventions) may constitute an obstacle for enrollment of patients in the non-invasive stimulation trials immediately after stroke. Therefore, by providing precise temporal and behavioral evidence, our results provide a window of opportunity for the researchers in which the current gap in our understanding of the clinical efficacy of contralesional inhibition in acute phase after stroke can be approached with more confidence.

Future direction

In continuum with our experiments, a mixed model of ET1 cortical ischemia and contralesional inactivation can be used to address the following behavioral questions in rats:

1- Is **delayed** inactivation of contralesional hemisphere by chronic microinfusions of Muscimol as efficacious in terms of functional recovery as the immediate inactivation protocols that were employed in the present study?

2- Can contralesional inactivation provide similar benefits in terms of functional recovery of the paretic forelimb function after **large** cortical lesions compared to the observed results of the present study with a small cortical lesion?

3- Can contralesional inactivation provide similar benefits in terms of functional recovery of paretic forelimb function after **subcortical** lesions compared to the results of the present study with a small cortical lesion?

4- Can **alternative** method of continuous inactivation (e.g. tDCS) provide similar benefits in terms of functional recovery of paretic forelimb function compared to the results of the present study that uses continuous GABA-A potentiation by means of chronic microinfusions of Muscimol?

5- Is **continuous vs. interrupted** inhibition with tDCS for 14 days starting immediately after the cortical lesion, providing different behavioral outcomes in terms of functional recovery of paretic forelimb function?

By comparing the behavioral outcomes of the groups 14D and 14Dslow, it is inferred that our mixed model of ET1 cortical ischemia and contralesional inactivation is able to provide reliable and reproducible behavioral results. Therefore we propose our rat model of ET1 cortical lesion and 14 day contralesional inactivation as a model of contralesional inactivation after stroke to investigate the following questions:

1- What is the difference of expression of activity dependent gene, Alivin-1, in the ipsilesional CFA and RFA of stroke rats with and without contralesional inactivation?

2- How electrophysiological indicators of potency of cortical inhibitory circuits including SICI are different between stroke rats with and without contralesional inactivation?

3- How electrophysiological indicators of cortical excitability including ICF are different between stroke rats with and without contralesional inactivation?

4- How electrophysiological indicators of transcallosal inhibition including SICI and IHI10 are different between stroke rats with and without contralesional inactivation?

5- How axonal sprouting and dendritic arborisation are different in ipsi and contralesional CFA and RFA of stroke rats with and without contralesional inactivation?

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Figures

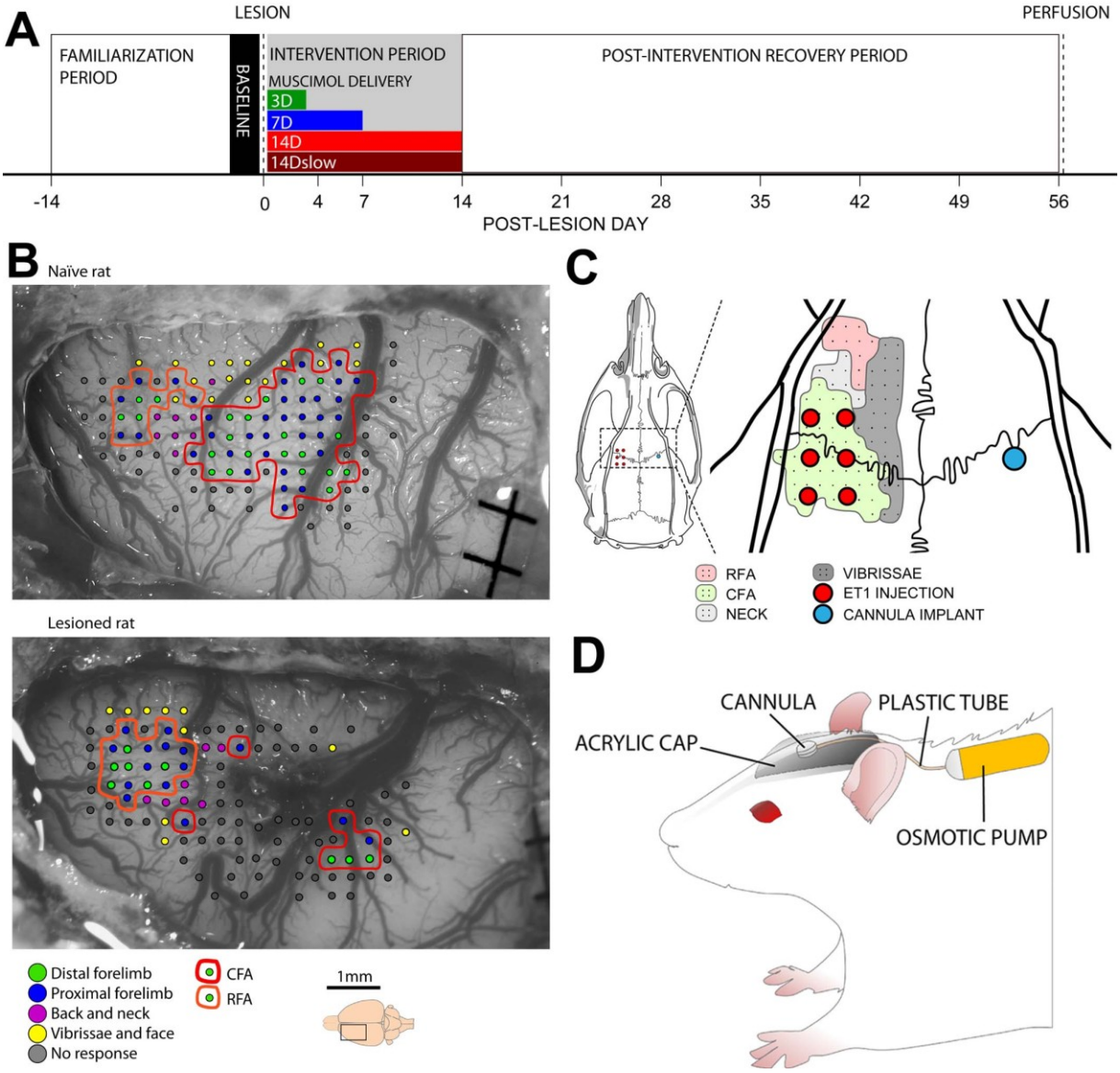

Figure 1. Experimental design.

A) Time course of events in reference to the lesion. Baseline data were collected on the 9th and 10th day of the familiarization period and averaged to establish a baseline performance score. Following the cortical lesion, data were collected twice in the first week, on days 4 and 7, and then once per week until day 56. Horizontal bars in the intervention period show the duration of inactivation in different experimental groups. B) Location of ET-1 lesion in relation to motor representations. The top panel shows a typical ICMS map in a naïve animal of comparable age and weight to animals used in the present study. Contours outline the caudal forelimb area (CFA; red) and the rostral forelimb area (RFA; orange). Each dot is a penetration site where

microstimulations were delivered. Bottom panel shows an example of the impact of the endothelin-1 lesion in a rat that spontaneously recovered for 56 days (Group $Lesion_{no\ pump}$). The lesion was in the CFA and spared the RFA. Evoked movements are color-coded. C) Cartoon showing the location of ET-1 injections based on stereotaxic coordinates in relation to the cortical motor map collected in the naïve animal shown in B. The red dots show the location of ET-1 injections and the blue dot the cannula. The ET-1 injections targeted the caudal forelimb area (CFA). D) Cartoon showing the location of the cannula and osmotic pump.

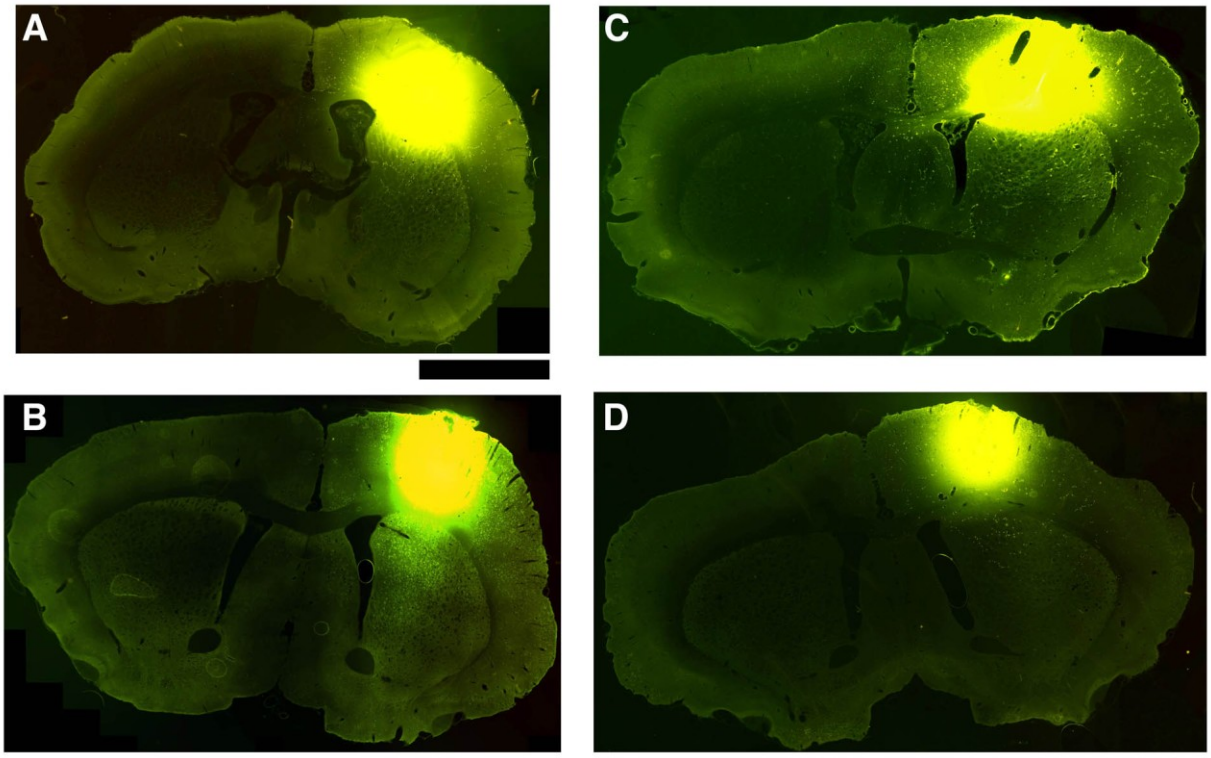

Figure 2. Spread of muscimol infusion in different experimental groups.

We infused fluorophore-conjugated Muscimol to evaluate the spread of muscimol in our different experimental groups. Coronal sections of animals in which Muscimol was infused for A) 3 days at 0.5µl/hour (Group 3D), B) 7 days at 0.5µl/hour (Group 7D), C) 14 days at 0.5µl/hour (Group 14D) and D) 14 days at 0.25µl/hour (Group $14D_{slow}$) are shown. We found that the radius of diffusion was less than 2mm in all animals. Scale bar = 5mm.

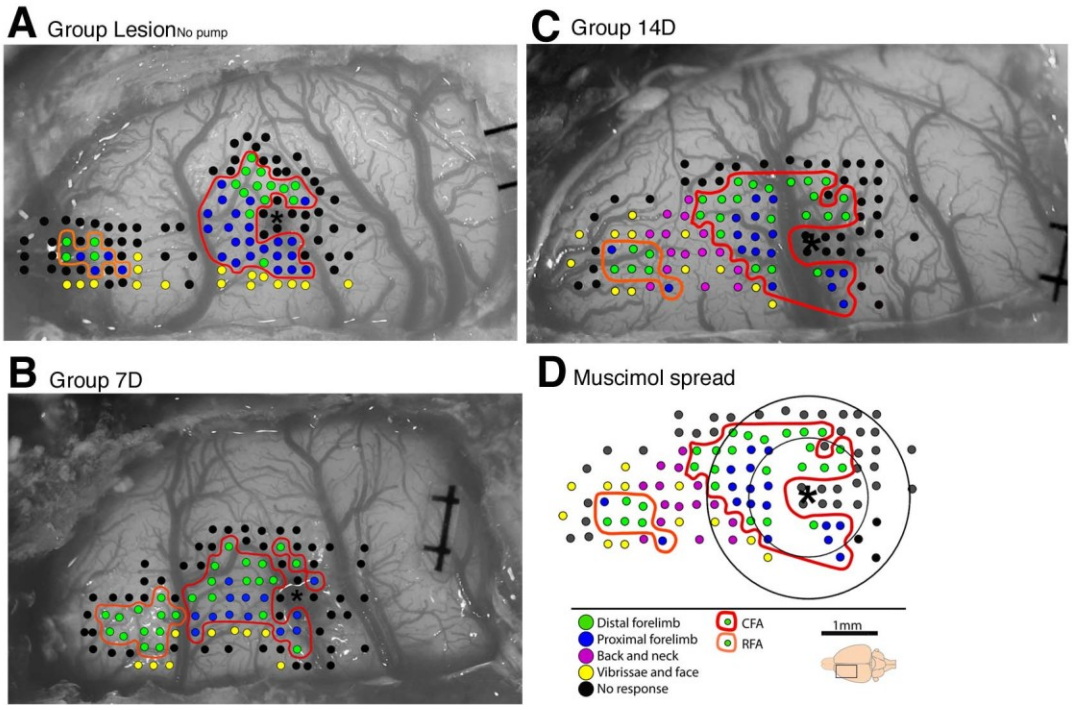

Figure 3. Responses to intracortical microstimulation following sustained muscimol infusion.

At the end of the recovery period, we derived motor maps in the contralesional hemisphere using intracortical microstimulation (ICMS) techniques in A) one animal from Groups $Lesion_{no\ pump}$, B) one animal from Group 7D and C) one animal from Group 14D. In all cases, only a small cortical lesion was visible on the surface of the cortex (asterisk). Evoked movements are color-coded and the two motor representations, the caudal (CFA) and the rostral forelimb area (RFA), are outlined. We found that the tissue in the CFA was responsive to stimulation, with no apparent difference between the animals. These data support that sustained infusion of Muscimol did not result in permanent damage of the tissue. D) ICMS map of the animal from Group 14D on which the range of Muscimol diffusion we found is superimposed (see Figure 2). Circles show the smallest (1.1mm, Group 14Dslow) and largest radius (1.9mm, Group 14D) of fluorescent Muscimol spread.

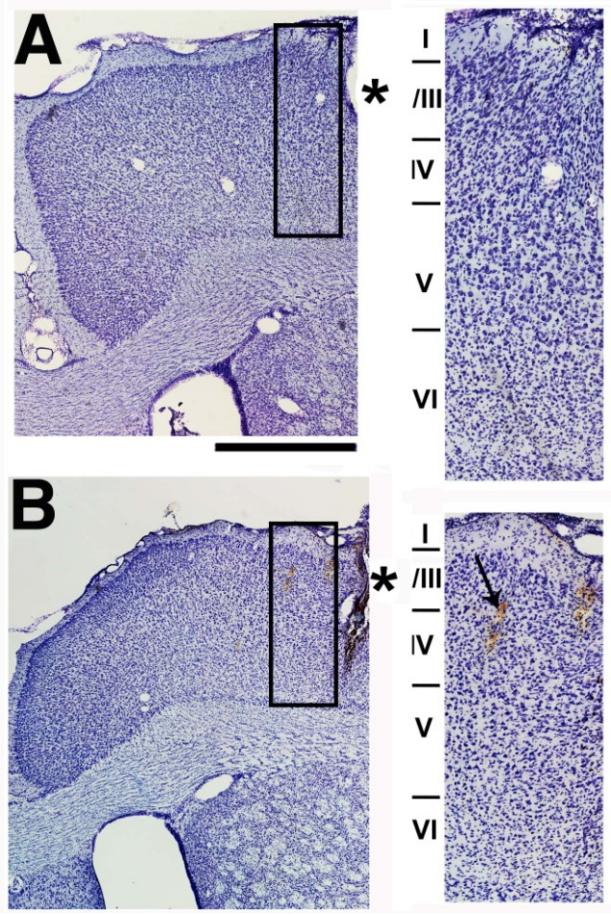

Figure 4. Histological evaluation of the tissue surrounding the cannula in the contralesional hemisphere.

A) Example of a Nissl stained coronal section of rat of Group $Lesion_{no\ pump}$ at +0.5mm AP. Asterisk shows the position of the cannula. The black box outlines the tissue shown at higher magnification on the left. Scale bar = 1mm. Inset width = 0.5mm. B) Example of a Nissl stained coronal section of rat of Group 14D at +0.5mm AP. Small arrow shows an example of ICMS electrode penetration track. There was no obvious difference in the tissue between animals infused with Muscimol and animals that had a metal rod in the Group $Lesion_{no\ pump}$. In all animals, the lesion made by the cannula was only visible in one Nissl-stained section, supporting that the lesion did not extend into the AP axis.

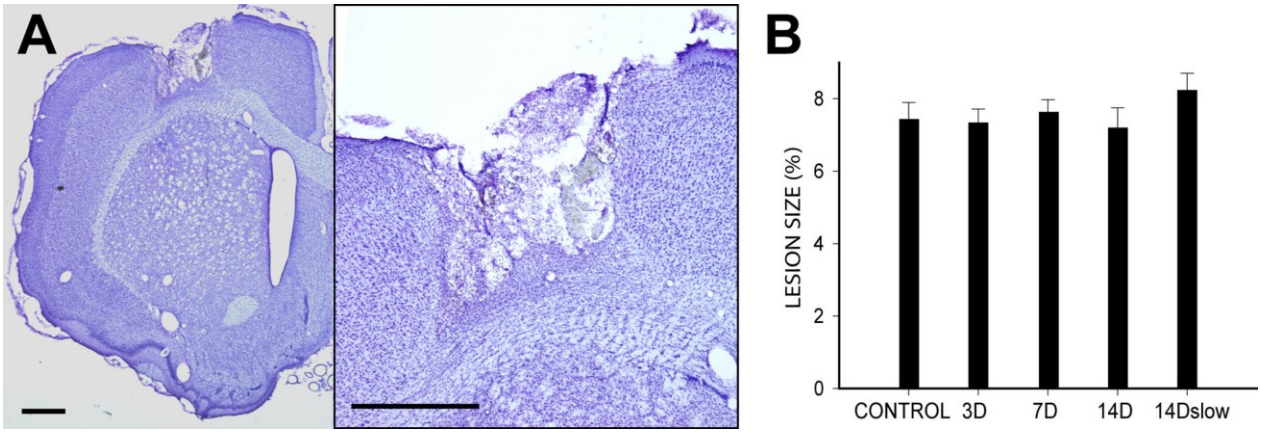

Figure 5. Histological evaluation of the endothelin-1 lesion size.

A) Example of a Nissl stained coronal section of rat brain at AP +0.5 mm from bregma of the ipsilesional hemisphere. B) A block of brain of identical size was reconstructed in Neurolucida (Microbrightfield, inc.) for each animal. To compare experimental groups, the lesion volume was normalized to the volume of contralesional hemisphere of the block (see Methods). There was no difference of lesion volume between experimental groups.

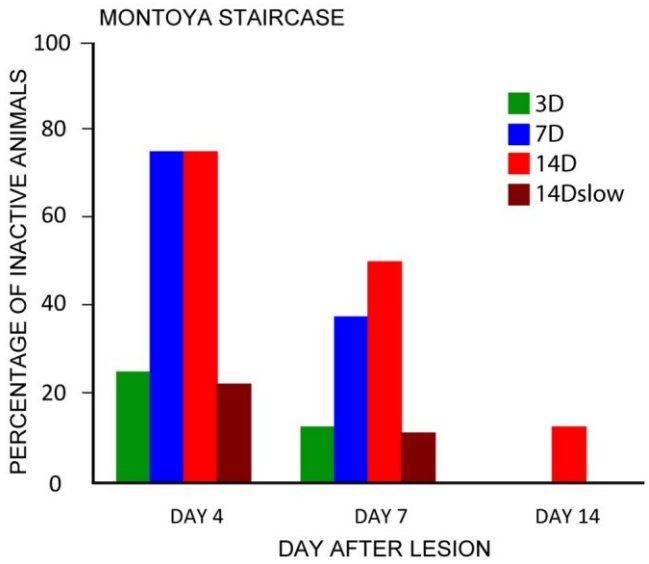

Figure 6. Side effects of contralesional inactivation.

During Muscimol delivery the different experimental groups showed variable degrees of inactivity in the Montoya staircase test. Rats were given 15 minutes in the Montoya to perform the task for each trial. The graphs show the percentage of animals in each experimental group that were inactive in the tests for that entire period and from which no behavioral data could be collected.

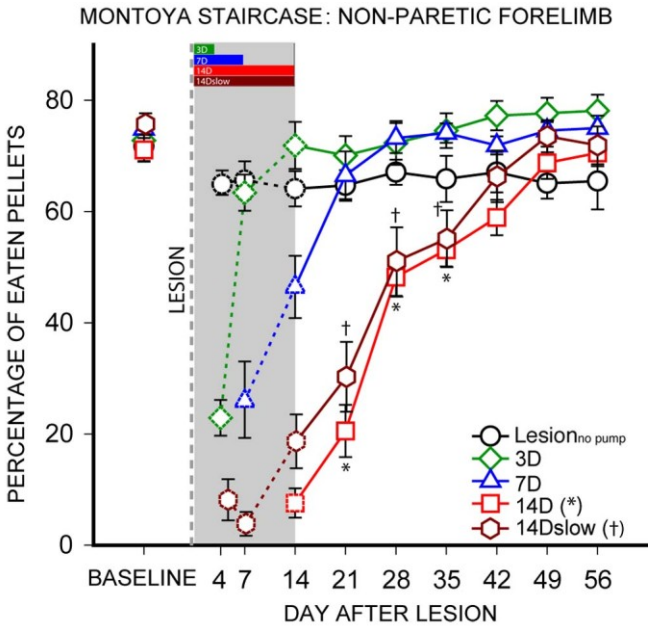

Figure 7. Non-paretic forelimb grasping performance on the Montoya test.

The intervention period is highlighted with gray background. In this period, data points with n≥5 are shown even if they were not included in the statistical analyses. Inactivation in Group 14D resulted in sustained deficits of the non-paretic forelimb until day 35 after the lesion. Slower infusion rate (Group $14D_{slow}$) induced similar deficits. Symbols show significant differences from Group $Lesion_{no\ pump}$.

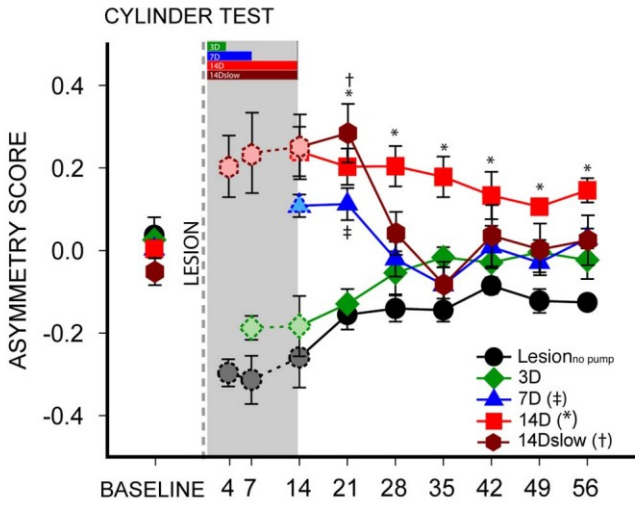

Figure 8. The effect of inactivation on spontaneous use of forelimbs.

Laterality scores in the cylinder test. Positive values highlight an increased use of the paretic forelimb in relation to the pre-lesion behavior of the animal. During the infusion period, only data points with n≥5 are shown even if they were not included in the statistical analyses. In controls, the ischemic lesion caused a bias for the non-paretic forelimb. In contrast, inactivation in Groups 7D, 14D and 14Dslow resulted in a bias to use the paretic forearm. The laterality index in Group 14D was significantly different from Group $Lesion_{no\ pump}$ throughout recovery. Symbols show significant differences from Group $Lesion_{no\ pump}$.

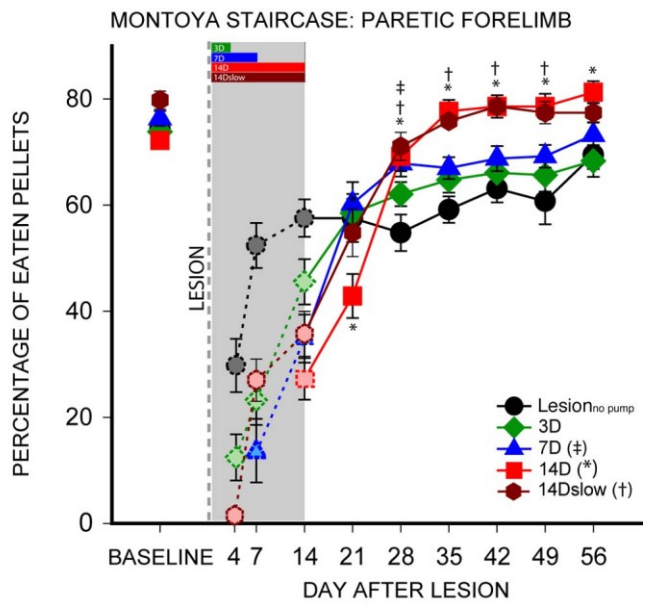

Figure 9. Effects of inactivation on the recovery of the paretic forelimb.

Grasping performance of the paretic forelimb in the Montoya test. Group $Lesion_{no\ pump}$ recovered to baseline level only by day 56. The performance was worsened by the inactivation during the intervention period. However, in the post-intervention recovery period, animals with inactivation recovered better than controls. Symbols show significant differences from Group $Lesion_{no\ pump}$.

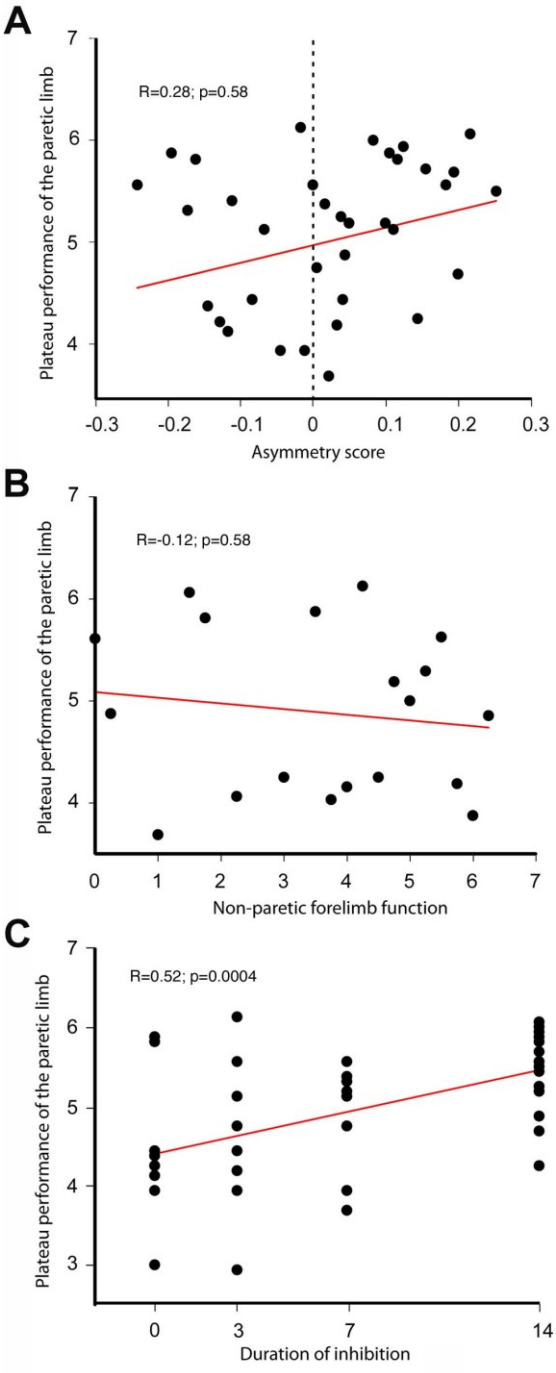

Figure 10. Correlation analyses of factors potentially involved in the recovery of the paretic forelimb.

A) Correlation between the spontaneous use of forelimbs at the beginning of the post-intervention period and level of recovery of the paretic forelimb. The plot shows the asymmetry scores at day 21 and the average Montoya score of the paretic forelimb between days 35 to 56 after the lesion (plateau

performance score). When more than one animal had the same laterality score, their plateau performance scores were averaged. B) Correlation between non-paretic forelimb function at the beginning of the post-intervention period and level of recovery of the paretic forelimb. The plot shows the Montoya score of non-paretic forelimb at day 21 and the plateau performance score of the paretic forelimb. When animals had the same Montoya score with the non-paretic forelimb, their plateau performance scores were averaged. C) Correlation between duration of inactivation and level of recovery of the paretic forelimb. The plot shows the duration of inactivation and the plateau performance score of the paretic forelimb. The plateau performance scores of the animals for each of the four time points (0, 3, 7, 14) are shown.

Tables

Table 1 - Animal groups and inactivation protocols

Group	Inactivation protocol	ET-1 Lesion	CL hemisphere	Osmotic Pump type	Muscimol concentration	Infusion rate (µl/hr)	n
No-pump	No inactivation	yes	Metal rod	-	-	-	9
3D	3 day	yes	Cannula	Alzet 1007D	10 mM	0.5	8
7D	7 day	yes	Cannula	Alzet 1007D	10 mM	0.5	8
14D	14 day	yes	Cannula	Alzet 2002	10 mM	0.5	8
14Dslow	14 day, slow rate	yes	Cannula	Alzet 1002	10 mM	0.25	9

Table 2 - A summary of currently used rat models of ischemic stroke

Model	Site of surgery	CFA involvement	Transcortical lesion	Intervention on skull	Mechanism of CVA	Reproducibility of lesion size	Localized lesion
MCA artery occlusion	Neck	No	Yes	N/A	Ischemia+reperfusion	No	No
MCA embolism	Neck	No	Yes	N/A	Ischemia	No	No
Peri MCA ET-1 injection	Skull	Yes	Yes	Craniotomy	Ischemia+reperfusion	No	No
Topical ET-1	Skull	Yes	No	Craniotomy	Ischemia+reperfusion	Good	Yes
Cortical microinjection of ET-1	Skull	Yes	Yes	Drilling holes	Ischemia+reperfusion	Excellent	Yes
Photothrombotic Model	Skull	Yes	No	Thinning the cranium	Ischemia	Good	variable
Devascularization	Skull	Yes	Yes	Craniotomy	Ischemia	Excellent	Yes