Maastricht University | universiteit hasselt

2016 | Faculty of Medicine and Life Sciences

DOCTORAL DISSERTATION

Neurostimulation to treat brain injury?

Doctoral dissertation is submitted to obtain the degrees of
- Doctor of Medicine and Life Science: Biomedical Science at Hasselt University
- Doctor at Maastricht University

Lisa-Maria Schönfeld

Promoters: Prof. Dr Sven Hendrix | UHasselt / tUL
Prof. Dr Yasin Temel | UM
Co-promoter: Dr Ali Jahanshahi | UM

D/2016/2451/23

universiteit hasselt | BIOMED BIOMEDISCH ONDERZOEKSINSTITUUT

The public defence will take place on Friday, 10 June 2016 at 10 a.m.

Examination board:

Prof. Dr Harry Steinbusch
Prof. Dr Ute Lindauer
Dr Govert Hoogland
Prof. Dr Peter Richard Schuurman
Prof. Dr Niels Hellings
Prof. Dr Jerome Hendriks

Für meine Eltern

TABLE OF CONTENTS

STATEMENTS

1. Electrical stimulation of brain tissue may have additional effects next to the acute reduction of disease symptoms. *This thesis*
2. The effect of motor cortex stimulation on stem cells *in vivo* may be interesting as a novel approach to treat tissue damage. *This thesis*
3. A careful choice of behavioral tests can reduce the level of discomfort experienced by experimental animals and increase data quality. *This thesis*
4. With the current paradigm, neurostimulation may not be effective to achieve functional recovery after severe tissue damage. *This thesis*
5. Changes in the experimental design may reveal benefits of neurostimulation to treat severe brain injury. *Valorization addendum*
6. "Your work is going to fill a large part of your life, and the only way to be truly satisfied is to do what you believe is great work." *Steve Jobs*
7. "Three things that can't be hidden. The sun, the moon and the truth." *Gautama Buddha*
8. "At the end of the day, we can endure much more than we think we can." *Frida Kahlo*

CHAPTER 1

GENERAL INTRODUCTION

AND AIMS

Brain injury: What are the current causes?

Modern life technology is advancing in a rapid speed and it is that speed that bears a certain conflict potential. Cities continue to urbanize and the population grows older, which increases interest in and reliance on machine technology.

Transportation by different kinds of motorized vehicles becomes faster and at the same time the risk for traffic accidents rises[1]. Every year, numerous vehicle crashes occur on roads worldwide that can result in death or sustained injury. Behaviors such as a willingness of risk-taking, impulsivity and misuse of alcohol or illicit drugs further increase the likelihood of accidents and are especially prone in young people. In addition, economic, political and ethnic conflicts around the world claim thousands of lives and can leave people traumatized, both psychologically and physically[2].

Traumatic brain injury

Traumatic brain injury (TBI) describes any insult caused by an external force that damages the brain. This force can be created through translational or rotational acceleration, in the case of traffic accidents, or through an object striking the skull, in case of missile or sports injuries[3]. These various external forces can then result in epidural hematomas, a concussion or skull fractures[3]. Causes of TBI reflect our modern lifestyle and valid discussions are ongoing about how to prevent TBI by using appropriate safety measures[4].

The development of TBI follows a simple causal course: An external mechanical input, for example a gun shot, causes a mechanical response by the affected tissue, in this case compression and rupture[3]. Depending in the severity of the mechanical input, the damage can be transient, only lasting a few hours or days, or permanent, resulting in sustained tissue damage and functional

impairment[1, 2]. Although the course of TBI in the long run depends on the type of mechanical insult and the tissue affected, the primary injury phase consists of cell membrane rupture as a direct result of the mechanical force[3]. This membrane damage either results in immediate cell death, or leads to abnormal ion flow through the membrane, which results in neurofilament disassembly and axonal conduction block[5, 6]. The secondary injury phase, which can last several months after the insult, is characterized by inflammation, excitotoxicity, ischemia and edema, which further exacerbate tissue damage[7]. The symptoms resulting from TBI depend on the affected brain region and type of insult, but often consist of aphasia and memory impairment, muscle weakness, gait disturbance and loss of coordination[8, 9].

Motor impairments caused by TBI interfere with a patient's activities of daily living and may create a complete dependence on family and professional caregivers. To restore motor functioning in the clinics, the traditional and still most widely used approach is physical rehabilitation therapy, with varying success rates. However, no therapy is available that successfully restores TBI directly at the tissue level, which strengthens the need to develop novel therapeutic approaches.

Preclinical research on TBI

In general, animal models are used to conduct preclinical research on behavior and motor functions. Concerning TBI, the use of primate models can lead to results with a high translational value due to the phylogenic similarities between primates and humans. In primates, the head and neck anatomy allows the induction of TBI through rapid acceleration, which closely resembles the majority of clinical TBI cases[10]. However, due to increasing ethical concerns,

limited availability and housing conditions, primate research has become substantially limited. Therefore smaller animals, such as rodents, are nowadays frequently used as model organisms.

Although rodents differ drastically from primates in the overt manifestation of motor functions, such as gait and fine motor skills, the underlying anatomy and functional organization of those behaviors can be surprisingly similar[11]. The motor cortex is the most important region controlling fine motor skills of the individual limbs and movement of other small parts of the body, such as the ears and face. Similar to humans, the primate motor cortex possesses clear functional representations of each respective bodypart, whereas those functional regions might overlap in the rat motor cortex[11]. Still, through electrophysiological mapping of the rat motor cortex through intracranial microstimulation, distinct functional representations of the whisker pad, ears, nose and individual limbs have been identified[12]. There is also a similarity among rats, primates and humans concerning action selection and motor learning by the basal ganglia[13]. However, in the case of gait, humans show a reliance on the motor cortex, whereas rodents mainly recruit subcortical structures such as the red nucleus[14, 15]. In addition, the adult rat brain shows a great amount of neuroplasticity and in case a certain motor pathway gets damaged, other functional regions take over and compensatory behavioral strategies are developed[16-20]. Therefore when using rodent models of TBI, the observed behavioral impairments might differ from the symptoms observed in human patients, which requires a careful selection of behavioral assessment tools.

Rodent models of TBI

To induce TBI in rodents, the most commonly used method is a focal contusion. The 'weight-drop contusion model' is probably the oldest rodent model of TBI and creates damage to the cortex and subcortical regions by guiding a free-falling weight towards the skull or exposed cortex[21]. This model creates tissue damage and functional deficits that last from several days to weeks, depending on the contusion severity[21, 22]. However, a major drawback of the weight-drop technique is, that the chance of a rebound injury rises with increasing weight and speed of the impact. Another widely used technique to induce TBI in rodents is the 'fluid percussion model', where a water pulse guided through a tube, placed on the exposed cortex, compresses the brain tissue[23]. Unfortunately, lesions created with the fluid percussion model vary in their severity because there is poor control over the exact amount of water pushing onto the brain tissue[24]. A TBI model with a minimal rebound injury and maximal control over contusion size can be created by using a 'controlled cortical impact' (CCI). With a CCI, a round impactor tip of a certain diameter is attached to a mechanical actuator and strikes the exposed cortex at a pre-defined velocity, depth and dwell time[25]. The resulting lesions are specific and reproducible, while animal mortality is low; therefore nowadays CCI is the most often used rodent model of experimental TBI [26-30].

Neurostimulation to restore motor functions

The sporadic usage of electrical current to influence the central nervous system can be traced back for hundreds of years and in the 19th century electrical stimulation was systematically applied to the brain to treat psychosis, seizures and chronic pain[31, 32]. Through invention of the stereotactic frame, it became

possible to selectively target deep brain structures with implantable electrodes[33]. Nowadays this technique is known as deep brain stimulation (DBS) and its effectiveness to alleviate symptoms caused by numerous neuropsychiatric diseases, such as Parkinson's disease, Huntington's disease, motor tics, Tourette syndrome, obsessive-compulsive disorder and depression is being investigated[34-39].

An increased understanding of the influence of electrical current on the brain at a macroscopic and microscopic level paved the way to develop a broad range of other neurostimulation techniques and applications with varying degrees of invasiveness. Motor cortex stimulation (MCS) delivered through implanted electrodes was developed as treatment to alleviate neuropathic pain by acting on ascending and descending pain pathways, for example decreasing the hyperactivity of pain-related thalamic nuclei[40]. Interestingly, patients treated with MCS to control central pain after stroke, also reported a subjective improvement in attenuating motor rigidity[41]. To investigate the potential of MCS to improve the function of a paretic limb, a condition often observed after stroke, several preclinical studies were conducted using rodent and primate models of stroke. In these studies, MCS did support the recovery of motor functions, possibly by enhancing neuronal survival and inducing a functional reorganization of cortical maps[42-45]. In additional studies on motor recovery after stroke, the role of stimulation-induced neuroplasticity has been confirmed by observing increased axonal sprouting or angiogenesis to repair damaged fiber tracts[46-48]. Occasionally, an increased amount of cell proliferation in neurogenic areas has been reported after electrical stimulation together with increased numbers of neural stem and progenitor cells (NSPCs) at the stimulated region[49, 50]. Since motor impairments resulting from stroke are

comparable to TBI, it may be suggested that electrical stimulation of the motor cortex could also exert beneficial effects in the latter; however, the therapeutic potential of MCS for TBI still needs to be investigated.

The influence of electrical stimulation at the cellular level

Unraveling the underlying cellular and molecular mechanisms of neurostimulation is essential to improve its therapeutic specificity, but to date the mechanisms *in vivo* are still not completely understood. *In vitro*, a broad diversity of cell types can respond to electrical fields with a directed migration towards one of the two electrical poles, a process called 'electrotaxis' [51, 52]. Several molecular mechanisms underlying this guided migration pattern have been identified[52] and electrotaxis *in vitro* is a well-documented and robust phenomenon. However, the question remains whether or not electrotaxis is inducible in the central nervous system of a living organism. Electrical fields are naturally present *in vivo*, for example they help to guide NSPCs from the neurogenic zone towards their destination during development[53] and abnormal electrical activity has been measured after stroke or TBI[54-56]. Interestingly, ischemic stroke also triggers proliferation of NSPCs in the subventricular zone (SVZ), followed by their migration towards the lesion side[57, 58]. The co-occurrence of altered electrical activity and stem cell migration observed in rodent models of stroke, suggests a role of the electrical activity in guiding migration of the cells.

Besides migration, cell proliferation and differentiation can also be altered in the presence of electrical fields[59]. In contrast to directed migration, the latter two processes are already described to result from electrical stimulation *in vivo*. Several studies reported an enhancement of NSPC proliferation either in the

SVZ, or the dentate gyrus, the second neurogenic area in adult animals, together with increased neurogenesis when applying different stimulation paradigms[50, 60-62].

In line with previous findings, we revealed that in healthy rats MCS enhanced cell proliferation in the SVZ[63]. Also an increased number of NSPCs and mature neurons was found at the stimulated cortical area[64]. Although it was not possible to selectively trace cells originating from the SVZ, our findings may imply that the NSPCs found in the stimulated cortex might have migrated from the SVZ towards the electrode, since evidence of NSPCs present in brain regions other than the SVZ and the hippocampus is sparse, but nonetheless existing[65].

Motivated by the mentioned findings and the observation that MCS has an effect on motor recovery, we suggest that the therapeutic success of MCS may be due to its influence on NSPC proliferation, migration towards the stimulation (and potentially lesion) side and differentiation into mature cortical cells, which have a positive influence on the damaged tissue.

Outlook to the aims of this research line

The application of neurostimulation may have various, beneficial effects on the injured brain. To establish and refine neurostimulation as a therapeutic approach, the effect of electrical current at the cellular level *in vivo* needs to be understood. In this thesis, we review the available data about cell migration within an electrical field *in vitro* and *in vivo* (Jahanshahi and Schönfeld[52]; Chapter 2). Thereafter, we describe the influence of chronic MCS on cell proliferation in the SVZ and a potential migration of NSPCs towards the stimulation electrode in healthy rats (Jahanshahi et al.[63]; Chapter 3).

To test potential new treatments in a subsequent experiment, we used a standardized rat model of TBI together with a number of behavioral tests to measure a long-lasting unilateral motor impairment (Schönfeld et al., under review[66]; Chapter 4). In general, experimental animals are still used to gain insight into disease development, progression and treatment. To reduce the number of experiments performed and to refine the experimental design, a careful choice of behavioral testing methods is essential. We review a number of behavioral tests to measure rodent motor functions and present recommendations on how to choose the most optimal test (Schönfeld et al., to be submitted; Chapter 5).

Lastly we tested the capacity of MCS as a standalone treatment to induce functional recovery of a unilateral motor deficit in a rat model of TBI (Schönfeld et al., to be submitted; Chapter 6). We then conclude with a summary of what can be learned from our scientific results and how to place the outcomes into a larger context (Chapter 7).

REFERENCES

1. Finfer, S.R. and Cohen, J. (2001). Severe traumatic brain injury. Resuscitation 48, 77-90.

2. Maas, A.I., Stocchetti, N. and Bullock, R. (2008). Moderate and severe traumatic brain injury in adults. Lancet neurology 7, 728-741.

3. LaPlaca, M.C., Simon, C.M., Prado, G.R. and Cullen, D.K. (2007). CNS injury biomechanics and experimental models. Progress in brain research 161, 13-26.

4. Reilly, P. (2007). The impact of neurotrauma on society: an international perspective. Progress in brain research 161, 3-9.

5. Pettus, E.H., Christman, C.W., Giebel, M.L. and Povlishock, J.T. (1994). Traumatically induced altered membrane permeability: its relationship to traumatically induced reactive axonal change. Journal of neurotrauma 11, 507-522.

6. Shi, R. and Pryor, J.D. (2002). Pathological changes of isolated spinal cord axons in response to mechanical stretch. Neuroscience 110, 765-777.

7. Park, E., Bell, J.D. and Baker, A.J. (2008). Traumatic brain injury: can the consequences be stopped? CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne 178, 1163-1170.

8. Drijkoningen, D., Caeyenberghs, K., Vander Linden, C., Van Herpe, K., Duysens, J. and Swinnen, S.P. (2015). Associations between Muscle Strength Asymmetry and Impairments in Gait and Posture in Young Brain-Injured Patients. Journal of neurotrauma 32, 1324-1332.

9. Steel, J., Ferguson, A., Spencer, E. and Togher, L. (2015). Language and cognitive communication during post-traumatic amnesia: A critical synthesis. NeuroRehabilitation 37, 221-234.

10. Cernak, I. (2005). Animal models of head trauma. NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics 2, 410-422.

11. Cenci, M.A., Whishaw, I.Q. and Schallert, T. (2002). Animal models of neurological deficits: how relevant is the rat? Nature reviews. Neuroscience 3, 574-579.

12. Fonoff, E.T., Pereira, J.F., Jr., Camargo, L.V., Dale, C.S., Pagano, R.L., Ballester, G. and Teixeira, M.J. (2009). Functional mapping of the motor cortex of the rat using transdural electrical stimulation. Behavioural brain research 202, 138-141.

13. Graybiel, A.M. (1995). Building action repertoires: memory and learning functions of the basal ganglia. Curr Opin Neurobiol 5, 733-741.

14. Muir, G.D. and Whishaw, I.Q. (2000). Red nucleus lesions impair overground locomotion in rats: a kinetic analysis. The European journal of neuroscience 12, 1113-1122.

15. Lee, S.Y., Kim, M.S., Chang, W.H., Cho, J.W., Youn, J.Y. and Kim, Y.H. (2014). Effects of repetitive transcranial magnetic stimulation on freezing of gait in patients with Parkinsonism. Restorative neurology and neuroscience 32, 743-753.

16. Carmichael, S.T., Wei, L., Rovainen, C.M. and Woolsey, T.A. (2001). New patterns of intracortical projections after focal cortical stroke. Neurobiology of disease 8, 910-922.

17. Starkey, M.L., Bleul, C., Zorner, B., Lindau, N.T., Mueggler, T., Rudin, M. and Schwab, M.E. (2012). Back seat driving: hindlimb corticospinal neurons assume forelimb control following ischaemic stroke. Brain : a journal of neurology 135, 3265-3281.

18. Whishaw, I.Q. (2000). Loss of the innate cortical engram for action patterns used in skilled reaching and the development of behavioral compensation following motor cortex lesions in the rat. Neuropharmacology 39, 788-805.

19. Whishaw, I.Q., Pellis, S.M., Gorny, B.P. and Pellis, V.C. (1991). The impairments in reaching and the movements of compensation in rats with motor cortex lesions: an endpoint, videorecording, and movement notation analysis. Behavioural brain research 42, 77-91.

20. Grillner, S. and Wallen, P. (1985). Central pattern generators for locomotion, with special reference to vertebrates. Annual review of neuroscience 8, 233-261.

21. Feeney, D.M., Boyeson, M.G., Linn, R.T., Murray, H.M. and Dail, W.G. (1981). Responses to cortical injury: I. Methodology and local effects of contusions in the rat. Brain research 211, 67-77.

22. Mychasiuk, R., Hehar, H., Candy, S., Ma, I. and Esser, M.J. (2015). The direction of the acceleration and rotational forces associated with mild traumatic brain injury in rodents effect behavioural and molecular outcomes. Journal of neuroscience methods 257, 168-178.

23. Kabadi, S.V., Hilton, G.D., Stoica, B.A., Zapple, D.N. and Faden, A.I. (2010). Fluid-percussion-induced traumatic brain injury model in rats. Nature protocols 5, 1552-1563.

24. Stalhammar, D., Galinat, B.J., Allen, A.M., Becker, D.P., Stonnington, H.H. and Hayes, R.L. (1987). A new model of concussive brain injury in the cat produced by extradural fluid volume loading: I. Biomechanical properties. Brain injury 1, 73-91.

25. Brody, D.L., Mac Donald, C., Kessens, C.C., Yuede, C., Parsadanian, M., Spinner, M., Kim, E., Schwetye, K.E., Holtzman, D.M. and Bayly, P.V. (2007).

Electromagnetic controlled cortical impact device for precise, graded experimental traumatic brain injury. Journal of neurotrauma 24, 657-673.

26. Combs, H.L., Jones, T.A., Kozlowski, D.A. and Adkins, D.L. (2015). Combinatorial Motor Training Results In Functional Reorganization Of Remaining Motor Cortex After Controlled Cortical Impact In Rats. Journal of neurotrauma.

27. Shijo, K., Ghavim, S., Harris, N.G., Hovda, D.A. and Sutton, R.L. (2015). Glucose administration after traumatic brain injury exerts some benefits and no adverse effects on behavioral and histological outcomes. Brain research 1614, 94-104.

28. d'Avila, J.C., Lam, T.I., Bingham, D., Shi, J., Won, S.J., Kauppinen, T.M., Massa, S., Liu, J. and Swanson, R.A. (2012). Microglial activation induced by brain trauma is suppressed by post-injury treatment with a PARP inhibitor. Journal of neuroinflammation 9, 31.

29. Harris, N.G., Mironova, Y.A., Hovda, D.A. and Sutton, R.L. (2010). Chondroitinase ABC enhances pericontusion axonal sprouting but does not confer robust improvements in behavioral recovery. Journal of neurotrauma 27, 1971-1982.

30. Vonder Haar, C., Friend, D.M., Mudd, D.B. and Smith, J.S. (2013). Successive bilateral frontal controlled cortical impact injuries show behavioral savings. Behavioural brain research 240, 153-159.

31. Kalinowsky, L.B. (1986). History of convulsive therapy. Annals of the New York Academy of Sciences 462, 1-4.

32. Hamani, C., Schwalb, J.M., Rezai, A.R., Dostrovsky, J.O., Davis, K.D. and Lozano, A.M. (2006). Deep brain stimulation for chronic neuropathic pain: long-term outcome and the incidence of insertional effect. Pain 125, 188-196.

33. Clarke, R.H. and Horsley, V. (2007). THE CLASSIC: On a method of investigating the deep ganglia and tracts of the central nervous system (cerebellum). Br Med J 1906:1799-1800. Clinical orthopaedics and related research 463, 3-6.

34. Jankovic, J. (2015). Therapeutic Developments for Tics and Myoclonus. Movement disorders : official journal of the Movement Disorder Society 30, 1566-1573.

35. Metman, L.V. and Slavin, K.V. (2015). Advances in functional neurosurgery for Parkinson's disease. Movement disorders : official journal of the Movement Disorder Society 30, 1461-1470.

36. Gonzalez, V., Cif, L., Biolsi, B., Garcia-Ptacek, S., Seychelles, A., Sanrey, E., Descours, I., Coubes, C., de Moura, A.M., Corlobe, A., James, S., Roujeau, T. and Coubes, P. (2014). Deep brain stimulation for Huntington's disease: long-term results of a prospective open-label study. Journal of neurosurgery 121, 114-122.

37. Schrock, L.E., Mink, J.W., Woods, D.W., Porta, M., Servello, D., Visser-Vandewalle, V., Silburn, P.A., Foltynie, T., Walker, H.C., Shahed-Jimenez, J., Savica, R., Klassen, B.T., Machado, A.G., Foote, K.D., Zhang, J.G., Hu, W., Ackermans, L., Temel, Y., Mari, Z., Changizi, B.K., Lozano, A., Auyeung, M., Kaido, T., Agid, Y., Welter, M.L., Khandhar, S.M., Mogilner, A.Y., Pourfar, M.H., Walter, B.L., Juncos, J.L., Gross, R.E., Kuhn, J., Leckman, J.F., Neimat, J.A., Okun, M.S., Tourette Syndrome Association International Deep Brain Stimulation, D. and Registry Study, G. (2015). Tourette syndrome deep brain stimulation: a review and updated recommendations. Movement disorders : official journal of the Movement Disorder Society 30, 448-471.

38. Kohl, S., Schonherr, D.M., Luigjes, J., Denys, D., Mueller, U.J., Lenartz, D., Visser-Vandewalle, V. and Kuhn, J. (2014). Deep brain stimulation for treatment-refractory obsessive compulsive disorder: a systematic review. BMC psychiatry 14, 214.

39. Schlaepfer, T.E., Bewernick, B.H., Kayser, S., Hurlemann, R. and Coenen, V.A. (2014). Deep brain stimulation of the human reward system for major depression--rationale, outcomes and outlook. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 39, 1303-1314.

40. Kim, J., Ryu, S.B., Lee, S.E., Shin, J., Jung, H.H., Kim, S.J., Kim, K.H. and Chang, J.W. (2015). Motor cortex stimulation and neuropathic pain: how does motor cortex stimulation affect pain-signaling pathways? Journal of neurosurgery, 1-11.

41. Katayama, Y., Oshima, H., Fukaya, C., Kawamata, T. and Yamamaoto, T. (2002). Control of post-stroke movement disorders using chronic motor cortex stimulation. Acta neurochirurgica. Supplement 79, 89-92.

42. Kleim, J.A., Bruneau, R., VandenBerg, P., MacDonald, E., Mulrooney, R. and Pocock, D. (2003). Motor cortex stimulation enhances motor recovery and reduces peri-infarct dysfunction following ischemic insult. Neurological research 25, 789-793.

43. Teskey, G.C., Flynn, C., Goertzen, C.D., Monfils, M.H. and Young, N.A. (2003). Cortical stimulation improves skilled forelimb use following a focal ischemic infarct in the rat. Neurological research 25, 794-800.

44. Plautz, E.J., Barbay, S., Frost, S.B., Friel, K.M., Dancause, N., Zoubina, E.V., Stowe, A.M., Quaney, B.M. and Nudo, R.J. (2003). Post-infarct cortical plasticity

and behavioral recovery using concurrent cortical stimulation and rehabilitative training: a feasibility study in primates. Neurological research 25, 801-810.

45. Adkins, D.L., Campos, P., Quach, D., Borromeo, M., Schallert, K. and Jones, T.A. (2006). Epidural cortical stimulation enhances motor function after sensorimotor cortical infarcts in rats. Experimental neurology 200, 356-370.

46. Carmel, J.B., Kimura, H., Berrol, L.J. and Martin, J.H. (2013). Motor cortex electrical stimulation promotes axon outgrowth to brain stem and spinal targets that control the forelimb impaired by unilateral corticospinal injury. The European journal of neuroscience 37, 1090-1102.

47. Zheng, J., Liu, L., Xue, X., Li, H., Wang, S., Cao, Y. and Zhao, J. (2013). Cortical electrical stimulation promotes neuronal plasticity in the peri-ischemic cortex and contralesional anterior horn of cervical spinal cord in a rat model of focal cerebral ischemia. Brain research 1504, 25-34.

48. Cheng, X., Li, T., Zhou, H., Zhang, Q., Tan, J., Gao, W., Wang, J., Li, C. and Duan, Y.Y. (2012). Cortical electrical stimulation with varied low frequencies promotes functional recovery and brain remodeling in a rat model of ischemia. Brain research bulletin 89, 124-132.

49. Kang, C., Yang, C.Y., Kim, J.H., Moon, S.K., Lee, S., Park, S.A., Han, E.H. and Zhang, L.Q. (2013). The effect of continuous epidural electrical stimulation on neuronal proliferation in cerebral ischemic rats. Annals of rehabilitation medicine 37, 301-310.

50. Morimoto, T., Yasuhara, T., Kameda, M., Baba, T., Kuramoto, S., Kondo, A., Takahashi, K., Tajiri, N., Wang, F., Meng, J., Ji, Y.W., Kadota, T., Maruo, T., Kinugasa, K., Miyoshi, Y., Shingo, T., Borlongan, C.V. and Date, I. (2011). Striatal stimulation nurtures endogenous neurogenesis and angiogenesis in chronic-phase ischemic stroke rats. Cell Transplant 20, 1049-1064.

51. Mycielska, M.E. and Djamgoz, M.B. (2004). Cellular mechanisms of direct-current electric field effects: galvanotaxis and metastatic disease. Journal of cell science 117, 1631-1639.

52. Jahanshahi, A., Schonfeld, L.M., Lemmens, E., Hendrix, S. and Temel, Y. (2013). In Vitro and In Vivo Neuronal Electrotaxis: A Potential Mechanism for Restoration? Molecular neurobiology.

53. McCaig, C.D., Song, B. and Rajnicek, A.M. (2009). Electrical dimensions in cell science. Journal of cell science 122, 4267-4276.

54. Dreier, J.P. and Reiffurth, C. (2015). The stroke-migraine depolarization continuum. Neuron 86, 902-922.

55. von Bornstadt, D., Houben, T., Seidel, J.L., Zheng, Y., Dilekoz, E., Qin, T., Sandow, N., Kura, S., Eikermann-Haerter, K., Endres, M., Boas, D.A., Moskowitz, M.A., Lo, E.H., Dreier, J.P., Woitzik, J., Sakadzic, S. and Ayata, C. (2015). Supply-demand mismatch transients in susceptible peri-infarct hot zones explain the origins of spreading injury depolarizations. Neuron 85, 1117-1131.

56. Hartings, J.A., Wilson, J.A., Hinzman, J.M., Pollandt, S., Dreier, J.P., DiNapoli, V., Ficker, D.M., Shutter, L.A. and Andaluz, N. (2014). Spreading depression in continuous electroencephalography of brain trauma. Annals of neurology 76, 681-694.

57. Vandeputte, C., Reumers, V., Aelvoet, S.A., Thiry, I., De Swaef, S., Van den Haute, C., Pascual-Brazo, J., Farr, T.D., Vande Velde, G., Hoehn, M., Himmelreich, U., Van Laere, K., Debyser, Z., Gijsbers, R. and Baekelandt, V. (2014). Bioluminescence imaging of stroke-induced endogenous neural stem cell response. Neurobiology of disease 69, 144-155.

58. Saha, B., Peron, S., Murray, K., Jaber, M. and Gaillard, A. (2013). Cortical lesion stimulates adult subventricular zone neural progenitor cell proliferation and migration to the site of injury. Stem cell research 11, 965-977.

59. Levin, M. (2007). Large-scale biophysics: ion flows and regeneration. Trends in cell biology 17, 261-270.

60. Liu, A., Jain, N., Vyas, A. and Lim, L.W. (2015). Ventromedial prefrontal cortex stimulation enhances memory and hippocampal neurogenesis in the middle-aged rats. eLife 4.

61. Liu, H.H., Xiang, Y., Yan, T.B., Tan, Z.M., Li, S.H. and He, X.K. (2013). Functional electrical stimulation increases neural stem/progenitor cell proliferation and neurogenesis in the subventricular zone of rats with stroke. Chinese medical journal 126, 2361-2367.

62. Jeong da, U., Lee, J.E., Lee, S.E., Chang, W.S., Kim, S.J. and Chang, J.W. (2014). Improvements in memory after medial septum stimulation are associated with changes in hippocampal cholinergic activity and neurogenesis. BioMed research international 2014, 568587.

63. Jahanshahi, A., Schonfeld, L., Janssen, M.L., Hescham, S., Kocabicak, E., Steinbusch, H.W., van Overbeeke, J.J. and Temel, Y. (2013). Electrical stimulation of the motor cortex enhances progenitor cell migration in the adult rat brain. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale.

64. Jahanshahi, A., Schonfeld, L., Janssen, M.L., Hescham, S., Kocabicak, E., Steinbusch, H.W., van Overbeeke, J.J. and Temel, Y. (2013). Electrical stimulation of the motor cortex enhances progenitor cell migration in the adult rat brain. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale 231, 165-177.

65. Rueger, M.A. and Androutsellis-Theotokis, A. (2013). Identifying endogenous neural stem cells in the adult brain in vitro and in vivo: novel approaches. Current pharmaceutical design 19, 6499-6506.

66. Schönfeld, L.M., Jahanshahi, A., Lemmens, E., Schipper, S., Dooley, D., Joosten, E.A., Temel, Y. and Hendrix, S. (2016). Long-term motor deficits after controlled cortical impact in rats can be detected by fine motor skill tests but not by automated gait analysis (*under revision*).

CHAPTER 2

IN VITRO AND *IN VIVO* NEURONAL ELECTROTAXIS: A POTENTIAL MECHANISM FOR RESTORATION?

Ali Jahanshahi*, Lisa-Maria Schönfeld*, Evi Lemmens, Sven Hendrix and Yasin Temel

*Ali Jahanshahi and Lisa-Maria Schönfeld contributed equally to this review.

Published in Molecular Neurobiology, 2014;49:1005-1016.

ABSTRACT

Electrical brain stimulation used to treat a variety of neurological and psychiatric diseases is entering a new period. The technique is well-established and the potential complications well-known and generally manageable. Recent studies demonstrated that electrical fields (EFs) can enhance neuroplasticity-related processes. EFs applied by electrical stimulation in the physiological range induce migration of different neural cell types in different species in *in vitro* conditions. There is some evidence that also the speed and directedness of cell migration is enhanced by EFs. However, it is still unclear how electrical signals from the extracellular space are translated into intracellular actions resulting in so-called electrotaxis phenomenon. Here, we aim to provide a comprehensive review of the data on responses of cells to electrical stimulation and the relation to functional recovery.

ABBREVIATIONS

AC: Alternating current

ACh: Acethylcholine

AChR: ACh receptor

BrdU: Bromodeoxyuridine

Cdc42: Cell division control protein 42 homolog

Ci-VSP: Ciona intestinalis voltage-sensor-containing phosphatase

CNS: Central nervous system

DBS: Deep brain stimulation

DC: Direct current

DRG: Dorsal root ganglion

DCX: Doublecortin

EF: Electrical field

EGFR: epidermal growth factor receptor

EMF: Electromagnetic field

GSK-3: glycogen synthase kinase-3

hiPS: human induced pluripotent cultured stem cells

ICMS: Intracortical microstimulation

MARCKS: Myristoylated alanine-rich C-kinase substrate

MTOC: microtubule-organizing center

NADPH: nicotinamide adenine dinucleotide phosphate

NaKa: Na^+/K^+-ATPase

NDNF: Neuron-derived neurotrophic factor

NHE: Na^+/K^+ exchanger

NHE3: Na^+/K^+ exchanger isoform 3

NSCs: Neural stem cells

NSPCs: Neural stem/progenitor cells

$O2^{-}$: Superoxide

pHi: Intracellular pH

PI3K: Phosphoinositide 3-kinase

PI3P: phosphatidylinositol-triphosphate

PKC: Protein kinase C

PLC: Phospholiase C

PTEN: Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase

RhoA: Ras homolog gene family, member A

SVZ: Subventricular zone

TBI: Traumatic brain injury

TTX: tetrodotoxin

VGCCs: voltage-gated Ca^{2+} channels

VGSCs: voltage-gated sodium channels

V_{mem}: Membrane potential

INTRODUCTION

Electrical brain stimulation through implanted electrodes is receiving a substantial interest in treating neurological and psychiatric disorders[1, 2]. Although electrical stimulation in the field of neurosciences is not a new concept[3], fundamental research has revealed novel mechanisms of action, going beyond the initial ideas of axonal excitation[4] and cellular inhibition when performed at higher frequencies[5]. Utilizing animal models, several studies have shown that deep brain stimulation (DBS) can induce neuroplasticity-related effects at the molecular level by up-regulating specific transcription factors[6], at the cellular level by augmenting the formation of newly born neurons and glia[7-9], and at the functional level leading to enhanced neurobehavioral performance[10]. These advances have motivated scientists to investigate the therapeutic potential of brain stimulation in central nervous system (CNS) diseases characterized by substantial tissue loss such as stroke and traumatic brain injury (TBI).

One of the fields, in which electrical stimulation has already been used in the clinical setting to enhance recovery, is hemiparetic stroke in patients. In contrast to DBS, which implies the penetration of brain tissue, electrical stimulation to promote recovery has classically been applied epidurally or subdurally. Patients who had suffered from stroke received epidural motor cortex stimulation (MCS) during the rehabilitation process[11-13]. These open-label studies found significant therapeutic effects on motor recovery. Unfortunately, those early clinical observations could not been reproduced by a large but prematurely terminated clinical trial, known as the Everest trial[14]. The primary aim of the Everest trial was to assess the efficacy of MCS in combination with rehabilitation therapy on upper limb recovery in patients with ischemic stroke.

Stimulation was turned on during the rehabilitation sessions with the following stimulation parameters: bipolar pulses, frequency of 50 Hz, pulse width of 250 μs and a stimulation amplitude of 6.5 mA. A rehabilitation session had typically a duration of 2.5 hours for 2-5 days a week during the first 6 weeks. Interim-analysis showed that the investigational subjects performed only 20% better than the control subjects. This was not considered satisfactory and the trial was terminated prematurely. The question that arises here is whether or not the stimulation paradigm applied in this study was the most appropriate one, since it was derived from pain-treatment studies, meant to interfere with the pathological neural activity below the electrode[15].

On the hand, in the preclinical setting MCS has been applied in rodents and primate models of stroke and therapeutic effects were observed[15-18]. For instance, in a rat model of electrocoagulation-induced stroke, Kleim and colleagues have found that 50Hz monopolar anodal or cathodal or bipolar stimulation for a period of 10 days, 20 minutes/day during performance of a skilled reaching task, resulted in positive superior reaching accuracy. The best effects were obtained with monopolar cathodal stimulation[16]. Similar results were also shown by Teskey *et al*. (2003). They reported an increase in grasping performance following MCS with similar stimulation parameters in a devascularization-based rat model of stroke[18]. In a non-human primate model of stroke induced by electrocoagulation, improved hand movements were found after a combination of MCS (50Hz, 35 minutes for a period of 11-24 days) and rehabilitation therapy. Interestingly, cortical mapping using the intracortical microstimulation (ICMS) technique after cortical stimulation therapy revealed an increase in ICMS-responsive cortical tissue underneath the electrode. These

findings suggested a newly-emerged hand representation in the peri-infarct region[17].

The mechanisms underlying the recovery-promoting effect of electrical stimulation are not fully understood. One potential mechanism might be the partial regeneration of neural tissue due to the migration of newborn neural cells towards the stimulation site. Electrical fields have the ability to attract progenitor cells towards the source of current *in vitro* conditions, which has been named as "electrotaxis" or "galvanotaxis"[19-21]. In an attempt to demonstrate electrotaxis *in vivo*, Morimoto and colleagues investigated the effect of DBS in the rat striatum after cerebral ischemia induced by middle cerebral artery occlusion[22]. Rats received continuous stimulation of the ischemic penumbra for one week starting immediately after electrode implantation. Combinations of stimulation parameters were applied, with currents of 0, 100 and 200μA and frequencies of 0, 2, 10 and 50Hz. The results were an increased volume of brain tissue in the infarcted area and increased number of bromodeoxyuridine/doublecortin (BrdU/DCX) double-labeled cells. These latter cells are widely accepted to represent migratory neuroblasts. In addition, an increased number of mature neurons was detected in the ischemic penumbra of treated animals[22]. In another study, the effect of electromagnetic field (EMF) stimulation was examined in a lysophosphatidylcholine-induced rat model of demyelination. Applied EMF led to a significant increase in BrdU-positive cells at the lesion site (corpus callosum) after 7 and 14 days of stimulation. These new cells are thought to originate from the SVZ[23]. Recently, we addressed the question whether epidural stimulation of the cortical regions exerted similar effects. Epidural stimulation was applied with a current of 100μA and frequencies of 30 and 330Hz at the level of the motor cortex in rats for a period

of one month. Results showed an enhanced cell migration from the subventricular zone (SVZ) towards the source of current. In addition, a considerable amount of the migrating cells exhibited a neuronal phenotype[24]. These findings suggest that neurogenesis and migration towards the stimulation electrode might play a role in the recovery induced by electrical brain stimulation. However, a key question is whether the newly formed cell population at the damaged brain area is beneficial or not? In this respect, more detailed investigations are required to determine the functional properties of the newly formed neurons. Here, we aim to provide a comprehensive review of the data on responses of cells to electrical stimulation (Table 1) and how this is potentially related to functional recovery.

STEM- AND PRECURSOR CELL RESPONSE TO CELL DAMAGE

Neural stem- and progenitor cells (NSPCs) are present at specific sites in the adult mammalian brain, including the SVZ[25, 26]. The SVZ contains the highest number of NSPCs[27]. After proliferation in the SVZ, newborn cells mainly migrate rostrally towards the olfactory bulb, where they incorporate into the existing neural circuit and function like regional cells[28]. During the recent years we have seen that the adult mammalian CNS shows far more widespread neural migration than previously known. Neuronal migration plays an important role in regulating a wide range of physiological and pathological processes in the CNS including brain development during embryogenesis [29] and neuron guidance[30, 31]. Conditions causing cell damage, such as stroke and TBI, are known to stimulate proliferation of endogenous NSPCs in the SVZ and their migration towards the damaged brain regions[25, 32-35]. However, this migration appears to be insufficient to supply enough cells to the lesion site to achieve appropriate tissue repair and

NSPC (-related) source	Stimulation parameters	Duration	Stimulation device	Results	Ref
Xenopus neural tube cells	1-10 mV/mm^2	6 hours	Sealed culture chamber, EF applied through salt bridges	More and longer neurites at cathodal stimulation site.	53
Rat embryonic lateral ganglionic eminence explant	50-250 mV/mm^2	3 hours	Microchamber build for EF application, EF applied through agar salt bridges	Migrating cells were NSPCs positive for Nestin and DCX. Increased directedness and speed of migration to cathode.	21
Primary hippocampal neurons	50-300 mV/mm^2	2 hours (polarity reversed after 1 hour)	Culture micro-chambers	Increased direction of cell migration towards cathode with EFs above 120mV/mm^2, also after polarity switch. No effect on migration speed.	60

NSPC (-related) source	Stimulation parameters	Duration	Stimulation device	Results	Ref
Primary hippocampal neurons	Biphasic current pulses, 50 µsec pulse width, 20 Hz, 30 µA	72 hours	Microelectrode arrays	Neuron-only cultures showed increased necrosis around electrode. Astrocyte co-culture: Migration of neurons towards cathode.	62
Adult rat NSC clone HCN-A94	250 mV/mm^2	n/a	Electrotactic chamber: NSCs plated out on culture dish and sealed with coverslip, EF application through salt bridges	Increased migration directed towards cathode.	64
Adult rat NSC clone HCN-A94	250-500 mV/mm^2	1 hour	Electrotactic chamber	NSCs stayed positive for Nestin Migration of NSCs towards cathode in spinal cord slices.	19

NSPC (-related) source	Stimulation parameters	Duration	Stimulation device	Results	Ref
Primary hippocampal neurons	50-300 mV/mm^2	1 hour	Electrotactic chamber, EF application through salt bridges	Polarization of Golgi apparatus and centrosome towards cathode. Cell division towards cathode. Neurite outgrowth and migration towards cathode.	65
Human induced pluripotent stem cells (hiPS)	30-200 mV/ mm^2	3-8 hours	hiPS-containing matrigel in electrotactic chamber	Colonies and single cells moved towards anode in medium and 3D matrigel. HiPS stayed positive for stem cell markers SSEA-4 and Oct-4.	100

NSPC (-related) source	Stimulation parameters	Duration	Stimulation device	Results	Ref
Human neural stem cells	30-200 mV/ mm^2	3-8 hours	Electrotactic chamber	Cathodal migration, also after field reversal; migration increased with field strength.	20
Co-culture of primary rat astrocytes and dorsal root ganglia	500 mV/ mm^2 (only to astrocytes)	24 hours	Salt bridges in culture chambers as well as electrotactic chamber	Astrocytes align perpendicular to electrical field. Neurons grow directionally on EF-aligned astrocytes, but non-directionally on randomly orientated astrocytes.	67

NSPC (-related) source	Stimulation parameters	Duration	Stimulation device	Results	Ref
Primary rat embryonic hippocampal neurons	Cathodic-first biphasic current pulses, 50 µs pulse width, 20 Hz, 15, 30, 60 µA	72 hours	Micro electrode arrays: Culture chamber with 32 electrodes	Aggregation of cells around electrodes with 30 en 60 µA. Increase in number of neural processes and total number of astrocytes.	63
Neural progenitor cells (NPCs) derived from periventricular region of adult mice	Direct current (dc-) EF of 250 mV/ mm^2	2.5 hours	Electrotactic chamber	NSPs increased displacement and directedness to cathode when exposed to dcEF	101

Table 1: **Overview of studies reporting electrotaxis**. Details including the type of cells, stimulation parameters, duration of stimulation, stimulation device and findings are provided.

ultimately an improved function. Therefore, there is a need to find a new, effective and safe way to enhance proliferation of these endogenous precursors and direct them towards the damaged CNS sites. For cell replacement therapy in neurodegenerative diseases as well as brain injuries, NSPCs are among the main candidates. However, successful therapy largely depends on how the endogenous and/or transplanted NSPCs respond to directional guidance cues leading them towards the damaged zone[36]. In recent years, the majority of studies have focused on directing the migration of NSPCs in *in vitro* conditions[19, 37, 38], whereas *in vivo* studies investigating the migration of NPSCs towards electrical fields (EFs) have not been widely performed thus far. In fact, the responses of brain tissue to TBI or to electrical stimulation might be different in *in vivo*. In addition to neuronal migration, a number of other processes can take place due to tissue damage and electrical stimulation in the brain. Among these are the release of neurotransmitters such as glutamate, dopamine, serotonin and noradrenalin[39, 40], pro- and anti-inflammatory cytokines as IL-6, IL-1β, TNF-α and INF-γ[41, 42] and growth factors and growth factors e.g., BDNF[43]. Whether these processes would be beneficial or detrimental for electrotaxis in brain injuries is still unpredictable.

PHYSIOLOGICAL ELECTROTAXIS OF NEURAL STEM/PROGENITOR CELLS

Physical factors such as EFs can promote substantial directional responses in a variety of cell types. Reacting to current gradients, cells can migrate towards the source of current[44, 45]. Interestingly, the existence of endogenous steady direct current (DC) EFs has been discovered in biological systems. These currents are known to regulate a variety of biological processes such as tissue regeneration, wound healing and embryogenesis[44-48]. During early life, natural

EFs exist in the wall of the primary neural tube in vertebrate embryos and play a substantial role in guiding neuronal migration during the cranio-caudal nervous system development. The magnitude of endogenous EFs has been measured to range from 3-500mV/mm^2 [49, 50]. EFs in the physiological range induce directed migration in more than 15 different cell types[51, 52]. For example, in *Xenopus* an applied DC gradient of physiological strength guides the growth of neurites projecting from neuroblasts towards the source of the EF[53] (Figure 1). EFs exist in the CNS in both normal and pathological conditions. For instance, EFs occur around damaged nerves in the spinal cord[54], where they are believed to contribute to axonal regeneration[44].

The existence of a voltage gradient (40mV, internally positive) has also been shown in the SVZ, a major neurogenic zone, suggesting an influence of electrical currents on neurogenesis[55]. These EFs are most likely involved in the proliferation and migration of neuroblasts in this area. They play a so-called 'switch-on' role in neuronal migration from the germinal layers of the SVZ[56]. In addition to neurons, also other cell types in or outside of the CNS, such as astrocytes and Schwann cells, are known to possess electrotactic properties[57, 58]. Interestingly, not only in the CNS but also in models of peripheral tissue damage, EFs can induce directional cues to guide cell migration to facilitate the wound healing processes[59].

In vivo, NSPCs are exposed to large EFs during early neural development, which are internally positive and inwardly directed ionic ectodermal transepithelial steady potentials (20-50mV/mm^2) control neural tube formation[50]. Interestingly, the magnitude of this potential is close to the current, which induces electrotaxis *in vitro*. For instance, Li and coworkers succeeded in showing electrotaxis of

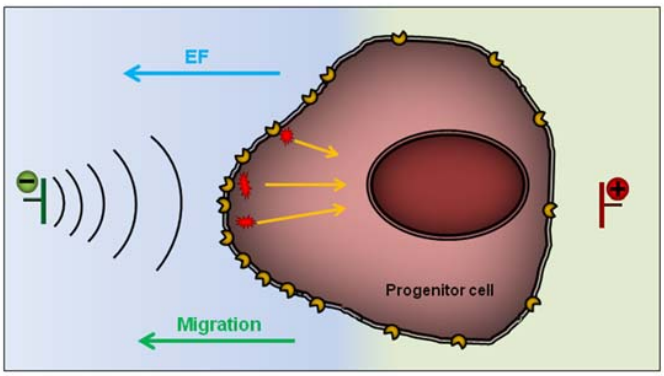


Figure 1. Schematic representation of migration of a neural stem/progenitor cell (NSPC), which is attracted by an extracellular electrical field (EF). The picture indicates that the cell is migrating along the current gradient towards the cathode. The blue and green arrows show the direction of the voltage gradient by the EF and the direction of cell migration, respectively. The EF induces an asymmetrical distribution and activation of the receptors in the membrane, which in turn activates intracellular signaling processes (orange arrows) leading to cell migration.

NSPCs *in vitro* in a culture of lateral ganglionic eminence-derived progenitor cells[38]. Voltage was delivered through agar-salt bridges connected to beakers of Steinberg's solution with silver/silver chloride electrodes. EFs ranging from 50 to 250mV/mm^2 were applied for a period of 3 hours. Time-lapse recording showed that application of EFs caused straight migration of NSPCs out of the explant towards the cathode and also increased migration velocity, when compared to the sham-stimulated NSPCs. Immunocytochemical labeling of explant cells showed that the majority of migrating cells were nestin- and DCX-positive, indicating that EFs may act as a directional guidance cue to control and expedite NSPCs[38]. Notably, the above-mentioned effects were intensity-dependent; the higher the current, the stronger the effect on direction and speed of migration[38].

Yao and colleagues demonstrated that applied DC electrical stimulation in the physiological range (50–300mV/mm^2) directed the migration of rat hippocampal-derived precursor neurons. At a field strength of 120mV/mm^2 and above, neurons exhibited a strong cathodal migration, which increased with higher field strength. However, the speed of neuron migration was not affected by applied EFs. Switching the field polarity switched the direction of neuron migration as well[60]. However, it is not reported whether higher current amplitudes resulted in any cell stress or damage, which can happen *in vivo*[61]. In primary hippocampal neuronal cell cultures, biphasic current pulses (50μs pulse-width, 20Hz, 30μA) delivered by microarray electrodes, directed neuronal migration towards the source of electrical current[62]. A wide range of stimulation parameters (15-60μA, 16-496μs and 5-220Hz) was effective without inducing cell damaging[63].

In vitro cathodal migration of NSPCs has also been observed in several other experiments using a similar stimulation setup. For instance, Arocena and associates applied EFs of 250mV/mm^2 on rat neural stem cells (NSCs), inducing neuronal migration[64]. EFs of 250-500mV/mm^2 applied on embryonic and adult NSCs enhanced both migration itself and migration speed, with higher amplitudes resulting in a more pronounced effect[19]. DC EFs (50–300mV/mm^2) were applied on neonatal hippocampal primary neurons and more effect was observed in 300 mV/mm^2 stimulated cultures[65]. In line with this, Zhang and colleagues found directional cues from EFs of 30-250mV/mm^2 in human induced pluripotent cultured stem cells (hiPS)[66]. Higher magnitudes of EFs induced stronger migration, while the best directional cue was induced by an EF of 30mV/mm^2. In a recent study, NSCs were injected into cultured mouse spinal

cord slices where they showed cathodal migration even in a three-dimensional environment close to *in vivo* conditions[66]. Despite the variety of *in vitro* research mentioned above, there is only a few convincing *in vivo* evidence concerning electrotaxis of NSPCs in the CNS[22-24].

With the eye on clinical applications in the future, it is of great importance to ensure that there are no differences in the electrotactic response of NSPCs from different species, at least not between animals and humans. Human NSCs, like rodent NSCs migrate towards the cathode, even using very small EFs, such as 16mV/mm^2 [20]. This finding strengthens the clinical implication of all previous electrotaxis experiments, suggesting a possible therapeutic role for NSPC guidance towards CNS lesion sites. EFs are able to guide neuronal migration in an indirect manner as well. Astrocytes, which are known to provide a structural framework for growing neurites during development, show strong alignment when exposed to an EF of physiological strength (500mV/mm^2)[67, 68]. However, in contrast to other cell types, astrocytes align to the axis perpendicular to the EF vector[69]. When dorsal root ganglion (DRG) cells were grown on electrically aligned astrocytes in the absence of an EF, DRG neurites were significantly longer compared to DRG cells grown on randomly aligned astrocytes[67]. However, in the presence of a strong EF, DRG cells grown on aligned astrocytes developed significantly shorter neurites than DRG cells grown on aligned astrocytes in the absence of an EF. As neurites usually tend to grow directly towards the cathode of an EF, they were confronted with two opposing forces (i.e. the EF versus perpendicular astrocyte alignment), which could have resulted in the lack of neurite growth[67]. The question remains whether neurite growth is functional and more beneficial when attracted directly by an EF or

when guided along astrocytes, both events leading to neurite growth in a different direction. Future studies should be conducted to look at quantitative differences in neurite growth speed and number to determine the most efficient way of neuronal migration. It seems that during early development neurons are programmed to respond to electrical directing cues and this response may be preserved in adult NSPCs.

In line with this, we found evidence for *in vivo* electrotaxis with stimulation parameters approaching the above-mentioned physiological ranges[24]. More specifically, we applied cathodic current based on *in vitro* observations that NSPCs show mobility responses to cathodic stimulation instead of anodic[64]. The stimulation frequencies and electrical currents were based on findings on cell motility and regenerative processes[44, 45, 62, 70].

HOW CAN NEURAL STEM/PROGENITOR CELLS SENSE AND RESPOND TO ELECTRICAL SIGNALS?

The abovementioned studies demonstrate consistently that electrotaxis is an existing phenomenon and is a potent inducer of neuronal migration. However, the exact underlying mechanisms of electrotaxis are not completely understood. To create an overview of the potential pathways that may play a role in electrotaxis, a summary of recent findings is provided in the following sections. Note that the cellular mechanisms, which are discussed below were not investigated exclusively in NSPCs but also in a few other non-neuronal cell types as well. However, these pathways involve general elements of cell biology, e.g. intracellular organelles, cytoskeleton and certain ion channels, which are known to exist in the majority of cell types.

Detecting the electrical fields

A crucial aspect in neuronal migration caused by electrotaxis is that the cell should be able to sense and respond to EFs. It has been hypothesized that EFs re-distribute cell surface receptors and in turn trigger several downstream signaling pathways linked to the cytoskeleton and different organelles. These in turn control directional sensing and motility, allowing cells to move towards the source of electrical current. Following the exposure to EFs cell membranes can undergo conformational alterations, which are not only directly involved in cell motility, but activate a variety of downstream intracellular signaling pathways leading to cell migration[55, 56, 71-73].

Signal transduction and motility

It has been suggested that asymmetric localization of cell membrane components takes place in response to an EF, which could contribute to transducing the EF signal to the intracellular machinery[56, 72]. During this process, the cytoskeletal network exerts an asymmetrical and ordered reorganization following the exposure of the cell to an electrical gradient[74]. Thereafter, Na/K-ATPase (NaKa) and phosphorylated Na^+/K^+ exchanger isoform 3 (NHE_3) co-localize with β-actin and vinculin on the leading edge of membrane protrusions in Calvaria cells, which also show cathodal migration. Furthermore, inhibition of NaKa or $NHE_{1/3}$ resulted in a decrease in directedness of migrating cells in response to an EF[73]. Moreover, the polarized membrane-associated protein CLIP-170 can grab the plus ends of microtubules, thereby defining the direction of the cytoskeleton-mediated cell migration[75]. Besides the cytoskeleton, dynamic reorientation of some other intracellular organelles such

as the Golgi apparatus has also been reported[65]. Relocation of the Golgi apparatus in the cytoplasm can affect the leading edge of the migrating cell, promoting cell motility[65]. Contrary to the view that electrotactic migration is caused by opening of voltage-gated channels or receptor dislocation, a recent study proposed the principle of electromechanical migration[71]. According to this principle, the EF would exert a mechanical pull on negatively charged components of the cytoskeleton. DC electrical stimulation (100V/m) was combined with different frequencies of alternating current (AC; 40V/m), which were thought to exert a dragging force on the extracellular glycocalyx of keratinocytes. With low-frequency ACs (1.6Hz) keratinocytes significantly slowed down migration, despite presence of a DC EF[71]. The finding that a superimposed AC was able to disrupt cell migration despite the presence of a constant DC EF rather supports the electromechanical model than a receptor-based mechanism of migration.

Downstream signaling pathways

A number of intracellular signal transduction cascades have been described to be involved in cytoskeletal reorganization, specifically Rho family GTPases and phosphoinositide 3-kinase (PI3K) and many of their upstream regulators and downstream effectors as well as the transmembrane subclass of guanylyl cyclases, all known to play an important role in cell migration[76-78]. Different types of cells including neurons react to EFs by voltage-sensitive membrane phosphatase proteins[79]. The *Ciona intestinalis* voltage-sensor-containing phosphatase (Ci-VSP), acts independently as a channel-like gate in response to the voltage signals and translates changes in membrane potential into the

modification of the cytoplasmic enzyme domain, which controls the turnover of phosphoinositides (PI3K and/or PIP3). It has been shown that phosphatidylinositol 3-phosphates play an important role in microtubule-related cell motility[79]. The involvement of PI3K/Akt signaling in the electrotaxis response of NSPCs has been explored the most. For example, there is evidence that EFs in the physiological range induce asymmetric localization of signaling molecules and the actin cytoskeleton, which ultimately directs migration of NSPCs[19]. It has been shown that EFs trigger the redistribution of the PI3K effector PIP3 and the co-localization with actin at the leading edge of the cell, which results in sensing electrical gradients. In addition, it is likely that EFs regulate cell migration at the single cell level directly rather than through modulation of cell–cell interactions. For instance, a cell, which was not in physical contact with surrounding cells, exerted the same migration pattern as a population of the cells in culture. Treatment with the PI3K inhibitor LY294002 reduced the cathodal bias of protrusions and the frequency of changes in direction towards EFs[64]. Furthermore, the amount of phosphorylated Akt, an indicator of PI3K activity, was markedly reduced after LY294002 treatment, despite the presence of growth factors and exposure to the EFs (Figure 2).

Some scientists have reported that activation of PI3K/Akt pathway by EFs influences proliferation of NSPCs besides promoting their migration as well[44], suggesting that EFs can trigger multiple responses of NSPCs through the PI3K/Akt signaling pathway. As mentioned above, dynamic reorientation of specific intracellular organelles, such as the Golgi apparatus also promotes cell motility[65]. ROCK, a downstream effector kinase of Rho A, which is a family member of the Rho GTPase involves cytoskeleton remodeling, cell adhesion and

membrane protrusion, has also been shown to mediate the migration of hiPS towards EFs[66]. Interestingly, Rho and PI3K do not only regulate cell membrane reorganization, but also play a major role in Golgi polarization following exposure to EFs[65].

NMDA- Ca^{2+} mediated responses

EFs activate NMDA receptors, which in turn enhance physical interaction of these channels with the Rac1/Tiam1/Pak1 signaling pathways resulting in conformational changes in actin cytoskeleton and eventually NSPC motility[38]. In line with this, it was shown that treatment with tetrodotoxin (TTX) prevents the Ca^{2+}-dependent migration by blocking this pathway mediated by NMDA receptors. As a result, electrical stimulation of TTX-treated neurons did not induce neuronal migration[62].

Ca^{2+}, is thought to be essential in electrotactic migration amongst a wide variety of cell types[45]. After preventing a rise in intracellular Ca^{2+} through administration of a Ca^{2+} channel blocker, electrotaxis was prevented although cell motility in general remained unaffected[80, 81]. Electrical fields can induce cell movement by fluctuations in Ca^{2+} in two ways, first, via passive ion influx; during the exposure to an EF the membrane at the cathodal site gets depolarized, whereas the membrane at the anodal site becomes hyperpolarized,

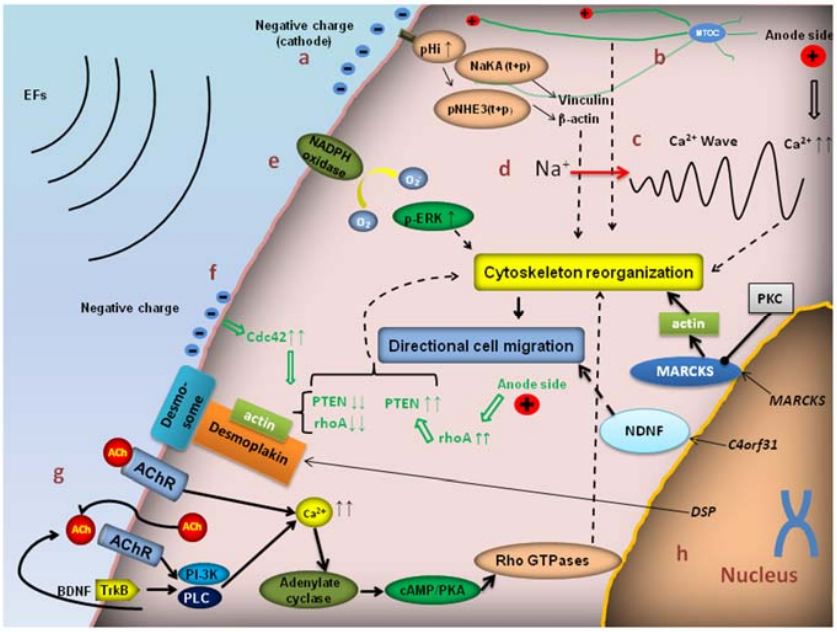


Figure 2: Molecular mechanisms underlying electrotaxis in different cell types. (a) Exposure to the EF might lead to hyperpolarization of the cell membrane of cathode-migrating NSPCs. Hyperpolarization causes a rise in intracellular pH (pHi) which is accompanied by active relocation of Na, K ATPase (NaKa) and phosphorylated Na+/K+ exchanger isoform 3 (NHE3) towards the leading edge. Both proteins co-localize with the cytoskeletal proteins *β*-actin and vinculin, which ultimately leads to reorganization of the cytoskeleton and polarization towards the cathode[73]. (b) The microtubule-organizing center (MTOC) orientates itself towards the site of migration[97]. (c) If the membrane at the cathodal site (leading edge) is depolarized, Ca^{2+} leaks into the cell at the hyperpolarized trailing edge. This creates an internal Ca^{2+} wave which pushes the cell towards the cathode, mainly by membrane contraction of the trailing edge caused by high Ca^{2+} concentrations[45, 82]. (d) Voltage-gated sodium channels (VGSCs) open at the cathodal site due to depolarization. The incoming Na^{+} pushes Ca^{2+} towards the anodal site where it leads to disassembly of the cytoskeleton[84, 87]. (e) The coenzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is activated by the EF and contributes to the production of intracellular superoxide (O_2^-). O_2^- activates the ERK signaling pathways, which play a role in reorganization and polarization of the cytoskeleton towards the current source[98]. (f) Cell division control protein 42 (Cdc42) activity is enhanced on the cathodal site, whereas activity of phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase **(**PTEN) and Ras homolog gene family, member A **(**rhoA) are attenuated. Conversely the latter two enzymes are activated by the anode. Activation of Cdc42 and inhibition of rhoA at the same time is associated with the switch from contact guidance to electrotaxis as migrational cue[76, 92]. (g) EFs cause asymmetric relocation of

membrane-bound receptors, like acetylcholine (ACh) receptors (AChRs). On the cathodal site intracellular ACh is released and binds to these receptors. Activation of AChRs leads to elevation of intracellular Ca^{2+} as AChRs are slightly permeable for Ca^{2+}, which in turn is essential to cathodal migration. AChRs also activate the phosphatidylinositide 3-kinase/phospholiase C (PI3K/PLC) pathway, which leads to intracellular rises in Ca^{2+}. The same effect accounts for the TrkB receptor after binding of its ligand BDNF. Increased intracellular Ca^{2+} leads to elevations in cAMP, interacting with protein kinase A (PKA). Signaling of rho GTPases, such as Cdc42 or rhoA, is stimulated which again affects the cytoskeleton[44]. (h) Application of an EF leads to increased expression of genes, which are eventually translated into proteins related to cell migration. Myristoylated alanine-rich C-kinase substrate (MARCKS) causes actin polymerization, which in turn leads to cytoskeletal reorganization. MARCKS itself is also a substrate for protein kinase C (PKC), which inhibits its activity. Fibronectin type-III domain-containing protein C4orf31, also known as neuron-derived neurotrophic factor (NDNF), is known to support migration in cultured neurons. Desmoplakin is responsible for anchoring the desmosome, which forms junctions with other cells (not shown). Desmoplakin itself is tightly linked to actin[44, 99].

the latter leading to Ca^{2+} influx. Ca^{2+}, as a cathion, then moves towards the anode. An intracellular Ca^{2+} wave builds up a leading to cell contraction, which at the end propels the cell towards the stimulation electrode[45, 82]. Neurotransmission of Ca^{2+}, however, does not solely depend on passive ion diffusion as voltage-gated Ca^{2+} channels (VGCCs) are known to exist[83]. Hence, the second way by which EFs induce cell movement is via influx of Ca^{2+} through VGCCs. As VGCCs open in response to membrane depolarization, Ca^{2+} enters at the cathodal site and the cell moves towards the anode[45]. As a wide range of different cell types move towards the cathode when exposed to an EF, it is almost certain that there are more molecules involved, guiding the cell towards the cathode. However, the importance of Ca^{2+} influx through VGCCs for electrotactic migration has been challenged recently[84]. Earlier investigation had shown that VGCCs are not involved in cathodal migration of Mat-Ly-Lu cells, but the electrotactic migration of those cells is attenuated following the blockade of

voltage-gated sodium channels (VGSCs) by TTX[85]. It was hypothesized that Na^+ ions entering at the depolarized cathodal site push away Ca^{2+} ions towards the anodal site, where Ca^{2+} would lead to actin depolymerization and myosin disassembly[84, 86, 87]. VGSCs might indeed be involved in cell motion as they possess a cell adhesion motif, the β_2 subunit, to adhere to the extracellular matrix and contribute to the extension of neuronal processes[88, 89]. EFs cause asymmetric relocation of membrane-bound receptors, like the acetylcholine (ACh) receptors (AChRs). On the cathodal site intracellular ACh is released and binds to these receptors. Activation of AChRs leads to elevation of intracellular Ca^{2+} as AChRs are slightly permeable for Ca^{2+}, which in turn is essential to cathodal migration. AChRs also activate the phosphatidylinositide 3-kinase/phospholiase C (PI3K/PLC) pathway, which leads to intracellular rises in Ca^{2+}. The same effect accounts for the TrkB receptor after binding of its ligand BDNF. Increased intracellular Ca^{2+} leads to elevations in cAMP, interacting with protein kinase A (PKA). Signaling of rho GTPases, such as Cdc42 or rhoA, is stimulated which again affects the cytoskeleton[44] (Figure 2). Therefore, it seems that both VGCCs and VGSCs are involved in cell motility, one being more important than the other depending on the cell type studied.

Golgi apparatus induced cell motility

Among intracellular organelles, dynamic reorientation of Golgi apparatus exerts a prominent role in cell motility[65]. A protein essential to Golgi polarization by EFs is glycogen synthase kinase-3 (GSK-3), which is involved in mediating cell polarization[90]. Under the influence of EFs, GSK-3β immediately reaches a phosphorylation maximum, which stays constant for one hour. Phosphorylation

of GSK-3β is mediated by the PI3K/Akt pathway as inhibition of PI3K results in a significant decrease of GSK-3β phosphorylation during EF exposure[91]. Inhibition of GSK-3β itself, results in a 50% decrease of Golgi polarization induced by an EF[91]. Blockade of GSK-3β also reduced directedness of Chinese hamster ovary cells by 50%. Therefore, GSK-3β exerts a direct effect on electrotaxis as well. Protein kinase C (PKC) is responsible for GSK-3β phosphorylation and blockade of PKC has the same net effect on electrotaxis, although with a different time course[91].

Hyperpolarization or depolarization?

In the majority of abovementioned studies, a positive intracellular charge has been suggested to explain the mechanisms of electrotaxis. However, Ozkucur and coworkers (2011) found a depolarization at the trailing edge of Calvaria cells, which also show cathodal migration. They used the depolarized membrane potential (V_{mem}) reporter dye DiBAC4(3), which increases fluorescence at the depolarized site[73]. Detecting hyperpolarization at the migration site is a finding opposite to other studies where membrane polarization was determined theoretically based on ion flow[45, 84, 85]. Based on this conflicting evidence, further research is needed to ultimately determine membrane polarization at both the leading and trailing edges of the migrating cells (Figure 2).

Mechanisms of electrotaxis in non-neuronal cell types

A considerable amount of research has been conducted to identify the underlying mechanisms of electrotaxis in other cell types. Interestingly, there are many similarities between neuronal and non-neuronal cell types with regard

to the mechanisms involved in electrotaxis. In wound healing studies, it has been shown that EFs mimic the endogenous current released from skin wounds leading to cathodal polarization of the epidermal growth factor receptor (EGFR) and integrin β4, which are both receptors interacting with the extracellular matrix[92]. Also the molecules PI3K/Akt and Pten, which are responsible for directional guidance in chemotaxis, polarize towards the site of the cathode in an EF and re-arrange after a polarity switch[72, 92]. During the process of EFs-induced migration in non-neuronal cell types, EFs activate endogenous growth factor receptors and alter their nuclear translocation. This in turn activates and redistributes these receptors to the front edge of the migrating cell[93-95]. Interaction between the Rac GTPase and the transmembrane guanylyl cyclase has also been shown to mediate growth factor-induced fibroblast migration[96]. The involvement of PI3K/Akt signaling and the roles of EGF and FGF-2 in the electrotactic response have also been suggested for NSPCs[19]. After being cultured in medium lacking the growth factors EGF and FGF-2, embryonal and adult NSPCs showed a significantly reduced migration to the cathode and migration speed. However, addition of FGF-2 restored cathodal migration completely whereas, EGF alone showed only a minor effect. Thus, it appears that EFs recruit endogenous growth factors to promote electrotactic signaling most likely by activating the PI3K/Akt pathway (Figure 2).

CONCLUSION

Electrical brain stimulation used to treat a variety of neurological or psychiatric diseases is entering a new period. The technique is well-established and the potential complications well-known and generally manageable. Recent studies

demonstrated that EFs can enhance neuroplasticity related processes. EFs applied in the physiological range induce migration of NSPCs and neurons from different species towards the cathode in *in vitro* conditions. There is some evidence that also the speed of migration and directedness of cell migration is enhanced by EFs[19-21, 52, 60, 62, 64]. However, it is still largely unclear how electrical signals from the extracellular space are translated into intracellular actions resulting in the electrotaxis effect. Potential mechanisms involve the redistribution of cell-surface receptors and cytoskeletal components at the site close to the cathode[56, 72, 73], changing membrane potentials leading to the activation of second messenger signaling[75, 77-79], and activating voltage-gated Ca^{2+} channels[45, 83]. Therefore, the potential of electrical brain stimulation to restore brain function is an intriguing concept and deserves further exploration.

ACKNOWLEDGEMENTS

SH and YT have received financial support for this research program by the Universities of Hasselt and Maastricht (Sandwich grant). In addition, YT and AJ received grants from the Dutch Brain Foundation (Hersenstichting Nederland) and European Society for Stereotactic and Functional Neurosurgery (ESSFN).

REFERENCES

1. Perlmutter, J.S. and Mink, J.W. (2006). Deep brain stimulation. Annual review of neuroscience 29, 229-257.

2. Creed, M.C., Hamani, C., Bridgman, A., Fletcher, P.J. and Nobrega, J.N. (2012). Contribution of decreased serotonin release to the antidyskinetic effects of deep brain stimulation in a rodent model of tardive dyskinesia: comparison of the subthalamic and entopeduncular nuclei. J Neurosci 32, 9574-9581.

3. Hariz, M.I., Blomstedt, P. and Zrinzo, L. (2010). Deep brain stimulation between 1947 and 1987: the untold story. Neurosurg Focus 29, E1.

4. Ranck, J.B., Jr. (1970). Electrical impedance changes in many sites of brain in paradoxical sleep, anesthesia, and activity. Experimental neurology 27, 454-475.

5. Benazzouz, A. and Hallett, M. (2000). Mechanism of action of deep brain stimulation. Neurology 55, S13-16.

6. Kadar, E., Lim, L.W., Carreras, G., Genis, D., Temel, Y. and Huguet, G. (2011). High-frequency stimulation of the ventrolateral thalamus regulates gene expression in hippocampus, motor cortex and caudate-putamen. Brain research 1391, 1-13.

7. Derrick, B.E., York, A.D. and Martinez, J.L., Jr. (2000). Increased granule cell neurogenesis in the adult dentate gyrus following mossy fiber stimulation sufficient to induce long-term potentiation. Brain research 857, 300-307.

8. Toda, H., Hamani, C., Fawcett, A.P., Hutchison, W.D. and Lozano, A.M. (2008). The regulation of adult rodent hippocampal neurogenesis by deep brain stimulation. Journal of neurosurgery 108, 132-138.

9. Encinas, J.M., Hamani, C., Lozano, A.M. and Enikolopov, G. (2011). Neurogenic hippocampal targets of deep brain stimulation. J Comp Neurol 519, 6-20.

10. Stone, S.S., Teixeira, C.M., Devito, L.M., Zaslavsky, K., Josselyn, S.A., Lozano, A.M. and Frankland, P.W. (2011). Stimulation of entorhinal cortex promotes adult neurogenesis and facilitates spatial memory. The Journal of neuroscience : the official journal of the Society for Neuroscience 31, 13469-13484.

11. Brown, J.A. (2003). Editorial. Neurol Res 25:115–117, 2003.

12. Brown, J.A., Lutsep, H.L., Weinand, M. and Cramer, S.C. (2006). Motor cortex stimulation for the enhancement of recovery from stroke: a prospective, multicenter safety study. Neurosurgery 58, 464-473.

13. Levy, R., Ruland, S., Weinand, M., Lowry, D., Dafer, R. and Bakay, R. (2008). Cortical stimulation for the rehabilitation of patients with hemiparetic stroke: a multicenter feasibility study of safety and efficacy. Journal of neurosurgery 108, 707-714.

14. Harvey, R.L. and Winstein, C.J. (2009). Design for the everest randomized trial of cortical stimulation and rehabilitation for arm function following stroke. Neurorehabilitation and neural repair 23, 32-44.

15. Tsubokawa, T., Katayama, Y., Yamamoto, T., Hirayama, T. and Koyama, S. (1991). Treatment of thalamic pain by chronic motor cortex stimulation. Pacing Clin Electrophysiol 14, 131-134.

16. Kleim, J.A., Bruneau, R., VandenBerg, P., MacDonald, E., Mulrooney, R. and Pocock, D. (2003). Motor cortex stimulation enhances motor recovery and reduces peri-infarct dysfunction following ischemic insult. Neurological research 25, 789-793.

17. Plautz, E.J., Barbay, S., Frost, S.B., Friel, K.M., Dancause, N., Zoubina, E.V., Stowe, A.M., Quaney, B.M. and Nudo, R.J. (2003). Post-infarct cortical plasticity and behavioral recovery using concurrent cortical stimulation and rehabilitative training: a feasibility study in primates. Neurological research 25, 801-810.

18. Teskey, G.C., Flynn, C., Goertzen, C.D., Monfils, M.H. and Young, N.A. (2003). Cortical stimulation improves skilled forelimb use following a focal ischemic infarct in the rat. Neurological research 25, 794-800.

19. Meng, X., Arocena, M., Penninger, J., Gage, F.H., Zhao, M. and Song, B. (2011). PI3K mediated electrotaxis of embryonic and adult neural progenitor cells in the presence of growth factors. Experimental neurology 227, 210-217.

20. Feng, J.F., Liu, J., Zhang, X.Z., Zhang, L., Jiang, J.Y., Nolta, J. and Zhao, M. (2012). Guided migration of neural stem cells derived from human embryonic stem cells by an electric field. Stem cells 30, 349-355.

21. Li, L., El-Hayek, Y.H., Liu, B., Chen, Y., Gomez, E., Wu, X., Ning, K., Li, L., Chang, N., Zhang, L., Wang, Z., Hu, X. and Wan, Q. (2008). Direct-current electrical field guides neuronal stem/progenitor cell migration. Stem cells 26, 2193-2200.

22. Morimoto, T., Yasuhara, T., Kameda, M., Baba, T., Kuramoto, S., Kondo, A., Takahashi, K., Tajiri, N., Wang, F., Meng, J., Ji, Y.W., Kadota, T., Maruo, T., Kinugasa, K., Miyoshi, Y., Shingo, T., Borlongan, C.V. and Date, I. (2011). Striatal stimulation nurtures endogenous neurogenesis and angiogenesis in chronic-phase ischemic stroke rats. Cell Transplant 20, 1049-1064.

23. Sherafat, M.A., Heibatollahi, M., Mongabadi, S., Moradi, F., Javan, M. and Ahmadiani, A. (2012). Electromagnetic Field Stimulation Potentiates Endogenous Myelin Repair by Recruiting Subventricular Neural Stem Cells in an

Experimental Model of White Matter Demyelination. Journal of molecular neuroscience : MN.

24. Jahanshahi, A., Schonfeld, L., Janssen, M.L., Hescham, S., Kocabicak, E., Steinbusch, H.W., van Overbeeke, J.J. and Temel, Y. (2013). Electrical stimulation of the motor cortex enhances progenitor cell migration in the adult rat brain. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale.

25. Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z. and Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. Nature medicine 8, 963-970.

26. Jahanshahi, A., Temel, Y., Lim, L.W., Hoogland, G. and Steinbusch, H.W. (2011). Close communication between the subependymal serotonergic plexus and the neurogenic subventricular zone. J Chem Neuroanat 42, 297-303.

27. Doetsch, F. and Alvarez-Buylla, A. (1996). Network of tangential pathways for neuronal migration in adult mammalian brain. Proceedings of the National Academy of Sciences of the United States of America 93, 14895-14900.

28. Curtis, M.A., Kam, M., Nannmark, U., Anderson, M.F., Axell, M.Z., Wikkelso, C., Holtas, S., van Roon-Mom, W.M., Bjork-Eriksson, T., Nordborg, C., Frisen, J., Dragunow, M., Faull, R.L. and Eriksson, P.S. (2007). Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. Science 315, 1243-1249.

29. Keller, R. (2005). Cell migration during gastrulation. Curr Opin Cell Biol 17, 533-541.

30. Ayala, R., Shu, T. and Tsai, L.H. (2007). Trekking across the brain: the journey of neuronal migration. Cell 128, 29-43.

31. Hatten, M.E. (2002). New directions in neuronal migration. Science 297, 1660-1663.

32. Kolb, B., Morshead, C., Gonzalez, C., Kim, M., Gregg, C., Shingo, T. and Weiss, S. (2007). Growth factor-stimulated generation of new cortical tissue and functional recovery after stroke damage to the motor cortex of rats. J Cereb Blood Flow Metab 27, 983-997.

33. Parent, J.M., Vexler, Z.S., Gong, C., Derugin, N. and Ferriero, D.M. (2002). Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Annals of neurology 52, 802-813.

34. Teramoto, T., Qiu, J., Plumier, J.C. and Moskowitz, M.A. (2003). EGF amplifies the replacement of parvalbumin-expressing striatal interneurons after ischemia. J Clin Invest 111, 1125-1132.

35. Lindvall, O., Kokaia, Z. and Martinez-Serrano, A. (2004). Stem cell therapy for human neurodegenerative disorders-how to make it work. Nature medicine 10 Suppl, S42-50.

36. Okano, H. and Sawamoto, K. (2008). Neural stem cells: involvement in adult neurogenesis and CNS repair. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 363, 2111-2122.

37. Mogilner, A. (2009). Mathematics of cell motility: have we got its number? Journal of mathematical biology 58, 105-134.

38. Li, L., El-Hayek, Y.H., Liu, B., Chen, Y., Gomez, E., Wu, X., Ning, K., Chang, N., Zhang, L., Wang, Z., Hu, X. and Wan, Q. (2008). Direct-current electrical field guides neuronal stem/progenitor cell migration. Stem Cells 26, 2193-2200.

39. Faden, A.I., Demediuk, P., Panter, S.S. and Vink, R. (1989). The role of excitatory amino acids and NMDA receptors in traumatic brain injury. Science 244, 798-800.

40. van Dijk, A., Klompmakers, A.A., Feenstra, M.G. and Denys, D. (2012). Deep brain stimulation of the accumbens increases dopamine, serotonin, and noradrenaline in the prefrontal cortex. Journal of neurochemistry 123, 897-903.

41. Zhang, R., Liu, Y., Yan, K., Chen, L., Chen, X.R., Li, P., Chen, F.F. and Jiang, X.D. (2013). Anti-inflammatory and immunomodulatory mechanisms of mesenchymal stem cell transplantation in experimental traumatic brain injury. Journal of neuroinflammation 10, 106.

42. Yang, S.H., Gangidine, M., Pritts, T.A., Goodman, M.D. and Lentsch, A.B. (2013). IL-6 Mediates Neuroinflammation and Motor Coordination Deficits After Mild Traumatic Brain Injury and Brief Hypoxia in Mice. Shock.

43. Yanamoto, H., Nakajo, Y., Kataoka, H. and Iihara, K. (2013). High voltage electric potentials to enhance brain-derived neurotrophic factor levels in the brain. Frontiers of neurology and neuroscience 32, 129-138.

44. McCaig, C.D., Rajnicek, A.M., Song, B. and Zhao, M. (2005). Controlling cell behavior electrically: current views and future potential. Physiological reviews 85, 943-978.

45. Mycielska, M.E. and Djamgoz, M.B. (2004). Cellular mechanisms of direct-current electric field effects: galvanotaxis and metastatic disease. Journal of cell science 117, 1631-1639.

46. Robinson, K.R. and Messerli, M.A. (2003). Left/right, up/down: the role of endogenous electrical fields as directional signals in development, repair and invasion. Bioessays 25, 759-766.

47. Nuccitelli, R. (1988). Ionic currents in morphogenesis. Experientia 44, 657-666.

48. Nuccitelli, R. (1988). Physiological electric fields can influence cell motility, growth and polarity. Adv. Cell Biol. 2, 213-233.

49. Borgens, R.B. and Shi, R. (1995). Uncoupling histogenesis from morphogenesis in the vertebrate embryo by collapse of the transneural tube potential. Dev Dyn 203, 456-467.

50. Shi, R. and Borgens, R.B. (1994). Embryonic neuroepithelial sodium transport, the resulting physiological potential, and cranial development. Developmental biology 165, 105-116.

51. Nuccitelli, R. (2003). A role for endogenous electric fields in wound healing. Current topics in developmental biology 58, 1-26.

52. Nuccitelli, R. (2003). Endogenous electric fields in embryos during development, regeneration and wound healing. Radiation protection dosimetry 106, 375-383.

53. Patel, N. and Poo, M.M. (1982). Orientation of neurite growth by extracellular electric fields. The Journal of neuroscience : the official journal of the Society for Neuroscience 2, 483-496.

54. Borgens, R.B., Jaffe, L.F. and Cohen, M.J. (1980). Large and persistent electrical currents enter the transected lamprey spinal cord. Proceedings of the National Academy of Sciences of the United States of America 77, 1209-1213.

55. Song, B., Zhao, M., Forrester, J.V. and McCaig, C.D. (2002). Electrical cues regulate the orientation and frequency of cell division and the rate of wound healing in vivo. Proceedings of the National Academy of Sciences of the United States of America 99, 13577-13582.

56. McCaig, C.D., Song, B. and Rajnicek, A.M. (2009). Electrical dimensions in cell science. Journal of cell science 122, 4267-4276.

57. McKasson, M.J., Huang, L. and Robinson, K.R. (2008). Chick embryonic Schwann cells migrate anodally in small electrical fields. Experimental neurology 211, 585-587.

58. Moriarty, L.J. and Borgens, R.B. (2001). An oscillating extracellular voltage gradient reduces the density and influences the orientation of astrocytes in injured mammalian spinal cord. J Neurocytol 30, 45-57.

59. Zhao, M. (2009). Electrical fields in wound healing-An overriding signal that directs cell migration. Semin Cell Dev Biol 20, 674-682.

60. Yao, L., Shanley, L., McCaig, C. and Zhao, M. (2008). Small applied electric fields guide migration of hippocampal neurons. Journal of cellular physiology 216, 527-535.

61. Temel, Y., Visser-Vandewalle, V., van der Wolf, M., Spincemaille, G.H., Desbonnet, L., Hoogland, G. and Steinbusch, H.W. (2004). Monopolar versus bipolar high frequency stimulation in the rat subthalamic nucleus: differences in histological damage. Neuroscience letters 367, 92-96.

62. Jeong, S.H., Jun, S.B., Song, J.K. and Kim, S.J. (2009). Activity-dependent neuronal cell migration induced by electrical stimulation. Medical & biological engineering & computing 47, 93-99.

63. Jun, S.B., Hynd, M.R., Smith, K.L., Song, J.K., Turner, J.N., Shain, W. and Kim, S.J. (2007). Electrical stimulation-induced cell clustering in cultured neural networks. Medical & biological engineering & computing 45, 1015-1021.

64. Arocena, M., Zhao, M., Collinson, J.M. and Song, B. (2010). A time-lapse and quantitative modelling analysis of neural stem cell motion in the absence of directional cues and in electric fields. Journal of neuroscience research 88, 3267-3274.

65. Yao, L., McCaig, C.D. and Zhao, M. (2009). Electrical signals polarize neuronal organelles, direct neuron migration, and orient cell division. Hippocampus 19, 855-868.

66. Zhang, J., Calafiore, M., Zeng, Q., Zhang, X., Huang, Y., Li, R.A., Deng, W. and Zhao, M. (2011). Electrically Guiding Migration of Human Induced Pluripotent Stem Cells. Stem Cell Rev.

67. Alexander, J.K., Fuss, B. and Colello, R.J. (2006). Electric field-induced astrocyte alignment directs neurite outgrowth. Neuron glia biology 2, 93-103.

68. Tamamaki, N. (2002). Radial glias and radial fibers: what is the function of radial fibers? Anatomical science international 77, 2-11.

69. Borgens, R.B., Shi, R., Mohr, T.J. and Jaeger, C.B. (1994). Mammalian cortical astrocytes align themselves in a physiological voltage gradient. Experimental neurology 128, 41-49.

70. Salimi, I., Friel, K.M. and Martin, J.H. (2008). Pyramidal tract stimulation restores normal corticospinal tract connections and visuomotor skill after early postnatal motor cortex activity blockade. J Neurosci 28, 7426-7434.

71. Hart, F.X., Laird, M., Riding, A. and Pullar, C.E. (2012). Keratinocyte galvanotaxis in combined DC and AC electric fields supports an electromechanical transduction sensing mechanism. Bioelectromagnetics.

72. Zhao, M., Song, B., Pu, J., Wada, T., Reid, B., Tai, G., Wang, F., Guo, A., Walczysko, P., Gu, Y., Sasaki, T., Suzuki, A., Forrester, J.V., Bourne, H.R., Devreotes, P.N., McCaig, C.D. and Penninger, J.M. (2006). Electrical signals control wound healing through phosphatidylinositol-3-OH kinase-gamma and PTEN. Nature 442, 457-460.

73. Ozkucur, N., Perike, S., Sharma, P. and Funk, R.H. (2011). Persistent directional cell migration requires ion transport proteins as direction sensors and membrane potential differences in order to maintain directedness. BMC cell biology 12, 4.

74. Bielas, S.L. and Gleeson, J.G. (2004). Cytoskeletal-associated proteins in the migration of cortical neurons. Journal of neurobiology 58, 149-159.

75. Fukata, M., Watanabe, T., Noritake, J., Nakagawa, M., Yamaga, M., Kuroda, S., Matsuura, Y., Iwamatsu, A., Perez, F. and Kaibuchi, K. (2002). Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170. Cell 109, 873-885.

76. Fukata, M., Nakagawa, M. and Kaibuchi, K. (2003). Roles of Rho-family GTPases in cell polarisation and directional migration. Curr Opin Cell Biol 15, 590-597.

77. Hannigan, M., Zhan, L., Li, Z., Ai, Y., Wu, D. and Huang, C.K. (2002). Neutrophils lacking phosphoinositide 3-kinase gamma show loss of directionality during N-formyl-Met-Leu-Phe-induced chemotaxis. Proceedings of the National Academy of Sciences of the United States of America 99, 3603-3608.

78. Ayoob, J.C., Yu, H.H., Terman, J.R. and Kolodkin, A.L. (2004). The Drosophila receptor guanylyl cyclase Gyc76C is required for semaphorin-1a-plexin A-mediated axonal repulsion. The Journal of neuroscience : the official journal of the Society for Neuroscience 24, 6639-6649.

79. Murata, Y., Iwasaki, H., Sasaki, M., Inaba, K. and Okamura, Y. (2005). Phosphoinositide phosphatase activity coupled to an intrinsic voltage sensor. Nature 435, 1239-1243.

80. Nuccitelli, R., Smart, T. and Ferguson, J. (1993). Protein kinases are required for embryonic neural crest cell galvanotaxis. Cell motility and the cytoskeleton 24, 54-66.

81. Onuma, E.K. and Hui, S.W. (1988). Electric field-directed cell shape changes, displacement, and cytoskeletal reorganization are calcium dependent. The Journal of cell biology 106, 2067-2075.

82. Perret, S., Cantereau, A., Audin, J., Dufy, B. and Georgescauld, D. (1999). Interplay between Ca2+ release and Ca2+ influx underlies localized hyperpolarization-induced [Ca2+]i waves in prostatic cells. Cell calcium 25, 297-311.

83. Turner, D.J. and Mulholland, M.W. (1999). Calcium signaling pathways in the enteric nervous system. International journal of surgical investigation 1, 87-97.

84. Borys, P. (2012). On the biophysics of cathodal galvanotaxis in rat prostate cancer cells: Poisson-Nernst-Planck equation approach. European biophysics journal : EBJ 41, 527-534.

85. Djamgoz, M.B.A., Mycielska, M., Madeja, Z., Fraser, S.P. and Korohoda, W. (2001). Directional movement of rat prostate cancer cells in direct-current electric field: involvement of voltagegated Na+ channel activity. Journal of cell science 114, 2697-2705.

86. Wehrle-Haller, B. and Imhof, B.A. (2003). Actin, microtubules and focal adhesion dynamics during cell migration. Int J Biochem Cell Biol 35, 39-50.

87. Rees, D.A., Charlton, J., Ataliotis, P., Woods, A., Stones, A.J. and Bayley, S.A. (1989). Myosin regulation and calcium transients in fibroblast shape change, attachment, and patching. Cell motility and the cytoskeleton 13, 112-122.

88. Sheng, M. and Kim, E. (1996). Ion channel associated proteins. Curr Opin Neurobiol 6, 602-608.

89. Isom, L.L., Ragsdale, D.S., De Jongh, K.S., Westenbroek, R.E., Reber, B.F., Scheuer, T. and Catterall, W.A. (1995). Structure and function of the beta 2 subunit of brain sodium channels, a transmembrane glycoprotein with a CAM motif. Cell 83, 433-442.

90. Etienne-Manneville, S. and Hall, A. (2003). Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. Nature 421, 753-756.

91. Cao, L., Pu, J. and Zhao, M. (2011). GSK-3beta is essential for physiological electric field-directed Golgi polarization and optimal electrotaxis. Cellular and molecular life sciences : CMLS 68, 3081-3093.

92. Zhao, M., Penninger, J. and Isseroff, R.R. (2010). Electrical Activation of Wound-Healing Pathways. Advances in skin & wound care 1, 567-573.

93. Zhao, M., Dick, A., Forrester, J.V. and McCaig, C.D. (1999). Electric field-directed cell motility involves up-regulated expression and asymmetric redistribution of the epidermal growth factor receptors and is enhanced by fibronectin and laminin. Mol Biol Cell 10, 1259-1276.

94. Fang, K.S., Ionides, E., Oster, G., Nuccitelli, R. and Isseroff, R.R. (1999). Epidermal growth factor receptor relocalization and kinase activity are necessary for directional migration of keratinocytes in DC electric fields. Journal of cell science 112 (Pt 12), 1967-1978.

95. Maher, P.A. (1996). Nuclear Translocation of fibroblast growth factor (FGF) receptors in response to FGF-2. J Cell Biol 134, 529-536.

96. Guo, D., Tan, Y.C., Wang, D., Madhusoodanan, K.S., Zheng, Y., Maack, T., Zhang, J.J. and Huang, X.Y. (2007). A Rac-cGMP signaling pathway. Cell 128, 341-355.

97. Etienne-Manneville, S. and Hall, A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. Cell 106, 489-498.

98. Li, F., Wang, H., Li, L., Huang, C., Lin, J., Zhu, G., Chen, Z., Wu, N. and Feng, H. (2012). Superoxide plays critical roles in electrotaxis of fibrosarcoma

cells via activation of ERK and reorganization of the cytoskeleton. Free radical biology & medicine 52, 1888-1896.

99. Hartwig, J.H., Thelen, M., Rosen, A., Janmey, P.A., Nairn, A.C. and Aderem, A. (1992). MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. Nature 356, 618-622.

100. Zhang, J., Calafiore, M., Zeng, Q., Zhang, X., Huang, Y., Li, R.A., Deng, W. and Zhao, M. (2011). Electrically guiding migration of human induced pluripotent stem cells. Stem cell reviews 7, 987-996.

101. Babona-Pilipos, R., Droujinine, I.A., Popovic, M.R. and Morshead, C.M. (2011). Adult subependymal neural precursors, but not differentiated cells, undergo rapid cathodal migration in the presence of direct current electric fields. PLoS One 6, e23808.

CHAPTER 3

ELECTRICAL STIMULATION OF THE MOTOR CORTEX ENHANCES PROGENITOR CELL MIGRATION IN THE ADULT RAT BRAIN

Ali Jahanshahi, Lisa-Maria Schönfeld, Marcus L.F. Janssen, Sarah-Anna Hescham, Ersoy Kocabicak, Harry W.M. Steinbusch, Jacobus J.van Overbeeke and Yasin Temel

Published in Experimental Brain Research, 2013;231:165-177.

ABSTRACT

Clinical and preclinical investigations suggest that epidural stimulation of the motor cortex (MC) can improve stroke-induced neurological deficits. The mechanisms involved in stimulation-induced recovery are not well understood and might involve neurogenesis-related processes. Here, we addressed the question whether MC stimulation influences processes of migration and differentiation of neuronal progenitor cells *in vivo*. Epidural stimulation electrodes were implanted at the level of the MC in rats and electrical current was applied for a period of one month. Increased cell proliferation was observed in the subventricular zone (SVZ). We also found evidence for enhanced cell migration towards the source of current, a process known as electrotaxis. Some of these cells expressed the neuronal marker NeuN. In addition, our results indicate that MC stimulation enhances neuronal activity of the dorsal raphe nucleus, leading to an increase in the expression of 5-hydroxytryptamine in the SVZ. It is known that such an increase can promote formation of new cells in the SVZ. Our findings suggest that epidural MC stimulation influences neurogenesis-related processes in animal models.

INTRODUCTION

Damage to the motor cortex (MC), resulting from stroke, traumatic brain injury or other causes, usually results in (hemi-) paresis or paralysis adversely impacting the ability to perform activities of daily living. Rehabilitation therapy can be effective in improving these neurological deficits. Nevertheless, a substantial number of patients are left with serious sensorimotor impairments[1]. At present, there is a strong need to develop an effective and safe treatment for these patients. Epidural electrical stimulation of the MC has been proposed as a potential therapy based on clinical studies in neuropathic pain syndromes due to stroke[2-5]. Patients not only showed reduction in pain, but showed enhanced recovery of the stroke-induced motor deficits. Motivated by these findings, open-label studies were conducted and epidural stimulation of the MC was performed in patients with hemiparetic stroke and found to be safe and effective during rehabilitation for recovery of the arm and hand function[6-8]. The stimulation parameters were: 3-second pulse trains, a pulse duration of 250 μsec, a frequency of 50 Hz, a stimulation amplitude, which was set at a level of 50% of the amplitude causing motor response (maximum 6.5 μA), and the duration of 90 minutes per day for a period of 6 months. The selection of these parameters was based on the clinical experience with studies in neuropathic pain syndromes[8]. Despite the encouraging findings in the open-label studies, a large randomized and controlled clinical trial, named as the Everest trial, failed to show a beneficial outcome on upper limb motor function in patients with ischemic stroke[9]. Patients underwent electrical stimulation with the above-mentioned parameters for 6 weeks following the implantation of epidural electrodes. MC stimulation was delivered during sessions of rehabilitation therapy of the affected upper limb. The interim-analysis showed that the

investigational subjects performed only 20% better than the control subjects. This was not considered satisfactory and led to early termination of the trial. The question that arises here is whether the stimulation paradigm applied in this study was the most appropriate one, since it was derived from pain-treatment studies, meant to interfere with the pathological neuronal activity below the electrode. In animal experiments, using both rodents and non-human primates, stimulation of the MC stimulation was found to be effective in promoting recovery of motor functions during rehabilitation from cortical damage[4, 10-12], which was partly based on neuroplasticity-related mechanisms[10, 11]. These data suggest that stimulation parameters and duration of stimulation, which can influence neurogenesis-related mechanisms, might be more appropriate to be used in stroke-related studies.

Traumatic and ischemic tissue damage to the cortex enhances neurogenesis in the subventricular zone (SVZ) and promotes the migration of these newborn cells towards the affected brain area[13-16]. Some of these migrated progenitor cells express the neuronal phenotype[13, 17, 18]. Interestingly, stroke-derived SVZ neural stem/progenitor cells (NSPCs) migrate faster than progenitor cells in the non-stroke counterpart[19]. The SVZ contains the highest number of NSPCs[13, 20]. Initially, it was thought that after proliferation in the SVZ, newborn cells migrate rostrally towards the olfactory bulb (OB)[21]. Nowadays, we know that a more widespread neural migration takes place, including a route to the cerebral cortex[22]. Therefore, the SVZ can be seen as a potentially important supplier of new cells to all areas in demand.

To date, there is not much known about the factors that can enhance migration from the SVZ to achieve a higher number of migrating NSPCs to the affected brain areas. Evidence from physical sciences suggests that factors such as

electrical current can promote directional responses in a variety of cell types. For instance, cells can migrate towards the source of an electrical current, a process termed as electrotaxis or galvanotaxis[23, 24]. In fact, electrical currents do exist in the central nervous system (CNS) in both physiological and pathological conditions. As evidenced, electrical currents have been detected around damaged nerves in the spinal cord[25], where they are believed to enhance axonal regeneration[23]. An *in vitro* study showed that cultured progenitor cells, derived from the rat CNS, migrate towards the area of electrical stimulation[26].

In terms of mechanisms involved in the formation of new cells in the SVZ, the challenge remains the identification of factors controlling this process. In this respect, an interesting but relatively unexplored factor is the presence of the subependymal serotoninergic (5-hydroxytryptamine, 5-HT) fiber plexus. A dense plexus of subependymal 5-HT fibers overlaps the SVZ, separating the latter from the neighboring striatum. These 5-HT fibers originate from the dorsal raphe nucleus (DRN), as well as the median raphe nucleus (MNR)[27]. This close anatomical relationship predicts an important functional role for 5-HT in the regulation of cell proliferation in the SVZ[28]. This is indirectly supported by pharmacological studies in which selective serotonin reuptake inhibitors (SSRIs) and other 5-HT active drugs, which enhance central 5-HT levels, induced neurogenesis in animal models[29-32]. Interestingly, it has recently been shown that SSRI treatment in patients with an ischemic stroke accelerates the recovery process[33].

Here, we hypothesized that stimulation of the MC can accelerate the migration of newborn cells. Since the above-mentioned evidence suggests that the 5-HT system regulates local neurogenesis in the SVZ, we reasoned that the 5-HT

system might also be involved in this process. We evaluated the proliferation of newborn cells in the SVZ after epidural stimulation of the MC at either high or low frequency in rats. Also, the presence and phenotype of the new cells were investigated. In addition, we measured the 5-HT levels in the SVZ and in the MC as well as the 5-HT neuronal activation in the DRN.

MATERIALS AND METHODS

Electrode construction

The electrodes were custom-made by the medical engineering department of the Maastricht University. They were 3x3 mm in size, consisted of 6 circular pads, each with a 0,75 mm diameter and made up of a base material and two layers of copper (top and bottom). The base material used, had a thickness of 0.36 mm and both copper layers with a thickness of 18 µm. The copper was galvanized with nickel/gold (Fig. 1A), which consists of one central rectangle (2,5x1 mm) for the connection of a wire and 6 rounds with a connection to the bottom side (Fig. 1B), which make the stimulating side that was placed on the dura mater.

Subjects

Eighteen male Sprague Dawley rats weighing approximately 300 g at the time of surgery were housed individually (Central Animal Facility of Maastricht University, Maastricht, the Netherlands) under a 12 h reversed light/dark cycle, with acidified water and food *ad libitum*. Rats were randomly assigned to one of the following groups (n = 6 per group): sham, low frequency stimulation (LFS) and high frequency stimulation (HFS). In order to minimize the number of

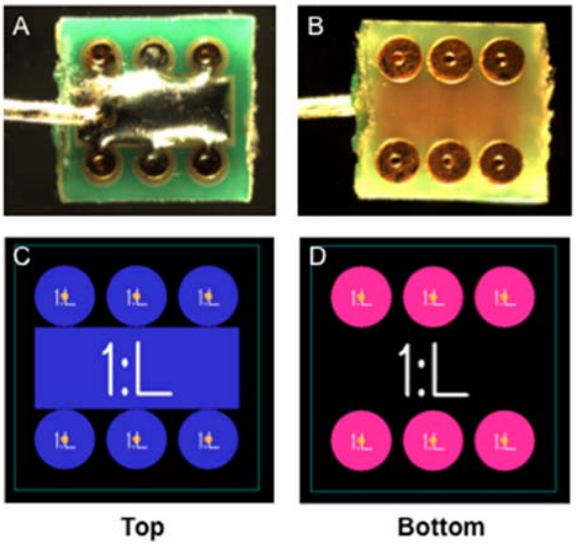


Figure 1
Photomicrographs showing the top (**A**) and bottom sides (**B**) of an electrode, used for motor cortex stimulation. Schematic representation of detailed electrode design (**C**: top and **D**: bottom), topside of the electrode consists of one central rectangle (2,5x1 mm) for the connection of a wire and 6 rounds with a connection to the bottom part. The bottom side of the electrode is the stimulating side and was placed epidurally. It consists of 6 circular pads (Ø = 0,75mm).

animals were used in this study a naive control group was not included. Instead, the contralateral hemispheres of sham subjects were considered as intact control. All experimental procedures were approved and carried out in accordance to the Animal Experiments and Ethics Committee of Maastricht University.

Surgical technique

The surgical procedures were performed under general anesthesia using isoflurane (IsoFlo®, Abbott Laboratories Ltd, Berkshire, UK). Animals were injected 30 min before surgery with buprenorphine (Temgesic®, 0.1 mg/kg,

s.c.) to ensure adequate analgesia. Body temperature was maintained at 37 °C during surgery with a temperature controller (DC Temperature controller 41-90-8D, FHC Inc. Bowdoin, USA). Rats underwent a craniotomy (3.5×3.5 mm) above the right hemisphere, at bregma levels (in mm) AP 0.0–3.5 and ML 0.0-3.5[34]. All animals received implantation of the stimulation electrode epidurally above the MC (coordinates from bregma in mm, AP 0.5–3.5 and ML 0.25-3.25) and a monopolar reference electrode was placed in the skull above the left hemisphere (coordinates from bregma in mm, AP 2.0 and ML 1.75)[34]. Stainless steel screws were positioned in the skull and to serve as anchoring arms. All electrodes were connected to pins of a lightweight miniature socket as a head-stage and fixed to the skull with dental acrylic (Paladur; Heraeus Kulzer GmbH, Hanau, Germany). Rats received a two-week recovery period.

Stimulation parameters

For a period of 31 consecutive days, each rat was stimulated in a freely moving condition in their homecage for four hours per day. During the stimulation session rats had free access to food and water. Stimulation was delivered using a stimulator (World Precision Instruments accupulser [WPI], A310, WPI, Berlin, Germany), which was connected to a stimulus isolator (A360, WPI). Sham operated animals were also connected for four hours to the stimulator, but were not stimulated. We developed a theoretical framework to establish specific stimulation parameters. We applied cathodic current based on *in vitro* observations that NSPCs show mobility responses to cathodic stimulation instead of anodic[35]. In addition, we used a monopolar setting and biphasic pulses to reduce the risk of tissue damage[36] and epileptic phenomena[37], respectively. Monopolar stimulation causes a more diffuse stimulation

influencing a larger area. The stimulation frequencies were low (30 Hz)[26] or high (330 Hz)[38], based on findings on cell motility and regenerative processes, respectively. We applied low amplitude of current, 100 µA/contact, in the range observed in physiological conditions in neuronal networks *in vivo*[23, 24]. With these stimulation parameters we did not observe contra-lateral motor responses. However, when we applied currents above 1 mA then we could clearly evoke a contra-lateral motor response in the forelimb.

BrdU administration and tissue processing

Before the stimulation period, all rats received five days, twice daily (minimum 6 hours apart) intra-peritoneal injections of the thymidine analog 5-bromo-2-deoxyuridine (BrdU, 50 mg/kg, Sigma, St. Louis, U.S.A) dissolved in 0.9% saline (pH 7.6). At the end of the stimulation period, rats received an overdose of pentobarbital (75 mg/kg, Nembutal®, Abbott Laboratories, North Chicago, USA), followed by a transcardial perfusion fixation with tyrode (0.1 M) and fixative containing 4% paraformaldehyde, 15% picric acid, 0,05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.6). Brains were removed and post-fixed for 2 h, followed by overnight immersion in 15% sucrose at 4 °C. Brain tissue was then quickly frozen with CO_2 and stored at -80 °C. Coronal sections (30 µm) were cut serially in 10 series using a cryostat (MICROM, Germany) and again stored at -80 °C.

Immunohistochemistry

To assess cell proliferation one series of brain sections was processed immunohistochemically for Ki-67, a well characterized protein used as a proliferation marker[39]. The migration of progenitor cells was accessed using an

antibody against doublecortin (DCX), which is known as a reliable marker for migratory neuroblasts[40]. In addition, to evaluate the density of 5-HT-immunoreactive (5-HT-ir) neuronal fibers in the SVZ and MC, two series of sections were processed for 5-HT immunohistochemistry. Another series of sections containing the olfactory bulbs (OB) was processed immunohistochemicaly for BrdU. Finally, to test the effect of MC stimulation on DRN cells, c-Fos (K-25) immunohistochemistry was carried out. C-Fos (K-25) is known to be a marker for chronic neuronal activation[41]. To further characterize c-Fos positive cells, we performed a double staining with 5-HT. Staining procedure and the antibody dilutions were the same as explained for the single stainings.

Stainings were performed using the following primary antibodies: rabbit anti-Ki-67 (diluted 1:1000, Vector laboratories Inc, city, USA), rabbit anti-5-HT antibody (59) (diluted 1:20000); mouse anti-BrdU (diluted 1:400, Roche, IN, USA), goat anti-DCX (dilution 1:500, Santa Cruz Biotechnology Inc, Santa Cruz, USA) and polyclonal rabbit anti-c-Fos (K-25; 1:10,000; Santa Cruz Biotechnology Inc). After the incubation period (three days for 5-HT and overnight for Ki-67, BrdU, c-Fos and DCX) and rinsing steps with Tris-buffered saline (TBS) and TBS-containing Triton X-100 (TBS-T), and incubation with the secondary antibody (diluted 1:800, donkey anti-rabbit biotin, donkey anti-mouse biotin and donkey anti-goat biotin, respectively, Jackson Immunoresearch Laboratories, West Grove, USA), the sections were incubated for two hours with an avidin-biotin-peroxidase complex (ABC) (diluted 1:800, Vector laboratories). To visualize the horseradish peroxide reaction product, sections were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB). Finally, sections were mounted and coverslipped using Pertex (Histolab,

Gothenburg, Sweden).

In order to evaluate neuronal differentiation, another series of tissue sections containing the MC was processed for BrdU and neuronal nuclear protein (NeuN) double labeling. Staining was performed using the primary antibodies; mouse anti-BrdU (1:400) and mouse anti-NeuN (biotin-conjugated; dilution 1:100, Temecula, California, USA). After overnight incubation and washing steps, brain sections were incubated with the secondary antibodies donkey anti-mouse Alexa 488 (1:200, Invitrogen, Oregon, USA) and streptavidin Alexa 594 (1:1000, Invitrogen). Sections were washed, mounted on gelatin-coated glasses and coverslipped with 80% glycerol in TBS.

Quantitative analysis of Ki-67-immunoreactive cells in the SVZ

The immunohistochemically stained sections were used to evaluate the total number of Ki-67 containing cells within the SVZ using designed based stereology. Stereological investigations were carried out with a stereological computer microscopy system (Stereo Investigator, Microbrightfield Bioscience, Williston, VT). In all sections showing the SVZ, the region comprising the Ki-67 containing cells within the SVZ, was delineated and the total numbers of Ki-67 containing cells was estimated with the optical fractionator[42-44], using previously described stereological counting method[45, 46].

Semi-quantitative analysis of BrdU and BrdU/NeuN double-labeled cells

Double-stained sections were evaluated using a spinning disk confocal microscope (Olympus, BX50, Tokyo, Japan) that was connected to a computer with analysis software (Stereo Investigator). Per brain, three coronal sections from three bregma levels (+3.20, +2.20 and +1.20) according to the atlas of

the rat brain[34], were subjected to cell counting. After delineating the MC, all BrdU-labeled cells were analyzed for double labeling with NeuN. The number of BrdU/NeuN labeled cells was expressed as a percentage of all BrdU-positive cells that were observed in the MC. A sample of cells that were identified as double labeled BrdU/NeuN cells, using conventional fluorescence microscopy was evaluated by spinning disk, confocal microscopy (Olympus DSU BX51WI) to confirm the co-localization. The number of BrdU-ir cells was counted at the same sections mentioned above. The average value of three sections was calculated for every rat and used for statistical analysis.

Semi-quantitative analysis of doublecortin-labeled cells

Photographs of the stained MC sections from 3 rostro-caudal anatomical levels (from bregma: +3.20, +2.20 and +1.20) were taken[34]. In these images the number of DCX-labeled cells were counted using ImageJ software. The average value of three sections was calculated and processed for statistical analysis.

Quantitative analysis of the density of 5-HT-immunoreactive fibers in the SVZ and motor cortex

The optical density of 5-HT-ir fibers in the in the SVZ and MC was quantified using an image analysis system (f-view; Olympus) connected to an Olympus AX70 brightfield microscope (analySIS; Imaging System, Münster, Germany) from digital photos taken by an Olympus U-CMAD-2 digital camera connected to an Olympus AX 70 microscope). Densitometric measurements (ImageJ software version 1.43u; National Institutes of Health, Bethesda, U.S.A.) were obtained at three rostro-caudal levels for each structure in both hemispheres. The anterio-posterior coordinates from bregma (in mm) were +0.48, +0.20 and -0.40 for

the SVZ and +3.20, +2.20 and +1.20 for the MC[34]. Data are expressed as an average optical density value of three levels in each region for each rat.

Quantification of c-Fos immunohistochemically labeled cells in the DRN

Photographs of the stained DRN sections from 3 rostro-caudal anatomical levels from bregma (AP -7.30, -7.64 and -8.0) were taken. In the images of the area of interest the number of c-Fos positive cells were counted using ImageJ. A cell was regarded positive when the intensity of the c-Fos positive cells was significantly higher than the surrounding background, as based on previous experience[47]. The average value of three sections was used for statistical analysis in each subject.

Olfactory Bulb Quantification

Digital photos were taken from ipsi- and contralateral sides of three rostro-caudal anatomical levels from the rat OB (+6.70, +7.0 and +7.30 mm from bregma) using an Olympus U-CMAD-2 digital camera connected to an Olympus AX 70 microscope and then quantified. Four OB areas (dorsal, ventral, medial, and lateral) from the granular layer of each section were randomly chosen from each side using the 20× magnification. Cells were counted in each photomicrograph using ImageJ. Quantification method was adopted from Sundholm-Peters et al.[16]

Statistical analysis

For all experiments, samples from MC stimulated and sham animals were processed in parallel. Quantitative data of the cell counts and optical density levels in the investigated areas were analyzed using one-way ANOVA and

statistically significant differences were evaluated further by a Tukey's post hoc test. All statistical analyses were performed with SPSS statistical software (SPSS version 15.0, IBM, Chicago, USA). Data are presented as means and standard errors of means (SEM). P-values lower than 0.05 were considered significant.

RESULTS

Enhanced cell proliferation in the SVZ

Stereological quantification of Ki-67-ir cells in the SVZ showed that proliferation of progenitor cells in animals with LFS and HFS of the MC was significantly higher in comparison to sham treated animals ($p < 0.05$ and $p < 0.01$, respectively; Fig. 2). However, there was no significant difference between animals receiving LFS and HFS. In addition, statistical analysis showed enhanced proliferation in the ipsilateral stimulated (right) hemisphere when compared to the contralateral non-stimulated side in both LFS and HFS treated animals ($p < 0.05$; Fig. 2).

Increased neurogenesis in the motor cortex

Semiquantitative analysis showed a higher number of BrdU-ir cells in the LFS and HFS groups as compared to the sham treated animals (Fig. 3F). To determine the phenotype of the migrated newborn cells to the MC, we characterized BrdU-positive cells by double labeling with NeuN. Results showed that the percentage of BrdU-positive cells in the MC, which are positive for NeuN, were higher in rats with LFS and HFS as compared to controls ($p < 0.05$). In addition, our findings indicated that the number of BrdU-positive cells double-labeled with NeuN was significantly higher in the stimulated hemisphere in comparison to the non-stimulated hemisphere in both LFS and HFS groups ($p <$

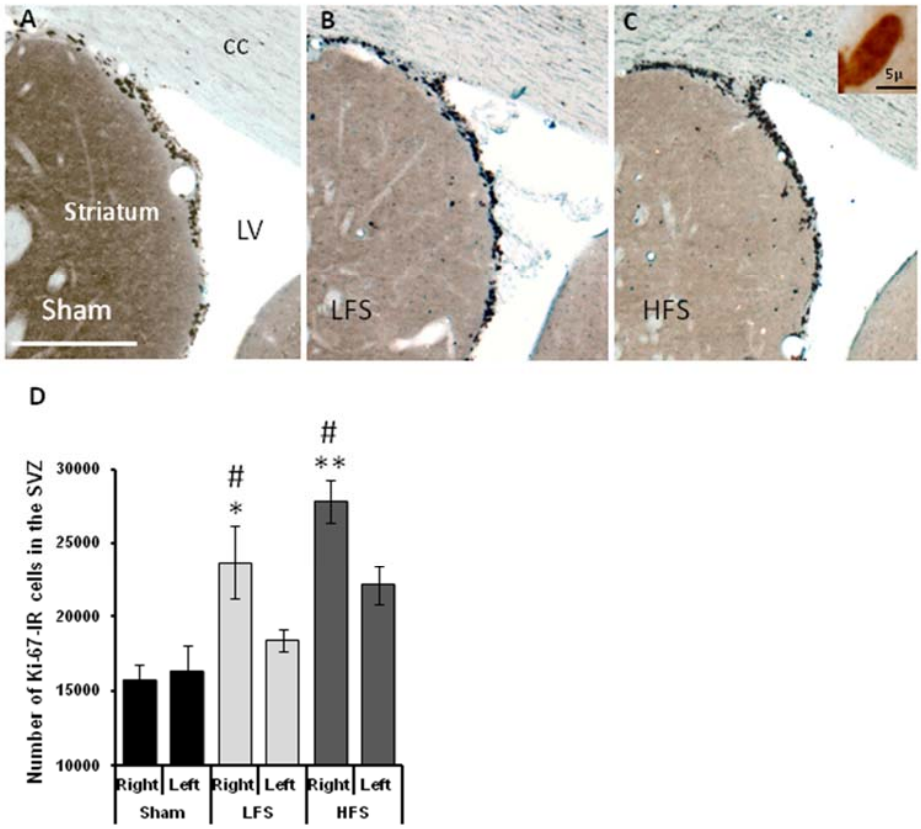


Figure 2
A-C: representative low-power photomicrographs of coronal brain sections containing the subventricular zone (SVZ) of animals with sham treatment and with motor cortex stimulation at low frequency (LFS), high frequency (HFS; scale bar = 400µm). **D**: substantial increase in the number Ki-67 immunoreactive cell in the SVZ of LFS and HFS animals in comparison to the sham treated subjects. In addition, both LFS and HFS groups have more Ki-67 immunoreactive cells in the right SVZ than the left one. Data are presented as mean ± standard error of the mean (SEM), #$p < 0.05$, *$p < 0.05$ and **$p<0.01$. Corpus callosum (cc), lateral ventricle (LV).

0.05). There was neither a significant difference between LFS and HFS groups, nor between the right and left hemispheres in controls (Fig. 4).

Enhanced migration of neuroblasts towards the motor cortex

Semiquantitative cell counting in the MC revealed a higher numbers of DCX-ir cells in the LFS and HFS groups in comparison to the sham treated animals (Fig. 3). The majority of DCX-ir cells were observed in V, VIa and VIb cortical layers[48]. Results showed that the number of DCX-ir cells was significantly higher in the stimulated hemisphere (right) when it was compared to the non-stimulated hemisphere in both the LFS and HFS groups ($p < 0.05$). Statistical analysis again did not reveal any significant difference between the LFS and HFS groups as well as between the right and left hemispheres of the sham treated subjects (Fig. 3C).

Migration to the olfactory bulbs

To test the hypothesis that the migration of progenitor cells to the MC is not at the expense of the physiological rostral migration, we examined the number of BrdU-labeled cells in the OB. No significant differences were found between the contra- and ipsilateral OB and therefore, the data were pooled. The mean number of BrdU-labeled cells in the OB of animals receiving LFS and HFS of the MC were not statistically different as compared to controls ($p > 0.05$; see supplementary data).

Innervation of 5-HT fibers to the SVZ and MC

Statistical analysis revealed no differences in the quantitative examination of 5-HT immunohistochemistry by optical density measurements in the SVZ between the contra- and ipsilateral sides in all groups. Therefore, data were pooled. A clear increase in the optical density of 5-HT containing fibers in the SVZ of animals with HFS and LFS of the MC was found as compared to controls ($p <$

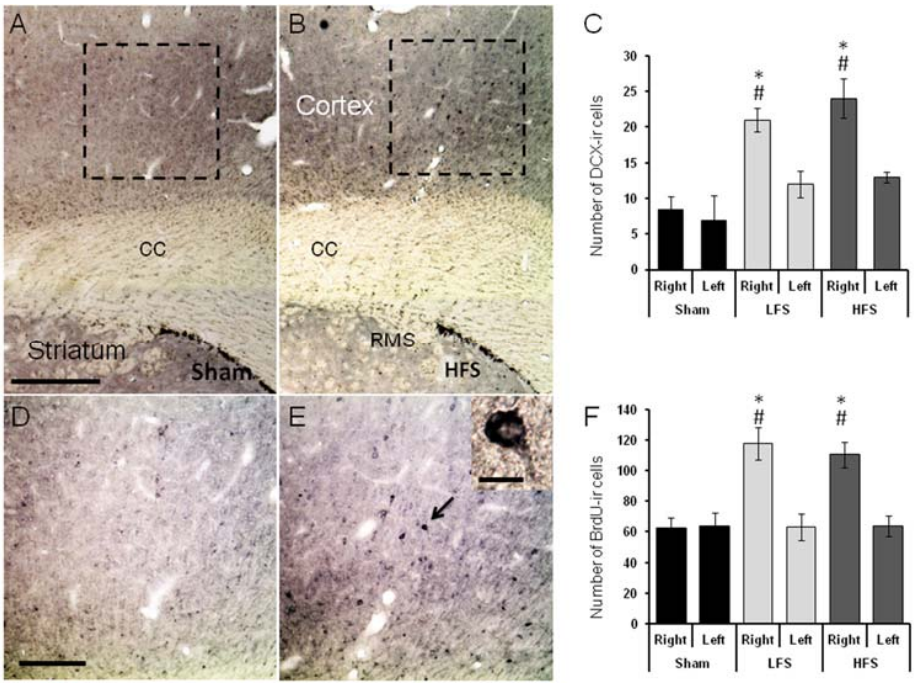


Figure 3
A-B: representative low-power photomicrographs of coronal brain sections containing the motor cortex (MC) of rats with sham treatment and with MC stimulation at high frequency (HFS; scale bar = 500 μm). **D-E**: a higher magnification of rectangles shown in **A** and **B** (scale bar = 250 μm). Note a substantial increase in the number DCX immunoreactive (DCX-ir) cells (an example is shown in the insertion in **E**; scale bar = 20 μm) in the MC of stimulated animals in comparison to the sham treated subjects. **C-F**: cumulative data on the effect of epidural stimulation of the MC at low and high frequency on neuronal migration in the MC. Note the significant increase in the number of BrdU and DCX containing cells in animals stimulated at both low and high frequency (LFS and HFS) in comparison to controls. In addition, there is a significant difference between stimulated (right) and non-stimulated sides (left) of the motor cortex, irrespective of the frequency of stimulation. Data are presented as mean ± standard error of the mean (SEM). *$p < 0.05$ and #$p < 0.05$ compared to the sham and non-stimulated side, respectively.

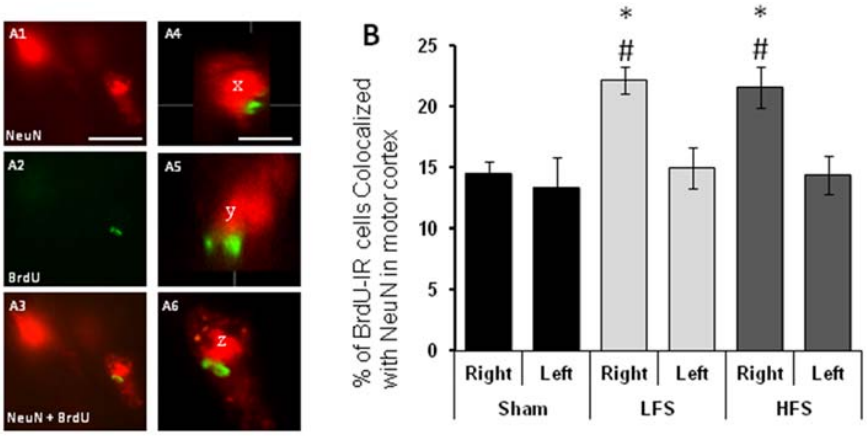


Figure 4
A: representative high-power photomicrograph taken from the motor cortex section of a rat with high frequency stimulation of the motor cortex (MC) using a confocal microscope. This figure shows a double labeled cell with BrdU and NeuN (scale bar = 20µm; A1-3), in a three dimensional constructed image in three anatomical planes (scale bar = 10µm; A4-6). **B**: cumulative data on the effect of epidural stimulation of the MC at low and high frequency on neuronal differentiation in the MC. Please note the significant increase in the percentage of BrdU containing cells, which are double labeled with NeuN in animals stimulated at both low and high frequency (LFS and HFS) in comparison to controls. In addition, there is a significant difference between stimulated (right) and non-stimulated sides (left) of the motor cortex, irrespective of the frequency of stimulation. Data are presented as mean ± standard error of the mean (SEM). *p < 0.05 and #p < 0.05 compared to the sham and non-stimulated side, respectively.

0.01 and p < 0.001, respectively). 5-HT expression was also higher in animals with HFS when compared to animals with LFS of the MC (p < 0.05; Fig. 5). Optical density measurement of the 5-HT fiber density in the MC (ipsilateral to the stimulation electrode) revealed no significant difference between groups (p > 0.05; data not shown).

Enhanced DRN activity

We found that LFS and HFS of the MC caused a robust increase of c-Fos

expression in the DRN as compared to control subjects ($p < 0.001$), indicative of an overall activation of DRN cells (Fig. 6). To further characterize these c-Fos positive cells, we performed a double staining with 5-HT. 5-HT-ir neurons were predominantly located in the dorso- and ventromedial portions of the DRN, as described earlier[47]. Fluorescent c-Fos expression was expressed throughout the DRN, equivalent to the above mentioned distribution (Fig. 6C). We found that c-Fos positive cells were double-labeled with 5-HT.

DISCUSSION

Our findings demonstrate that electrical stimulation of the MC enhances cell proliferation in the SVZ and at the same time enhances the density of 5-HT-containing fibers in the same area. This 5-HT innervation is derived from the raphe nuclei, in particular the DRN. In this respect, we found enhanced neuronal activation in the 5-HT cells in the DRN, which can explain the increase in 5-HT innervation in the SVZ. Furthermore, our results showed an increased number of DCX-ir cells in the cortical area below the electrode, when compared to either non-stimulated site or to the sham-stimulated subjects. DCX is a well-known marker to characterize migratory neuroblasts[49]. These findings most probably reflect electrotaxis, meaning that epidural MC stimulation is able to attract NSPCs from the SVZ to the site of the stimulation. Interestingly, we also found an increased number of BrdU-ir cells in the cortical area below the electrode, in comparison to the contralateral hemisphere and to the controls as well. In the stimulated animals these BrdU positive cells showed more often the neuronal phenotype.

We performed stimulation of the MC with low current and low and high frequencies, based on *in vitro*, *in vivo* and clinical evidence. With regard to

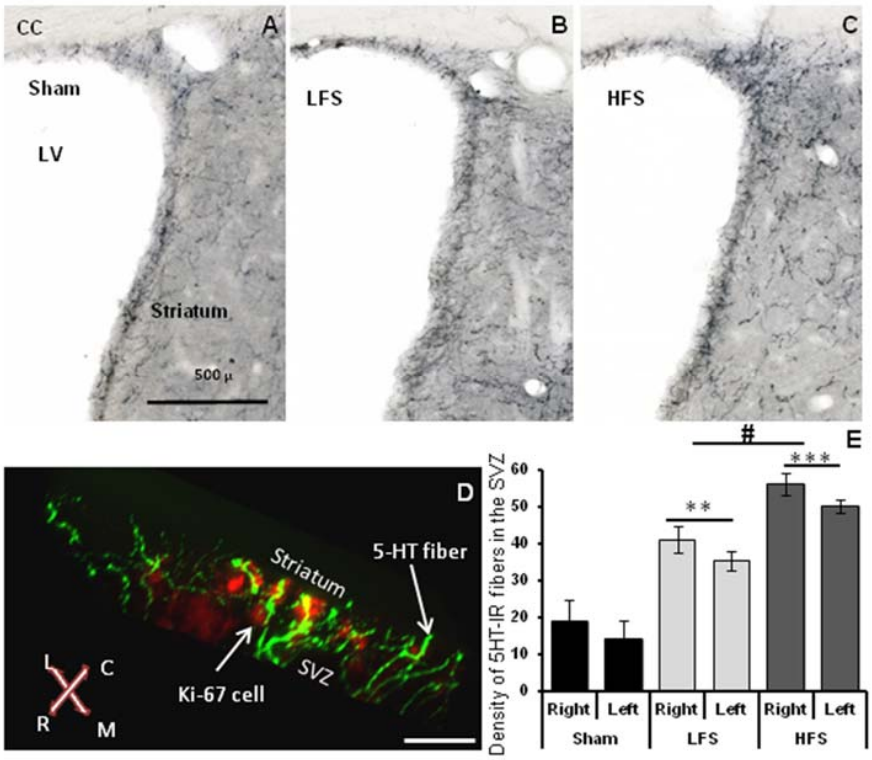


Figure 5
A-C: representative low-power photomicrographs of coronal brain sections containing the subventricular zone (SVZ) immunostained for 5-HT, of animals with motor cortex stimulation at low frequency (LFS), high frequency (HFS), and animals with sham treatment (scale bar = 500). **D**: proliferating (Ki-67 containing) cells are in close contact with 5-HT containing fibers in the SVZ as demonstrated three dimensional reconstruction (scale bar 30µm). **E**: graph showing a significant increase in optical density value measured for 5-HT signal in dorsal SVZ of LFS and HFS animals in comparison to the sham.
Corpus callosum (cc), lateral ventricle (LV), values are mean ± standard error of the mean (SEM) (n = 6). ** $p < 0.01$ and *** $p<0.001$ when compared to sham, # $p<0.05$.

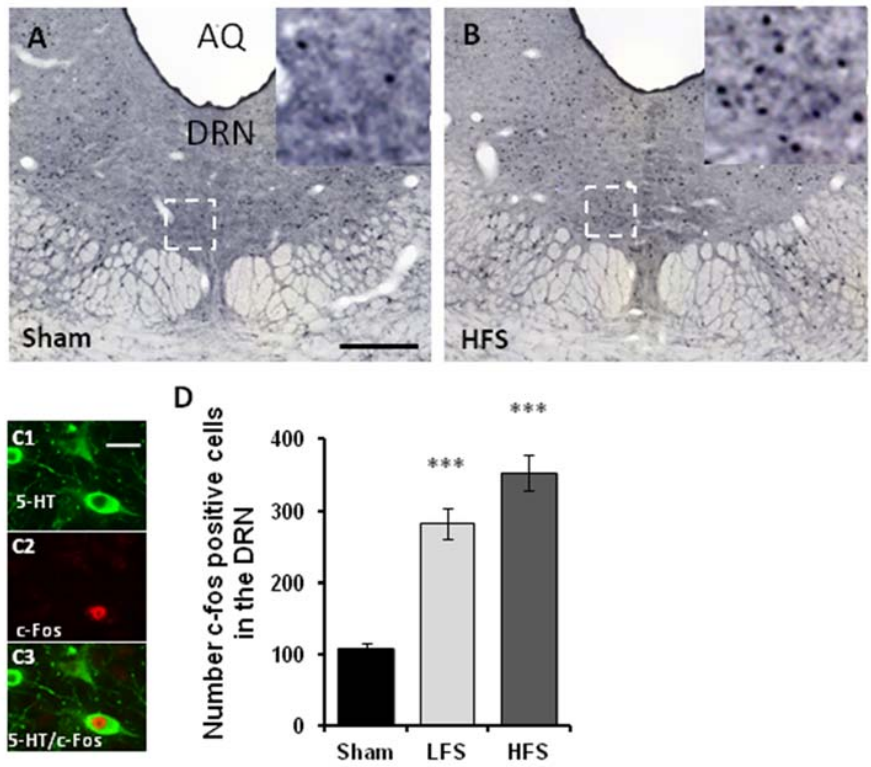


Figure 6
A-B: low-power photomicrographs taken from coronal rat brain sections containing the dorsal raphe nucleus (DRN) of animals with motor cortex stimulation at high frequency (HFS), and animals with sham treatment, stained for c-Fos (K-25; scale bare = 500 μm). **C1-C3**: photomicrographs of an activated (c-Fos) 5-HT containing cell in the DRN (scale bar = 25μm) show the evidence for double-labeling of c-Fos with 5-HT in the same cell. **D**: graph shows a robust increase in the number c-Fos immunoreactive cells in the DRN of LFS and HFS groups in comparison to the sham.
Aqueduct (AQ), values are mean ± standard error of the mean (SEM; n = 6), ***p < 0.001 compared to the sham.

the LFS, beneficial effects have been reported by cortical stimulation with 30-40 Hz for pain relief as well as in rehabilitation therapies in stroke patients[2-5]. In addition, *in vitro* studies using rat CNS derived cultured progenitor cells showed that 20-50 Hz electrical stimulation induced neuronal migration[26, 50, 51]. In

addition, we applied 330 Hz as HFS based on the beneficial effects of this frequency on motor function in an animal model of stroke[38]. No statistically significant differences between animals with LFS and HFS were found concerning the number of Ki-67 positive cells in the SVZ. Therefore, it seems that this frequency range (30-330 Hz) does not play a major role in the effectiveness of electrical stimulation on proliferation.

Low amplitude of electrical current was applied, below the intensity to evoke action potentials in neurons and in a range, which has been observed in physiological conditions *in vivo*[52, 53]. Interestingly, electrical currents in this range are involved in physiological electrotaxis in neural tissue[54]. Moreover, monopolar cathodal stimulation was applied based on the observations, indicating that neuronal newborn cells migrate towards the cathode instead of anode[35]. In line with this, our previous study has also revealed that monopolar pulses induce less tissue damage than bipolar pulses[36]. We found unilateral effects of unilateral stimulation of the MC on migration and differentiation, providing additional evidence for the specificity of the findings reported here. Since a good correlation between BrdU and Ki-67 immunohistochemistry has been reported before[55], cell proliferation in the SVZ was quantified using the Ki-67 staining to reduce the number of animals used in this study. Otherwise, two additional groups of rats would have been needed to be injected with BrdU at the end of the stimulation in order to evaluate proliferation. Quantitative cell counting of Ki-67 cells in the SVZ showed a significant increase in cell proliferation in stimulated animals in comparison to the sham group. Perhaps, enhanced proliferation of newborn cells in the stimulated side is secondary to the migration of these cells from the SVZ towards the MC. We found significantly higher number of both BrdU-ir and DCX-ir cells in the stimulated MC compared

to the controls. In addition, in stimulated animals a higher percentage of BrdU cells were expressing the neuronal marker (NeuN), indicating that neural progenitor cells have been attracted by stimulation of the MC and differentiated into mature neurons. We were not able to perform BrdU/DCX double labeling in this study. This was because of the facts that: i; BrdU was administered 31-36 days before sacrificing the animals and ii; DCX only labels up to two weeks old neuroblasts[40, 49]. Therefore, DCX-ir cells at the time of sacrifice were not born in the SVZ yet to pick up the injected BrdU. In other words, the neuronal migration, which was observed in this study, has most probably occurred during the second half of the stimulation period. In order to be able to detect also earlier migration, BrdU should be administered more times during the stimulation period. In this study, the contralateral hemispheres of the sham subjects were considered as intact control. Our data showed no significant difference in neurogenesis between ipsilateral and contralateral sides in sham stimulated group.

Electrical stimulation can alter different aspects of cellular plasticity such as nerve growth[56, 57], cell migration[58], cell adhesion[59], DNA synthesis[60] and protein secretion[61]. It is known that electrical stimulation enhances peripheral nerve regeneration after nerve injuries in the CNS[62]. Toda and colleagues showed that deep brain stimulation of the rodent thalamus at parameters similar to those used in the clinical settings, induced neurogenesis in the adult brain[63]. In cerebral ischemia rat models, electric stimulation of the affected cortex promoted functional recovery[64, 65]. Our data in combination with the data mentioned above, suggest that electrical stimulation has a potential role in regulating neurogenesis in the CNS. In the current study, however, as far as we are aware of, we suggest for the first time the existence of *in vivo* electrotaxis in

the rat adult MC.

The mechanisms underlying *electrotaxis* are still under investigation. Electrical stimulation alters the balanced membrane electrical state[66], which is characterized by the electrostatic surface charge of membrane and electrodynamic ions fluxes through the membrane channels[67, 68]. This may result in cell membrane permeability[67], and changes in the structure of the cytoskeleton[68]. *In vitro* studies showed that electrical stimulation promoted the production of neuronal growth factors such as brain derived neurotrophic factor (BDNF)[66]. In addition, it has been shown that adult neural progenitor cells alter specific genes, containing cell differentiation programs, such as *Hes1* and *Id2*, and enhance the expression of *NeuroD*, a positive regulator of neuronal differentiation, in response to excitatory stimuli. They introduced this as an excitation-neurogenesis coupling phenomenon[69].

Findings from our experiments revealed that the overall activity of the 5-HT system is increased following stimulation of the MC, evidenced by an enhanced density of 5-HT fibers in the SVZ and cellular activity of 5-HT producing neurons in the DRN. Accumulating evidence has shown that electrical stimulation induces axonal sprouting[70-73]. Therefore, one might argue that enhanced density of 5-HT-ir fibers in the SVZ occurs as a result of electrical currents. Nonetheless, measuring the density of 5-HT-ir fibers in the MC, which is even closer to the stimulating electrode, did not show any significant increase of 5-HT-ir fiber density in the stimulated groups. Therefore, we suggest that the increased 5-HT-ir fibers density takes place in response to a higher proliferation rate in the SVZ, which demands more trophic factors such as 5-HT. In this respect, we found a comparable increase of 5-HT-ir fiber density in the right and left SVZ following MC stimulation. This suggests that enhanced 5-HT innervation to the

SVZ is due to an increased input from the DRN and not axonal sprouting.

Our result demonstrated that migration of neuroblasts from the SVZ to the ipsilateral MC following the electrical stimulation of that area is not compromising the physiological migration to the OB. Neuroblasts migrate to the OB through the rostral migratory stream (RMS) in a chain along channels surrounded by astrocytes[74]. Our data suggest that migration of neuroblasts from the SVZ to the MC can potentially occur outside of the RMS system, as a parallel stream. The existence of parallel migratory streams has been shown before[16].

CONCLUSION

Our results showed that epidural electrical stimulation of the MC increases neurogenesis in the SVZ, which is linked to an enhancement of the 5-HT system, and induces new cells in the cortical area below the electrode. Some of these new cells show the neuronal phenotype. These observations predict the existence of *in vivo* electrotaxis. This mechanism might have the potential to open a new window for treating disorders characterized by tissue loss. The next step is to evaluate the functional properties of these attracted neurons and perhaps glia.

ACKNOWLEDGEMENTS

This study was funded by a grant from the Hersenstichting Nederland (Dutch Brain Foundation). In addition, YT and AJ received grants from the European Society for Stereotactic and Functional Neurosurgery (ESSFN).

REFERENCES

1. Hurst, W. (2002). The Heart, Arteries and Veins. 10 ed. Mc Graw-Hill: New York.

2. Nguyen, J.P., Lefaucheur, J.P., Decq, P., Uchiyama, T., Carpentier, A., Fontaine, D., Brugieres, P., Pollin, B., Feve, A., Rostaing, S., Cesaro, P. and Keravel, Y. (1999). Chronic motor cortex stimulation in the treatment of central and neuropathic pain. Correlations between clinical, electrophysiological and anatomical data. Pain 82, 245-251.

3. Tsubokawa, T., Katayama, Y., Yamamoto, T., Hirayama, T. and Koyama, S. (1991). Chronic motor cortex stimulation for the treatment of central pain. Acta neurochirurgica. Supplementum 52, 137-139.

4. Tsubokawa, T., Katayama, Y., Yamamoto, T., Hirayama, T. and Koyama, S. (1991). Treatment of thalamic pain by chronic motor cortex stimulation. Pacing Clin Electrophysiol 14, 131-134.

5. Tsubokawa, T., Katayama, Y., Yamamoto, T., Hirayama, T. and Koyama, S. (1993). Chronic motor cortex stimulation in patients with thalamic pain. J Neurosurg 78, 393-401.

6. Brown, J.A. (2003). Editorial. Neurol Res 25:115–117, 2003.

7. Brown, J.A., Lutsep, H.L., Weinand, M. and Cramer, S.C. (2006). Motor cortex stimulation for the enhancement of recovery from stroke: a prospective, multicenter safety study. Neurosurgery 58, 464-473.

8. Levy, R., Ruland, S., Weinand, M., Lowry, D., Dafer, R. and Bakay, R. (2008). Cortical stimulation for the rehabilitation of patients with hemiparetic stroke: a multicenter feasibility study of safety and efficacy. Journal of neurosurgery 108, 707-714.

9. Harvey, R.L. and Winstein, C.J. (2009). Design for the everest randomized

trial of cortical stimulation and rehabilitation for arm function following stroke. Neurorehabilitation and neural repair 23, 32-44.

10. Kleim, J.A., Bruneau, R., VandenBerg, P., MacDonald, E., Mulrooney, R. and Pocock, D. (2003). Motor cortex stimulation enhances motor recovery and reduces peri-infarct dysfunction following ischemic insult. Neurol Res 25, 789-793.

11. Plautz, E.J., Barbay, S., Frost, S.B., Friel, K.M., Dancause, N., Zoubina, E.V., Stowe, A.M., Quaney, B.M. and Nudo, R.J. (2003). Post-infarct cortical plasticity and behavioral recovery using concurrent cortical stimulation and rehabilitative training: a feasibility study in primates. Neurological research 25, 801-810.

12. Teskey, G.C., Flynn, C., Goertzen, C.D., Monfils, M.H. and Young, N.A. (2003). Cortical stimulation improves skilled forelimb use following a focal ischemic infarct in the rat. Neurological research 25, 794-800.

13. Arvidsson, A., Collin, T., Kirik, D., Kokaia, Z. and Lindvall, O. (2002). Neuronal replacement from endogenous precursors in the adult brain after stroke. Nature medicine 8, 963-970.

14. Darsalia, V., Heldmann, U., Lindvall, O. and Kokaia, Z. (2005). Stroke-induced neurogenesis in aged brain. Stroke; a journal of cerebral circulation 36, 1790-1795.

15. Lichtenwalner, R.J. and Parent, J.M. (2006). Adult neurogenesis and the ischemic forebrain. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 26, 1-20.

16. Sundholm-Peters, N.L., Yang, H.K., Goings, G.E., Walker, A.S. and Szele, F.G. (2005). Subventricular zone neuroblasts emigrate toward cortical lesions. Journal of neuropathology and experimental neurology 64, 1089-1100.

17. Parent, J.M., Vexler, Z.S., Gong, C., Derugin, N. and Ferriero, D.M. (2002). Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. Annals of neurology 52, 802-813.

18. Jin, K., Sun, Y., Xie, L., Peel, A., Mao, X.O., Batteur, S. and Greenberg, D.A. (2003). Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. Molecular and cellular neurosciences 24, 171-189.

19. Zhang, R., Zhang, Z., Wang, L., Wang, Y., Gousev, A., Zhang, L., Ho, K.L., Morshead, C. and Chopp, M. (2004). Activated neural stem cells contribute to stroke-induced neurogenesis and neuroblast migration toward the infarct boundary in adult rats. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 24, 441-448.

20. Doetsch, F. and Alvarez-Buylla, A. (1996). Network of tangential pathways for neuronal migration in adult mammalian brain. Proceedings of the National Academy of Sciences of the United States of America 93, 14895-14900.

21. Curtis, M.A., Kam, M., Nannmark, U., Anderson, M.F., Axell, M.Z., Wikkelso, C., Holtas, S., van Roon-Mom, W.M., Bjork-Eriksson, T., Nordborg, C., Frisen, J., Dragunow, M., Faull, R.L. and Eriksson, P.S. (2007). Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. Science 315, 1243-1249.

22. Hatten, M.E. (2002). New directions in neuronal migration. Science 297, 1660-1663.

23. McCaig, C.D., Rajnicek, A.M., Song, B. and Zhao, M. (2005). Controlling cell behavior electrically: current views and future potential. Physiological reviews 85, 943-978.

24. Mycielska, M.E. and Djamgoz, M.B. (2004). Cellular mechanisms of direct-current electric field effects: galvanotaxis and metastatic disease. Journal of cell

science 117, 1631-1639.

25. Borgens, R.B., Jaffe, L.F. and Cohen, M.J. (1980). Large and persistent electrical currents enter the transected lamprey spinal cord. Proc Natl Acad Sci U S A 77, 1209-1213.

26. Jeong, S.H., Jun, S.B., Song, J.K. and Kim, S.J. (2009). Activity-dependent neuronal cell migration induced by electrical stimulation. Medical & biological engineering & computing 47, 93-99.

27. Steinbusch, H.W. (1981). Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. Neuroscience 6, 557-618.

28. Jahanshahi, A., Temel, Y., Lim, L.W., Hoogland, G. and Steinbusch, H.W. (2011). Close communication between the subependymal serotonergic plexus and the neurogenic subventricular zone. J Chem Neuroanat 42, 297-303.

29. Banasr, M. and Duman, R.S. (2007). Regulation of neurogenesis and gliogenesis by stress and antidepressant treatment. CNS Neurol Disord Drug Targets 6, 311-320.

30. Banasr, M., Hery, M., Printemps, R. and Daszuta, A. (2004). Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-HT receptor subtypes in the dentate gyrus and the subventricular zone. Neuropsychopharmacology 29, 450-460.

31. Nandam, L.S., Jhaveri, D. and Bartlett, P. (2007). 5-HT7, neurogenesis and antidepressants: a promising therapeutic axis for treating depression. Clin Exp Pharmacol Physiol 34, 546-551.

32. Newton, S.S. and Duman, R.S. (2007). Neurogenic actions of atypical antipsychotic drugs and therapeutic implications. CNS Drugs 21, 715-725.

33. Chollet, F., Tardy, J., Albucher, J.F., Thalamas, C., Berard, E., Lamy, C.,

Bejot, Y., Deltour, S., Jaillard, A., Niclot, P., Guillon, B., Moulin, T., Marque, P., Pariente, J., Arnaud, C. and Loubinoux, I. (2011). Fluoxetine for motor recovery after acute ischaemic stroke (FLAME): a randomised placebo-controlled trial. Lancet neurology 10, 123-130.

34. Paxinos, G. and Watson, C. (1998). The rat brain in stereotaxic coordinates. Academic Press San Diego.

35. Arocena, M., Zhao, M., Collinson, J.M. and Song, B. (2010). A time-lapse and quantitative modelling analysis of neural stem cell motion in the absence of directional cues and in electric fields. Journal of neuroscience research 88, 3267-3274.

36. Temel, Y., Visser-Vandewalle, V., van der Wolf, M., Spincemaille, G.H., Desbonnet, L., Hoogland, G. and Steinbusch, H.W. (2004). Monopolar versus bipolar high frequency stimulation in the rat subthalamic nucleus: differences in histological damage. Neuroscience letters 367, 92-96.

37. Osorio, I. and Frei, M.G. (2009). Seizure abatement with single dc pulses: is phase resetting at play? Int J Neural Syst 19, 149-156.

38. Salimi, I., Friel, K.M. and Martin, J.H. (2008). Pyramidal tract stimulation restores normal corticospinal tract connections and visuomotor skill after early postnatal motor cortex activity blockade. J Neurosci 28, 7426-7434.

39. Scholzen, T. and Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. J Cell Physiol 182, 311-322.

40. Gleeson, J.G., Lin, P.T., Flanagan, L.A. and Walsh, C.A. (1999). Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23, 257-271.

41. Budzikowski, A.S., Vahid-Ansari, F. and Leenen, F.H. (1998). Chronic activation of brain areas by high-sodium diet in Dahl salt-sensitive rats. Am J

Physiol 274, H2046-2052.

42. Schmitz, C. and Hof, P.R. (2000). Recommendations for straightforward and rigorous methods of counting neurons based on a computer simulation approach. J Chem Neuroanat 20, 93-114.

43. Schmitz, C. and Hof, P.R. (2005). Design-based stereology in neuroscience. Neuroscience 130, 813-831.

44. West, M.J., Slomianka, L. and Gundersen, H.J. (1991). Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. Anat Rec 231, 482-497.

45. Temel, Y., Visser-Vandewalle, V., Kaplan, S., Kozan, R., Daemen, M.A., Blokland, A., Schmitz, C. and Steinbusch, H.W. (2006). Protection of nigral cell death by bilateral subthalamic nucleus stimulation. Brain research 1120, 100-105.

46. Westin, J.E., Janssen, M.L., Sager, T.N. and Temel, Y. (2011). Automated gait analysis in bilateral Parkinsonian rats and the role of l-DOPA therapy. Behavioural brain research.

47. Tan, S.K., Janssen, M.L., Jahanshahi, A., Chouliaras, L., Visser-Vandewalle, V., Lim, L.W., Steinbusch, H.W., Sharp, T. and Temel, Y. (2011). High frequency stimulation of the subthalamic nucleus increases c-fos immunoreactivity in the dorsal raphe nucleus and afferent brain regions. J Psychiatr Res.

48. Rosen, G.D., Jacobs, K.M. and Prince, D.A. (1998). Effects of neonatal freeze lesions on expression of parvalbumin in rat neocortex. Cereb Cortex 8, 753-761.

49. Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., Bogdahn, U., Winkler, J., Kuhn, H.G. and Aigner, L. (2005). Doublecortin expression levels in adult brain reflect neurogenesis. Eur J Neurosci

21, 1-14.

50. Jun, S.B., Hynd, M.R., Smith, K.L., Song, J.K., Turner, J.N., Shain, W. and Kim, S.J. (2007). Electrical stimulation-induced cell clustering in cultured neural networks. Medical & biological engineering & computing 45, 1015-1021.

51. Li, S., Li, H. and Wang, Z. (2010). Orientation of spiral ganglion neurite extension in electrical fields of charge-balanced biphasic pulses and direct current in vitro. Hear Res 267, 111-118.

52. McCaig, C.D., Song, B. and Rajnicek, A.M. (2009). Electrical dimensions in cell science. Journal of cell science 122, 4267-4276.

53. Borgens, R.B. and Shi, R. (1995). Uncoupling histogenesis from morphogenesis in the vertebrate embryo by collapse of the transneural tube potential. Dev Dyn 203, 456-467.

54. Yao, L., Shanley, L., McCaig, C. and Zhao, M. (2008). Small applied electric fields guide migration of hippocampal neurons. Journal of cellular physiology 216, 527-535.

55. Reif, A., Fritzen, S., Finger, M., Strobel, A., Lauer, M., Schmitt, A. and Lesch, K.P. (2006). Neural stem cell proliferation is decreased in schizophrenia, but not in depression. Mol Psychiatry 11, 514-522.

56. Rajnicek, A.M., McCaig, C.D. and Gow, N.A. (1994). Electric fields induce curved growth of Enterobacter cloacae, Escherichia coli, and Bacillus subtilis cells: implications for mechanisms of galvanotropism and bacterial growth. J Bacteriol 176, 702-713.

57. McCaig, C.D., Allan, D.W., Erskine, L., Rajnicek, A.M. and Stewart, R. (1994). Growing Nerves in an Electric Field. Neuroprotocols, 4, 134–141

58. Li, X. and Kolega, J. (2002). Effects of direct current electric fields on cell migration and actin filament distribution in bovine vascular endothelial cells. J

Vasc Res 39, 391-404.

59. Pullar, C.E., Isseroff, R.R. and Nuccitelli, R. (2001). Cyclic AMP-dependent protein kinase A plays a role in the directed migration of human keratinocytes in a DC electric field. Cell Motil Cytoskeleton 50, 207-217.

60. Brown, M.J. and Loew, L.M. (1994). Electric field-directed fibroblast locomotion involves cell surface molecular reorganization and is calcium independent. J Cell Biol 127, 117-128.

61. McBain, V.A., Forrester, J.V. and McCaig, C.D. (2003). HGF, MAPK, and a small physiological electric field interact during corneal epithelial cell migration. Invest Ophthalmol Vis Sci 44, 540-547.

62. Al-Majed, A.A., Brushart, T.M. and Gordon, T. (2000). Electrical stimulation accelerates and increases expression of BDNF and trkB mRNA in regenerating rat femoral motoneurons. Eur J Neurosci 12, 4381-4390.

63. Toda, H., Hamani, C., Fawcett, A.P., Hutchison, W.D. and Lozano, A.M. (2008). The regulation of adult rodent hippocampal neurogenesis by deep brain stimulation. Journal of neurosurgery 108, 132-138.

64. Baba, T., Kameda, M., Yasuhara, T., Morimoto, T., Kondo, A., Shingo, T., Tajiri, N., Wang, F., Miyoshi, Y., Borlongan, C.V., Matsumae, M. and Date, I. (2009). Electrical stimulation of the cerebral cortex exerts antiapoptotic, angiogenic, and anti-inflammatory effects in ischemic stroke rats through phosphoinositide 3-kinase/Akt signaling pathway. Stroke; a journal of cerebral circulation 40, e598-605.

65. Buitrago, M.M., Luft, A.R., Thakor, N.V., Blue, M.E. and Hanley, D.F. (2004). Effects of somatosensory electrical stimulation on neuronal injury after global hypoxia-ischemia. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale 158, 336-344.

66. Huang, J., Hu, X., Lu, L., Ye, Z., Zhang, Q. and Luo, Z. (2009). Electrical regulation of Schwann cells using conductive polypyrrole/chitosan polymers. J Biomed Mater Res A 93, 164-174.

67. Puc, M., Corovic, S., Flisar, K., Petkovsek, M., Nastran, J. and Miklavcic, D. (2004). Techniques of signal generation required for electropermeabilization. Survey of electropermeabilization devices. Bioelectrochemistry 64, 113-124.

68. Kojima, J., Shinohara, H., Ikariyama, Y., Aizawa, M., Nagaike, K. and Morioka, S. (1992). Electrically promoted protein production by mammalian cells cultured on the electrode surface. Biotechnol Bioeng 39, 27-32.

69. Deisseroth, K., Singla, S., Toda, H., Monje, M., Palmer, T.D. and Malenka, R.C. (2004). Excitation-neurogenesis coupling in adult neural stem/progenitor cells. Neuron 42, 535-552.

70. Sharma, N., Coughlin, L., Porter, R.G., Tanzer, L., Wurster, R.D., Marzo, S.J., Jones, K.J. and Foecking, E.M. (2009). Effects of electrical stimulation and gonadal steroids on rat facial nerve regenerative properties. Restor Neurol Neurosci 27, 633-644.

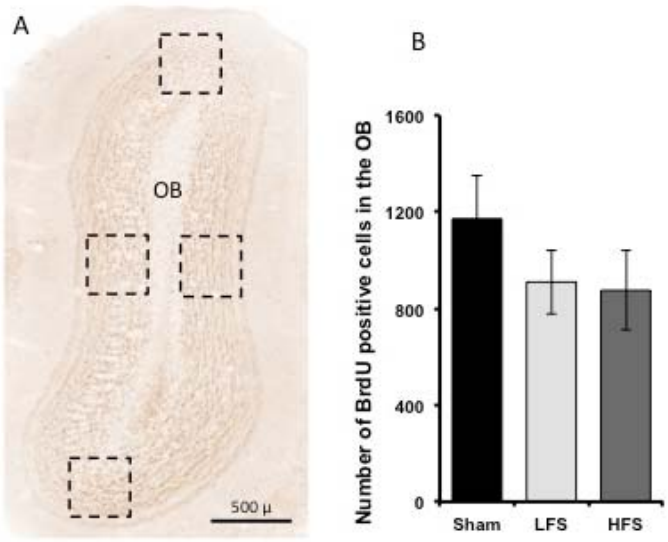
71. Kim, I.S., Song, Y.M., Cho, T.H., Pan, H., Lee, T.H., Kim, S.J. and Hwang, S.J. Biphasic electrical targeting plays a significant role in schwann cell activation. Tissue Eng Part A 17, 1327-1340.

72. Brus-Ramer, M., Carmel, J.B., Chakrabarty, S. and Martin, J.H. (2007). Electrical stimulation of spared corticospinal axons augments connections with ipsilateral spinal motor circuits after injury. J Neurosci 27, 13793-13801.

73. Maehlen, J. and Nja, A. (1982). The effects of electrical stimulation on sprouting after partial denervation of guinea-pig sympathetic ganglion cells. J Physiol 322, 151-166.

74. Whitman, M.C. and Greer, C.A. (2009). Adult neurogenesis and the olfactory

system. Prog Neurobiol 89, 162-175.



Supplementary figure 1
A: Representative low-power photomicrograph taken from a coronal brain section containing the olfactory bulb (OB) of a sham treated rat, stained for BrdU and a light hematoxylin-eosin counter staining, to show the method the quantification method (scalebar = 500µm). **B**: cumulative data on the number of BrdU immunoreactive cells in animals with motor cortex stimulation at low frequency (LFS), high frequency (HFS), and animals with sham treatment. We found no significant differences. Data are presented as mean ± standard error of the mean (SEM).

CHAPTER 4

LONG-TERM MOTOR DEFICITS AFTER CONTROLLED CORTICAL IMPACT IN RATS CAN BE DETECTED BY FINE MOTOR SKILL TESTS BUT NOT BY AUTOMATED GAIT ANALYSIS

Lisa-Maria Schönfeld, Ali Jahanshahi, Evi Lemmens, Sandra Schipper, Dearbhaile Dooley, Elbert Joosten, Yasin Temel[#] and Sven Hendrix[#]

[#] Yasin Temel and Sven Hendrix contributed equally to this article

Under review at the Journal of Neurotrauma

ABSTRACT

Animal models with constant, long-lasting motor deficits together with the right tests to assess behavioral abnormalities are needed to study the effectiveness of potential therapies to restore motor functions. In the current study, controlled cortical impact (CCI) was applied in rats to induce damage to the forelimb area of the motor cortex and the dorsal striatum. Motor behavior was assessed before and after CCI using fine motor skill tests such as the adhesive removal test, the cylinder test and the Montoya staircase test as well as the automated gait analysis system CatWalk XT over a 6 week period.

CCI caused a variety of unilateral motor deficits, which were characterized in detail by using the selected fine motor skill tests. In striking contrast to previous studies on CCI in mice, neither forelimb impairments, nor general changes in gait were detected with the CatWalk XT.

These data suggest that the adhesive removal test, the cylinder test and the Montoya staircase test are the methods of choice to detect long-term unilateral motor deficits in rats after CCI, whereas the use of automated gait analysis systems might not be suitable to measure these behavioral deviations.

INTRODUCTION

Brain injuries caused by stroke or trauma can result in long-lasting motor impairments, which severely affect the patients' quality of life and prevent them from performing daily activities independently. Current research approaches to treat cortical lesions may include electrical stimulation, stem cell transplantation or neuroprotection[1-3]. To investigate whether these therapies are able to restore motor functions in animal models, suitable paradigms with a robust and long-lasting behavioral phenotype have to be used. Nonetheless, in the majority of animal models it is difficult to detect chronically persistent motor deficits opposed to what is observed in the clinics. A chronic behavioral phenotype is essential to test new treatments relevant for the patient population, since those treatments are usually administered chronically after the insult. To produce motor deficits in rodents, the motor cortex is among the main targets for lesion induction. In the present study, a controlled cortical impact (CCI) model was used in rats to create long-lasting motor deficits specific to the dominant or, in other words, preferred forelimb. CCI is a widely used method to model traumatic brain injury (TBI) in rodents, in which an impactor tip strikes the exposed cortical surface at a pre-defined depth, velocity and dwell time. Advantages of this model are the well-defined lesion and high reproducibility due to a low mortality and low variability[4, 5]. However, it is a notorious problem to reliably detect behavioral deficits that last longer than two to four weeks after the lesion[6].

Previously, Zörner *et al.* thoroughly profiled impairments in gross locomotion and coordinated limb movements after different kinds of central nervous system damage, among which unilateral cortical stroke[7]. Our study aims at creating a comparable profile of forelimb impairment after a CCI to the forelimb area of the

motor cortex, with a special focus on a variety of fine motor skills. Previous studies provide some indications, which behavioral testing methods can reliably detect behavioral deficits after CCI, but the overall picture remains inconclusive. We compared and validated four behavioral tests after CCI in rats. The behavioral tests used in this study all measure a distinct aspect of forelimb motor behavior: Integration of sensorimotor cues and purposeful forelimb motion are measured by the adhesive removal test, exploration along a vertical surface is assessed by the cylinder test and specific deviations in grasping small objects can be evaluated with the Montoya staircase test. These three tests have been used in previous publications on CCI in rats to detect motor deficits[8-11]. To increase readability, we refer to the tests as 'fine motor skill tests' in the remainder of the manuscript although the cylinder test also contains elements of balance and posture. In addition, we included the automated gait analysis system CatWalk XT, which has been used previously to show functional impairments after CCI in mice that were assessed up to 31 days post-surgery[12-14]. The CatWalk XT provides quantitative information about different aspects of gait with regard to all limbs in concert as well as each limb on its own.

We used a systematic approach to measure the presence and duration of forelimb impairment after CCI. Our data show, which motor deficits are still detectable in rats six weeks after injury induced by a standard model of TBI and which behavioral tests can be used to reliably measure those deficits.

MATERIALS & METHODS

Subjects

All animal experiments were conducted according to the EU Directive 2010/63/EU for animal experiments and were further approved by the local

ethical committee for animal experiments at Maastricht University. Seventeen male Sprague-Dawley rats (Charles River, L'Arbresle, France), weighing approximately 400 g at the time of surgery, were housed in pairs under a reversed 12 h light/dark cycle. Animals received standard laboratory chow (Sniff, Soest, Germany) and acidified water (pH 2.3 - 2.7) *ad libitum*. Animal housing took place under IVC conditions at a constant temperature of 22° C and a humidity of 40 – 60%.

Surgical procedures

Surgical procedures were performed as previously described with minor modifications[15]. Anesthesia was induced and maintained with 1.5-3% isoflurane and rats were fixed in a stereotactic frame (Stoelting, Wood Dale, IL, USA). A craniotomy of 3.5 x 3.5 mm was made, exposing the forelimb area of the motor cortex contralaterally to the preferred paw, as determined prior to surgery using the Montoya staircase test (see description below), at the coordinates AP 0-3.5 mm anterior to bregma and ML 0.5–4 mm lateral to bregma. The exact location of the forelimb area on the motor cortex was derived from a cortical mapping study and a rat brain atlas[16, 17]. CCI was created by an electromagnetically driven impactor device (Leica Impact One, Leica Biosystems, Richmond, IL, USA). Ten rats received a lesion using an impactor tip of 3 mm diameter with an impact depth of 5 mm and a velocity of 3 m/s. Seven rats received only a craniotomy using the same coordinates as the CCI rats to function as controls taking the potentially damaging effect by the craniotomy itself into account. The order of CCI and control surgeries was randomly alternated throughout the day. After receiving CCI or a craniotomy, a bioabsorbable coagulant mesh was placed on the lesion to stop the bleeding. The skull was closed with a thin layer of bone

wax covering the craniotomy, which was reinforced with dental cement (Paladur, Heraeus, Hanau, Germany). The dental cement was anchored by placing two miniature screws on top of the skull close to the craniotomy and the incision was sutured. After the surgery rats were left to recover for two weeks.

Behavioral testing

Behavioral testing was performed at three timepoints: Before surgery, two weeks and six weeks after surgery. All behavioral assessments took place during the dark phase of the reversed night-day cycle (between 7 am and 7 pm), which is the active period of the rats.

Before conducting the adhesive removal test, rats first were habituated to the testing environment, a Perspex cage measuring 40 x 40 x 50 cm, by placing them inside the cage for an increasing amount of time once daily during one week. On testing days, adhesive color coding stickers with a diameter of 8 mm (Avery, Holzkirchen, Germany) were placed on both forepaws covering the thenar and hypothenar muscles. Rats were placed in the testing cage and filmed from below (Logitech HD Pro C920, Lausanne, Switzerland; Debut Video Capture Software, NCH Software, Greenwood Village, Colorado, USA). A trial was finished when the rat had removed both stickers from its forepaws and one testing session consisted of three consecutive trials. Based on the video footage, time was stopped when the rat noticed the sticker under each paw (*time to contact*, visible by shaking the paw or bringing it to the nose) and when the rat achieved to remove the sticker (*time to remove*, usually with the teeth; VCL media player, VideoLAN Organization, Paris, France). Data are presented in seconds for both forelimbs separately and by showing the *difference score,*

which is calculated by subtracting the score of the non-preferred forelimb from the score of the preferred forelimb.

Asymmetry of paw use was assessed using the cylinder test as previously described[18]. In short, rats were placed in 37.5 cm high Perspex cylinders with a diameter of 19 cm and recorded from above during 10 minutes (EthoVision XT, Noldus, Wageningen, the Netherlands). The first 20 wall contacts with the forepaws were counted and used for analysis (VCL media player, VideoLAN Organization, Paris, France). It was differentiated between individual paw contacts of either the contralesional or ipsilesional paw or one simultaneous contact with both forepaws. An individual paw contact was counted as such when the rat initiated a rear or when both paws had left the wall before. In a case where one paw remained on the wall and the second paw also made contact, this was counted as 'both' paws making contact. When both paws remained on the cylinder wall, but crossed over irregularly, it was still counted as one contact ('both'), to keep a focus on the individual paws. After 20 paw contacts were counted, scores for the individual forepaws were calculated as a percentage of the total 20 paw contacts. To assess asymmetry between the preferred and the non-preferred paw after surgery, post-surgery scores were set relative to their corresponding baseline scores, followed by subtracting the score of the non-preferred paw from the score of the preferred paw[11]. A lower value thus indicated a decreased usage of the preferred paw in leaning against the cylinder wall.

To be tested in the Montoya staircase test, rats were trained to crawl into translucent boxes, with a staircase located on the left and on the right hand side of a central, narrow platform. In the first week, rats were familiarized to the testing boxes by allowing them to freely enter and leave the box followed by

closing the box for an increasing amount of time, gradually habituating the rats towards the testing duration of 15 minutes. During three weeks, rats were trained twice daily to reach a stable grasping performance that was considered sufficient when more than 55% of the pellets were eaten with at least one forelimb. Training conditions were similar to testing conditions. All steps, except the upper two, were baited with three sucrose pellets (Test Diet, Richmond, USA), resulting in a total of 15 sucrose pellets per site. The pellets were mixed with food coloring powder (Crazy Colors, Warburg, Germany) diluted in water and each step was baited with pellets of a distinct color. The upper two steps were left empty, since the rats were able to retrieve the pellets with their tongue, which would prevent initiating the desired grasping behavior. During the training phase and on the testing days, rats were food restricted to increase their motivation to retrieve the pellets. One test trial consisted of 15 minutes and the average score from the four trials was calculated at baseline, two weeks and six weeks after surgery. Individual test trials took place with a time interval between trials of at least three hours, which in most cases resulted in one test trial per day for each rat. Furthermore the fourth test trial was recorded using cameras placed next to the stairs on both sides to allow offline qualitative assessment of performance (Logitech HD Pro C920, Lausanne, Switzerland; Canon LEGRIA HF R16, Canon, Canon Nederland N.V., 's-Hertogenbosch, the Netherlands; Debut Video Capture Software, NCH Software, Greenwood Village, Colorado, USA). After the rats finished the test, grasping performance was assessed three-fold. First, the total number of eaten pellets, regardless of their color, was calculated for each side. Baseline values in the total number of pellets eaten were used to determine the preferred limb for each rats, i.e. the side where more pellets were eaten. Second, based on pellet colors, the following

parameters were assessed for each separate step: *pellets remained* for pellets still lying on their original step, *pellets misplaced* for pellets, which had been knocked off their original step onto a different (mostly lower) step and *pellets lost* for pellets thrown on the floor at the back of the staircase test, thereby being unreachable[19]. Based on those observations the parameters *pellets taken* (*pellets originally placed* minus *pellets remained*) and *pellets eaten* [*pellets originally placed* minus (*pellets remained* plus *pellets misplaced* plus *pellets lost*)] were calculated. The parameters *pellets remained* and *pellets taken* are considered to measure general reaching activity and reaching motivation, whereas the parameters *pellets misplaced, pellets lost* and *pellets eaten* provide more specific information about forelimb reaching and grasping success[19]. Third, using video footage, reaching attempts with the preferred limb were quantified based on absence or presence of 12 typical behaviors observed in a successful reaching action, similar to earlier research (VCL media player, VideoLAN Organization, Paris, France)[20]. Since we observed a high number of reaching attempts, which did not result in a pellet being grasped, reaching behavior to be included for qualitative assessment was defined as the forelimb being brought towards the pellets and either touching the steps or performing palpitating movements in search of pellets. For each rat, five such reaches were analyzed for the absence or presence of typical reaching behaviors after surgery and scores from the five reaches were averaged at baseline, two and six weeks, respectively. Each behavior was scored 1 when being present, 0,5 when being abnormal and 0 when being absent or severely abnormal[20]. Individual components of a typical reaching action included: (1) Inserting the snout into the staircase compartment, (2) advancing the limb towards the steps, (3) pronating the paw, while (4) extending the digits, (5) palpitating on the steps in

search of pellets, (6) flexing the digits, (7) closing all digits to form a tight first ("power grip"), (8) directing the limb to the snout, (9) supinating the paw 90 degrees towards the body, (10) bringing the paw to the mouth, (11) supinating the paw another 90 degrees for the palm to face the mouth and (12) eating the pellet from the paw. Data are presented showing the number of pellets with regard to the parameter at hand for each forelimb separately and by calculating the *difference score,* which is again calculated by subtracting the score of the non-preferred forelimb from the score of the preferred forelimb. Qualitative scores of reaching attempts were assessed for the preferred forelimb only.

Changes in gait were measured by the CatWalk XT (Noldus, Wageningen, the Netherlands), an automated gait analysis system. Rats were habituated to the system and trained to run down the walkway daily for one week. If a rat successfully crossed the entire walkway, it was rewarded with sugar pellets (TestDiet, Richmond, USA) at the end of the walkway. On the last day of training, baseline recordings for each rat were taken. In general, one successful test recording consisted of an average of three uninterrupted runs having a comparable running speed with a maximum variation of 30%.

The following twenty static and dynamic parameters assessing individual paw functioning and gait patterns were analyzed: stand, mean intensity, print area, print length, print width, swing mean, swing speed, stride length, maximum intensity at maximum contact, maximum intensity, minimum intensity, step cycle, duty cycle, regularity index, base of support of the forelimbs, base of support of the hindlimbs, three-limb support, speed, cadence and couplings between the ipsilateral forelimb (non-preferred) and the contralateral hindlimb. Data are presented for each forelimb as well as using the *difference score,*

defined as the score of the non-preferred forelimb subtracted from the score of the preferred forelimb in the respective units.

Tissue processing

The day after the last behavioral testing session, thus six weeks after CCI, rats were sacrificed by transcardial perfusion with Tyrode's buffer, followed by fixative containing 4% paraformaldehyde, 15% picric acid and 0.05% glutaraldehyde in 0.1M phosphate buffer (pH 7.6). Brains were post-fixed in the same fixative lacking glutaraldehyde for 2 h at 4°C, followed by immersion in 15% and 20% sucrose for cryoprotection. Brains were frozen using CO_2 and stored at -80°C until further processing. For histochemical analysis, brains were cut in ten series of 30 µm thick coronal sections, mounted on glass slides and fixed with 4% paraformaldehyde for 20 minutes. To measure the CCI lesion volume, one series of sections was stained with standard hematoxylin-eosin (Merck, Darmstadt, Germany).

Assessment of lesion volume

The effect of CCI on the motor cortex was assessed by quantifying lesion volumes using an Olympus BX50 microscope (Zoeterwoude, the Netherlands) and StereoInvestigator software (MBF Bioscience, Magdeburg, Germany). First, both hemispheres were delineated. The area of the hemisphere, which received a CCI or a craniotomy, was subtracted from the area of the contralateral hemisphere to calculate the lesion area in mm^2 of the CCI rats (in control rats the same calculation was performed although there was no lesion). The lesion volume in mm^3 per rat was calculated based on the sum of the lesion areas of all sections in mm^2 (A) and the distance between consecutive brain sections

(300 µm, *D*) using the following formula: $V = \Sigma A \times D$. In case one brain section could not be delineated due to tissue damage or folding, the average of the preceding and subsequent section was used to replace the single missing value. Animals were excluded from analysis if more than one brain section in a row was missing or could not be delineated. Groups consisted of n = 8 for CCI rats and n = 5 for control rats, with 10 brain sections being analyzed for each rat.

Statistical analysis

The behavioral data were analyzed with SPSS statistical software (version 20, IBM) using repeated-measures ANOVA. Behavioral scores at the three time points of testing were treated as repeated measures for each parameter, whereas the two groups (CCI vs. control rats) served as between-subjects factor. In case repeated-measures ANOVA showed that CCI and control rats differed significantly from each other after surgery, but not at baseline, Oneway ANOVA was performed comparing both groups at each separate timepoint. Outliers were defined as "extreme values" identified by SPSS and excluded.

To illustrate the differences in lesion size between CCI and control rats at the tissue level, average lesion volumes were compared between groups using Oneway ANOVA. Data are presented as mean ± standard error of the mean (SEM). Differences were considered significant at $p < 0.05$.

RESULTS

CCI led to severe cortical and striatal tissue damage

CCI on the forelimb area of the motor cortex contralateral to the preferred paw caused a substantial lesion at the motor cortex and striatum six weeks after CCI

Figure 1: Controlled cortical impact (CCI) on the forelimb motor cortex causes severe tissue damage
The impactor tip with a diameter of 3 mm was positioned on top of the exposed forelimb area of the motor cortex contralateral to the preferred limb and hit the tissue with a target depth of 5 mm (scheme created at 2.2 mm anterior to bregma, **A**). A representative hematoxylin-eosin stained section showing the severe tissue damage six weeks after CCI (approximately 1.7 mm anterior to bregma, **B)** further supported by quantitative analysis of the lesion volume in CCI rats compared to control rats with a craniotomy (*** $p < 0.001$, **C**).

(Fig. 1 A, B). Lesion volumes differed significantly between CCI and control rats, which only received a craniotomy above the target brain region ($p < 0.001$, Fig. 1 C).

Deficits in tactile recognition

Sensorimotor deficits were evaluated using the adhesive removal test. After CCI, the *time to contact* the sticker underneath the preferred paw was significantly increased compared to control rats at both timepoints after surgery ($p < 0.05$ and $p < 0.05$, Fig. 2 A2). However, there were no differences observed between CCI and control rats in the *time to remove* the sticker (Fig. 2 B1-3). Although rats were distributed randomly between the groups, after excluding outliers we observed a significant difference in baseline values between the groups with regard to the *time to contact* the sticker underneath the non-preferred paw ($p < 0.05$ and $p < 0.01$, Fig. 2 A1 and 2 A3,

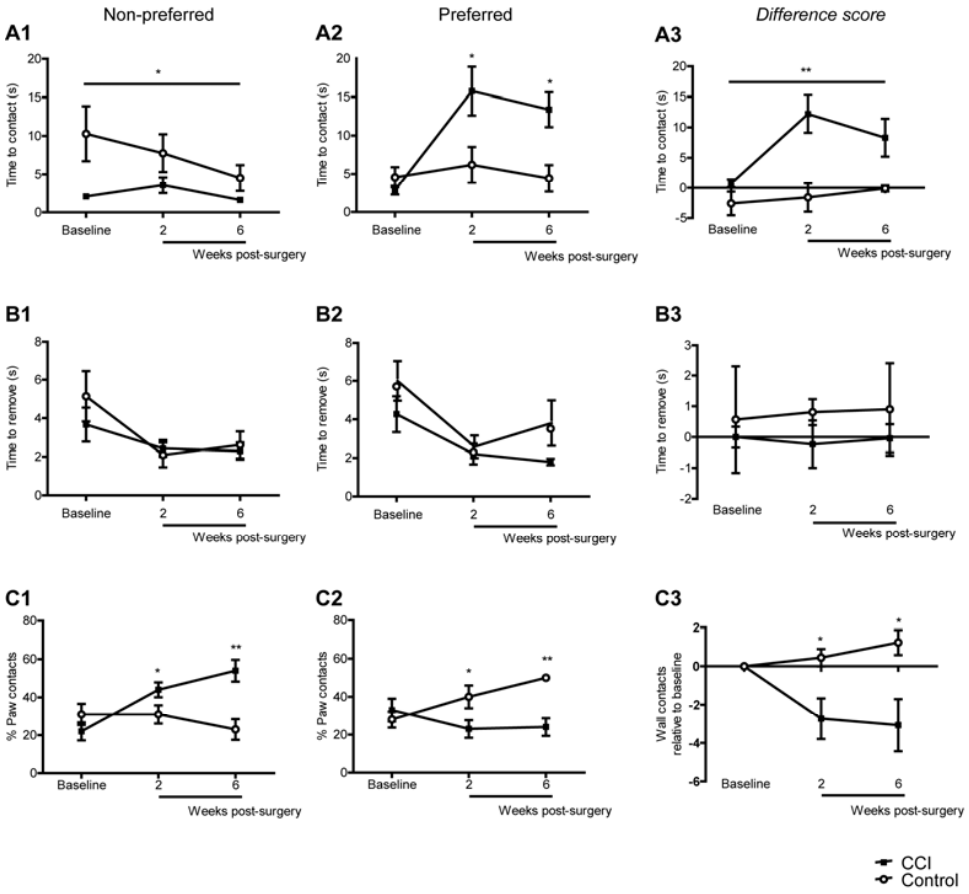


Figure 2: CCI impairs tactile recognition of adhesive stickers underneath the preferred paw and causes reliance on the non-preferred paw during vertical exploration

In the adhesive removal test, rats designated to the CCI group were significantly faster in contacting the sticker underneath their non-preferred paw at all three timepoints, thus even before CCI (**A1**). The time to contact the sticker underneath the preferred paw was significantly increased two and six weeks after CCI (**A2**). Using the *difference score*, both animal groups again differed significantly from each other at all three timepoints (**A3**). CCI did not affect the time taken to remove the stickers (**B1-3**).

After CCI, rats exhibit an increased percentage of wall contacts with their non-preferred paw while exploring the cylinder (**C1**). At the same time, the percentage of wall contacts with the preferred paw was significantly lower compared to the control group (**C2**). Also, the *difference score* (here set relative to baseline values) showed a progressive asymmetry in paw use in the CCI group over time (* $p < 0.05$, ** $p < 0.01$, **C3**).

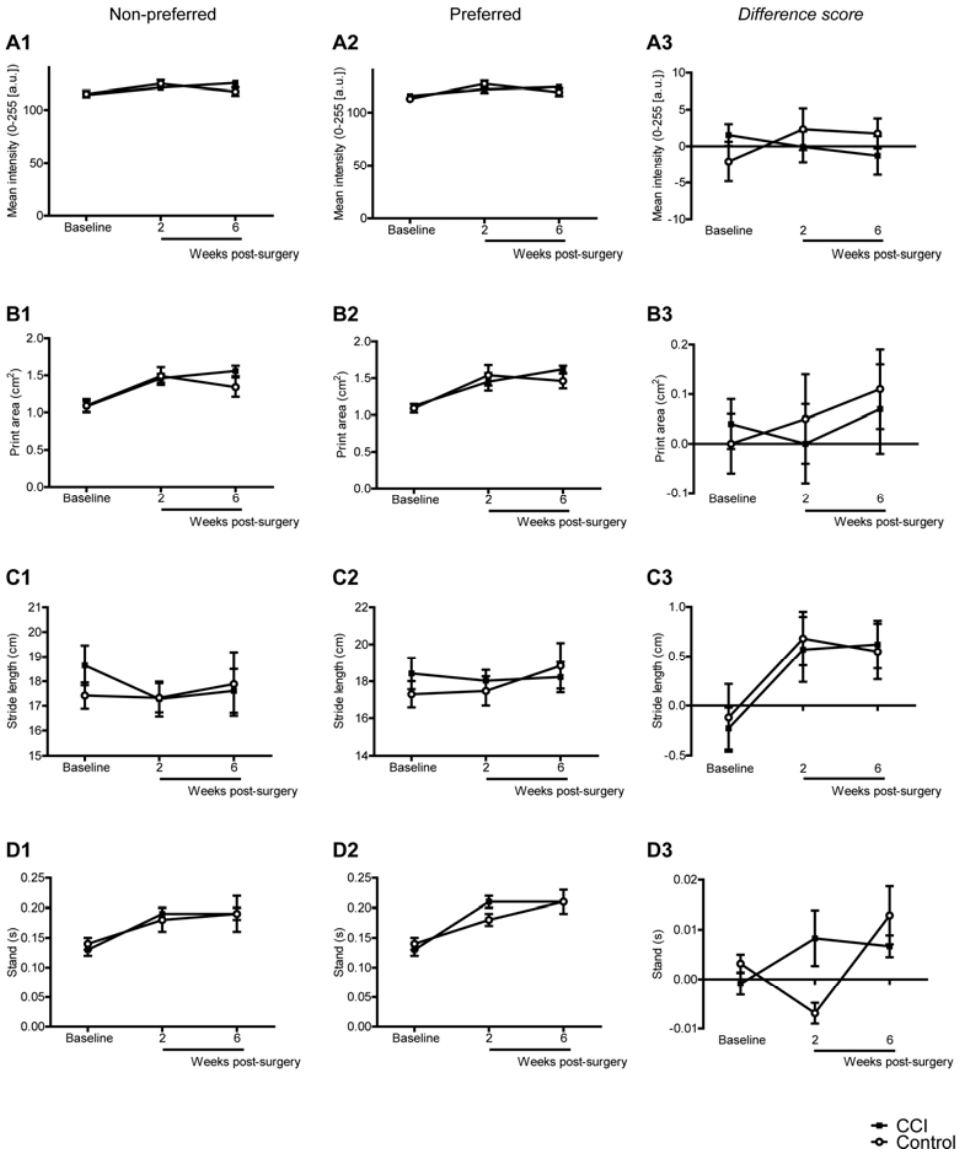
respectively). Therefore, data including the *time to contact* the sticker underneath the non-preferred paw should be interpreted with caution.

Using the non-preferred paw for vertical exploration

With the cylinder test, paw use during exploration of a vertical wall was measured. After CCI, rats relied mainly on their non-preferred, unaffected paw during vertical exploration and differed in their behavior significantly from control rats, which made use of both forelimbs at two and six weeks after surgery ($p < 0.05$ and $p < 0.01$, Fig. 2 C1-3).

CCI did not cause gait-related motor deficits

The automated gait analysis system CatWalk XT was used to detect gait-related motor deficits after CCI. No significant differences in functioning of the individual forelimbs were found when comparing CCI rats with control rats at all timepoints. Data of representative parameters such as mean paw print intensity and print dimensions are shown (Fig. 3 A-H), which were significantly changed in a study using a different model of TBI in rats[21]. One exception was the parameter maximum paw print intensity of the non-preferred paw, which was significantly higher in CCI rats six weeks after surgery ($p < 0.05$, Supplementary Fig. 1 A1). The non-preferred paw may compensate for the impairment in the preferred paw, but given the rather small difference in intensity values, the biological relevance of this result is questionable. Analysis of the *difference score* neither showed any significant changes after CCI. Values related to the majority of parameters increased over time for both paws, but this increase was equally present in CCI and control rats. Additionally, no significant differences in gait were found between the groups. All analyzed


Non-preferred
Preferred
Difference score
A1
A2
A3
Mean intensity (0-255 [a.u.])
B1
B2
B3
Print area (cm²)
C1
C2
C3
Stride length (cm)
D1
D2
D3
Stand (s)
Baseline
2
6
Weeks post-surgery
CCI
Control

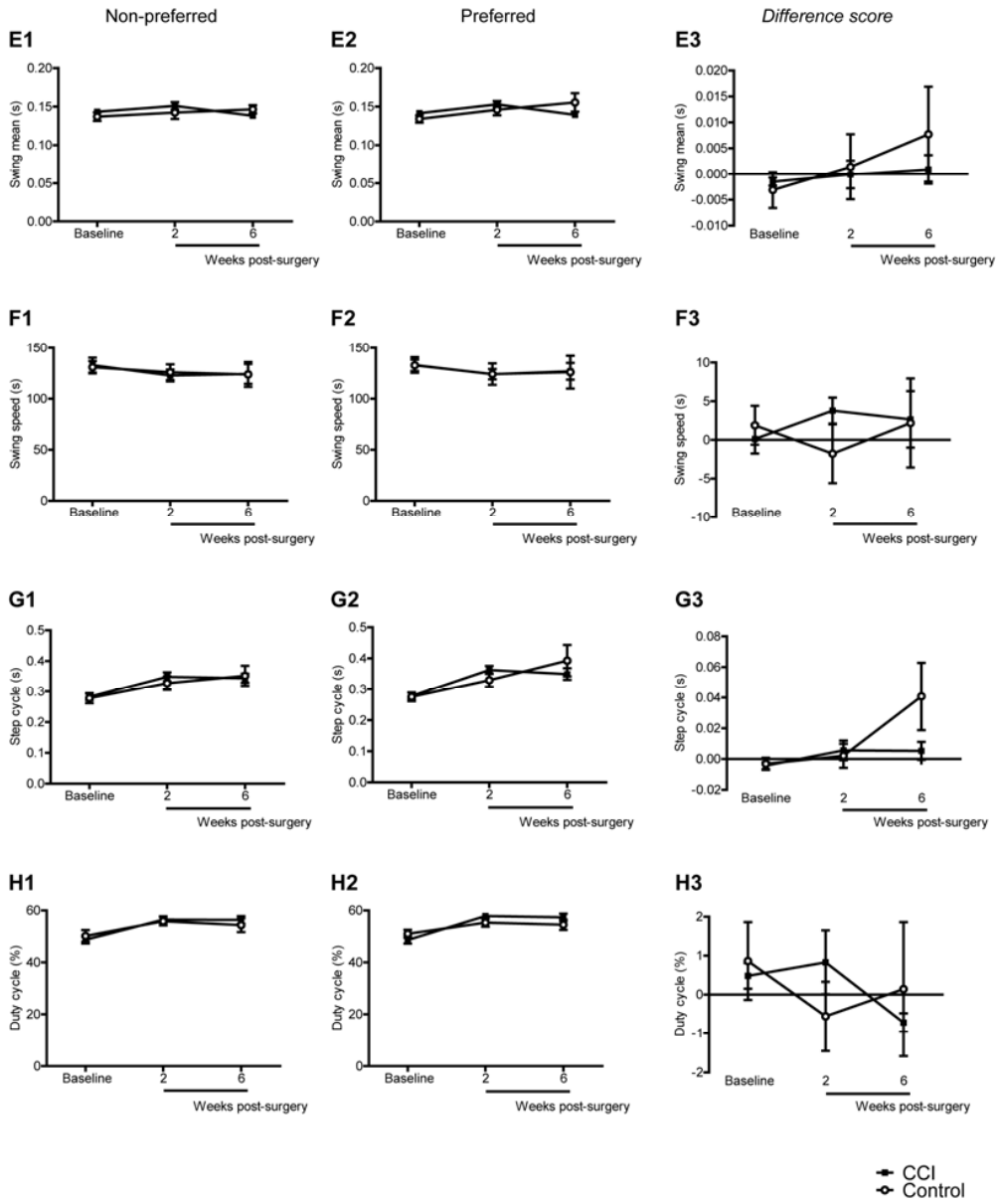


Figure 3: CatWalk XT analysis did not reveal any impairment in the preferred forelimb

Eight selected parameters (out of 13 parameters analyzed) describing individual paw functioning are shown (**Fig. 3.1 A1-3**: mean intensity, **B1-3**: print area, **C1-3**: stride length, **D1-3**: stand; **Fig. 3.2 E1-3**: swing mean, **F1-3**: swing speed, **G1-3**: step cycle, **H1-3**: duty cycle). No significant differences between

groups could be detected after CCI concerning the individual forelimbs and the *difference score*.

general gait parameters are shown in Figure 4 and correspond to parameters significantly changed up to seven days after TBI in an earlier study[21].

Severe disturbance of reaching and grasping behavior

The Montoya staircase test was used to assess grasping of small objects and coordinated paw-to-mouth movements. Two and six weeks after CCI, rats ate significantly fewer sucrose pellets with their preferred paw across all stairs (both $p < 0.001$, Fig. 5 A2). Analyzing the color composition of the pellets for each individual step revealed that significantly less pellets were eaten with the preferred paw from each individual step at six weeks after CCI, indicating that the height of the steps was unimportant for the number of *pellets eaten* ($p < 0.05$ or lower, Fig. 5 B2). CCI rats exhibited a general decrease in reaching attempts with their preferred paw, reflected by an increase in *pellets remained* for lower steps 5 until 7 ($p < 0.001$, Fig. 5 C2) together with a decrease in *pellets taken* for steps 5 until 7 ($p < 0.001$, Supplementary Fig. 2.4 C2). The number of *pellets misplaced* with the preferred paw was higher in CCI rats for the upper steps 3 and 4 ($p < 0.001$ and $p < 0.01$, Fig. 5 D2) whereas the number of *pellets lost* with the preferred paw was lower only at step 7 ($p < 0.01$, Supplementary Fig. 2.3 C2). Increases in *pellets misplaced* and *pellets lost* both reflect unsuccessful reaching performance, however, *pellets lost* is thought to result from more undirected searching for pellets. On the other hand, *pellets misplaced* may also result from successfully grasping a pellet with an inability to eat the pellet using the preferred paw.

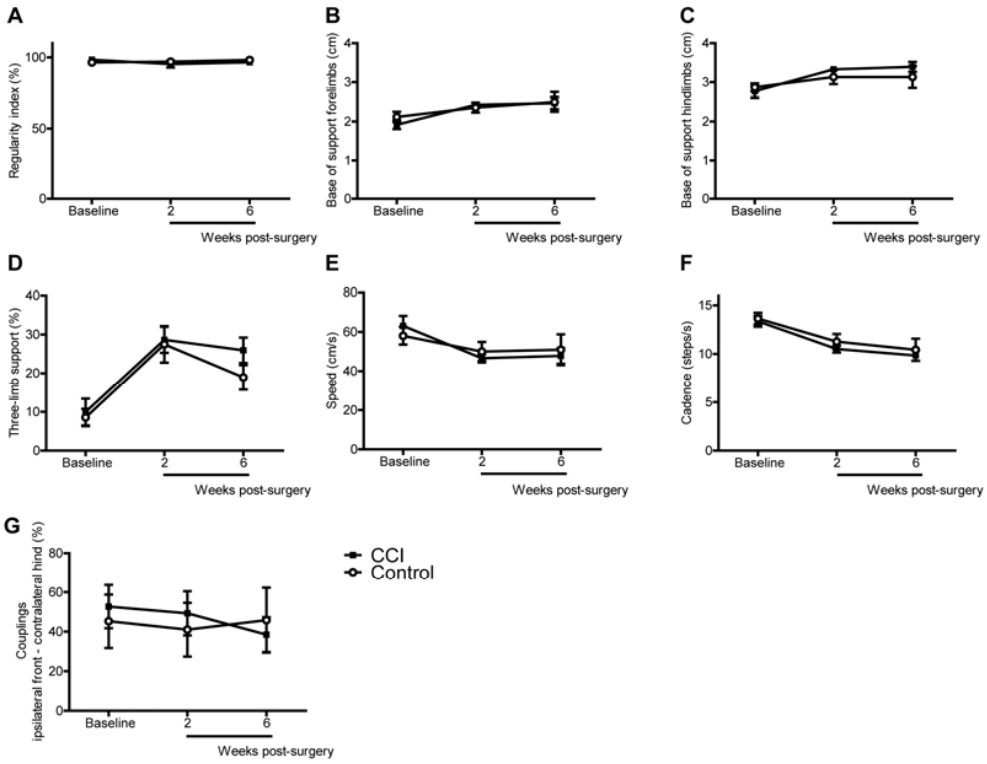


Figure 4: No changes in gait were measured after CCI using the CatWalk XT
All analyzed gait-related parameters, which describe coordinated movements of the four paws in concert, are shown (**A**: regularity index, **B**: base of support forelimbs, **C**: base of support hindlimbs, **D**: three-limb support, **E**: speed, **F**: cadence, **G**: couplings isilateral front – contralateral hind). None of the parameters was significantly changed after CCI when comparing CCI rats to controls.

To determine, which exact component of a typical reaching action prevented the rats from successfully eating a pellet after CCI, slow motion video analysis was done of reaching attempts with the preferred paw. Two weeks after CCI, rats exhibited specific grasping impairments shown by abnormal digit flexing and holding the pellet in a closed fist ("power grip"; $p < 0.05$, Supplementary Fig. 3 F and $p < 0.01$, Fig. 6 A). Often an inability to flex the digits when touching a

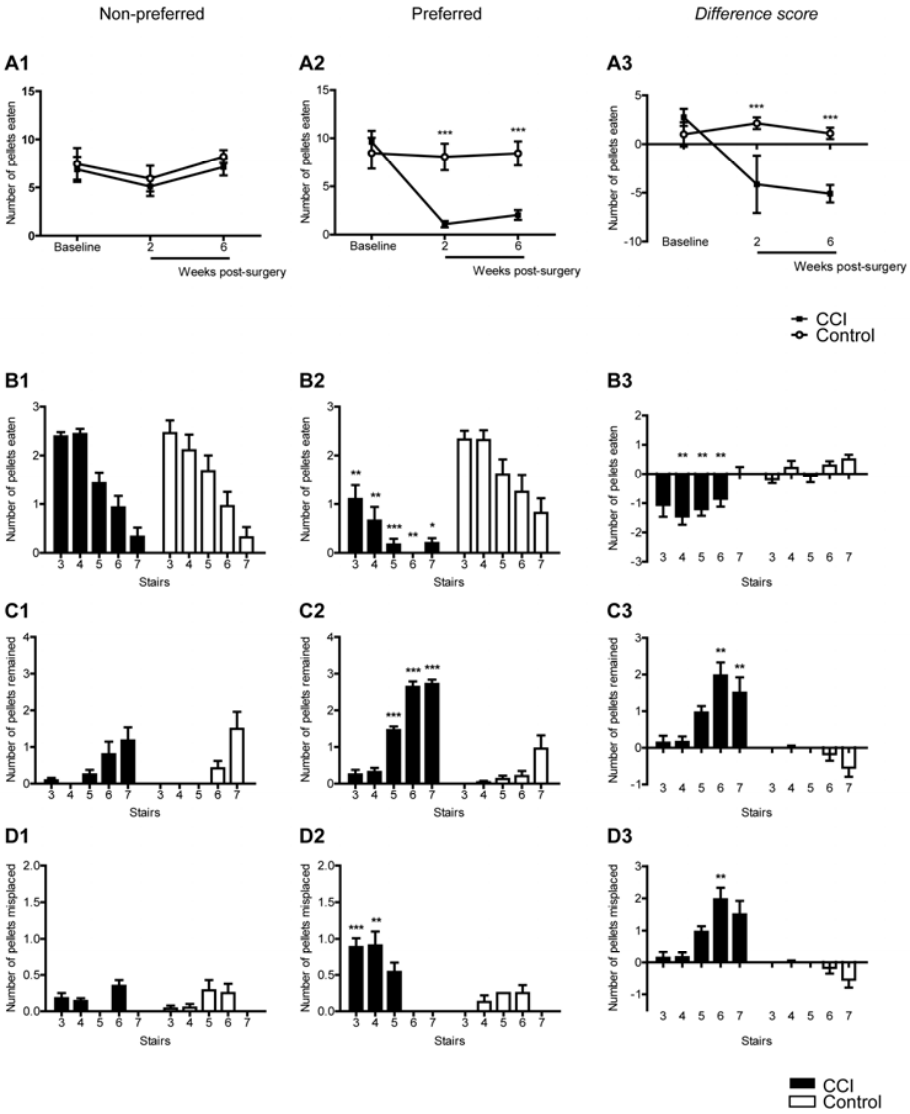


Figure 5: Pellet eating with the preferred paw is significantly deteriorated after CCI

In the Montoya staircase test pellet eating with the non-preferred paw did not differ between CCI and control rats (**A1**), whereas after surgery CCI rats ate significantly fewer pellets from the staircase close to their preferred paw (**A2**). The pellet consumption deficit in CCI rats was also measurable when analyzing the *difference score* comparing the number of eaten pellets between the preferred and non-preferred paw (**A3**).

Three additional parameters are shown at six weeks after surgery that provide more details about deficits in pellet retrieval (**B1-3**: number of pellets eaten, **C1-3**: number of pellets remained, **D1-3**: number of pellets misplaced). After

CCI, rats ate significantly fewer pellets from each individual step with their preferred limb (**B2**) while leaving more pellets untouched at their original location (**C2**). When reaching for the pellets with the preferred limb, there was an increase in the number of pellets misplaced after CCI (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **D2**).

pellet was observed, or, in the case where digits could be flexed, the pellet was either dropped or held by fewer than five digits. Also two weeks after CCI, rats showed deficits in bringing the paw towards the mouth and eating the pellet ($p < 0.01$, Fig. 6 B). Instead of bringing the paw towards the mouth, rats advanced their head towards the paw that was holding the pellet. The most prominent observation was that CCI rats showed severe impairments in opening the grip to release the pellet in order to eat it, an observation still present at six weeks after CCI ($p < 0.01$ and $p < 0.001$, Fig. 6 C). In many cases, the non-preferred paw had to hold the preferred limb in place, while rats tried to grab the pellet from the paw with their teeth.

DISCUSSION

Effective treatment opportunities, which target motor deficits are essential, given the high number of patients suffering from TBI. A wide variety of animal models for TBI and tests for postlesional motor performance are available[22], however, only a very limited number of studies were able to detect behavioral deficits exceeding two to four weeks. In order to use a standardized animal model of TBI that induces chronic motor deficits, we chose to damage the motor cortex by a CCI as this way of lesion induction is well defined in terms of impact location, depth, width and velocity and therefore highly reproducible. In the

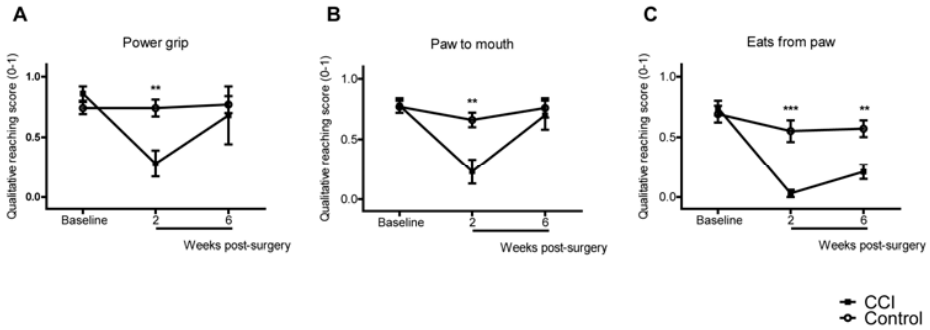


Figure 6: Reaching behavior with the preferred paw is severely altered after CCI
Rats showed overt abnormalities in a number of reaching behaviors related to pellet grasping and eating after CCI. Two weeks after CCI, rats had deficits in forming a "power grip", i.e. closing all digits around the pellet when achieving to grasp a pellet (**A**), followed by a problematic return of the paw towards the mouth (**B**). Pellet eating from the paw was significantly impaired both two and six weeks after CCI (** $p < 0.01$, *** $p < 0.001$, **C**).

present study, a severe lesion was created to increase the intensity of long-lasting behavioral deficits.

At the level of the cortex and the dorsal striatum, a considerable lesion was still evident six weeks after CCI, whereas the craniotomy performed in control animals did not cause measurable damage to the parenchyma. Using several fine motor skill tests, a clear phenotype of unilateral forelimb impairment could be identified. Quantifying forelimb function with a combination of fine motor skill tests is essential to describe impairment from several angles. As in humans, fine motor skills in rodents are controlled by the motor cortex and therefore have the same underlying neural basis. However, the selected muscle groups create behavior overtly different from humans, which stresses the need for distinct specialized tests[23]. Each of the fine motor skill tests used in the present study described different manifestations of the CCI-induced unilateral forelimb

impairment. The adhesive removal test allows a distinction between sensory deficits caused by a unilateral lesion, i.e. a decreased ability to feel the adhesive underneath the paw, and deteriorated motor skills, such as holding the paw in front of the mouth and extending the digits to remove the adhesive[18, 24, 25]. However, it should be noted that assessment of sensory deficits in the adhesive removal test requires intact gross motor function of the forelimb, since to count as a contact the rat has to shake its limb or raise the paw towards the nose. A significantly worsened sensory ability after CCI highlights the necessity of more than one behavioral test to describe impairment.

The cylinder test measures forelimb usage during exploration of unknown vertical surfaces, with a special focus on weight-bearing wall touches used to keep balance while standing on the hindlimbs[26]. We showed that rats no longer relied on their preferred paw to explore the cylinder walls after CCI. This asymmetry may either reflect a weaker muscle tone causing a decreased ability to stabilize weight while standing, or a sensory deficit, meaning a decreased capacity to feel the cylinder wall, or a combination of both types of deficits. The Montoya staircase test provides a detailed evaluation of pellet grasping and eating, which requires actions like digit extension, flexion and coordinated paw-to-mouth movement[20, 27]. After CCI, rats consumed less pellets on the steps contralateral to the lesion. Further analysis showed that this deviation was caused by less frequent attempts to reach with their preferred paw as well as deficient grasping while trying to obtain a pellet.

No behavioral deficits were detected with the automated gait analysis system CatWalk XT. Occasionally, a change in parameter values occurred over time, but this effect could be attributed to maturation of the rats, since no significant differences between the groups were detected. The CatWalk XT measures

changes in gait, focusing on coordinated movement of all four limbs together, as well as gait-related attributes of each paw separately[28]. Undisturbed gait in the presence of severe brain damage can be explained by central pattern generators (CPGs) located in the spinal cord, which regulate locomotion without conscious intention to move, whereas goal-directed actions such as grasping require conscious integration of spatial and proprioceptive cues as provided by the motor cortex[29, 30]. Additionally, a considerable portion of the striatum was spared in the present study. In the case where some connections from the motor cortex remain intact, the striatum is still able to guide gross movements, which explains the absence of deficits in gait-related parameters. In the majority of studies using animal models for cortical damage, behavioral abnormalities are assessed with fine motor skill tests comparable to the ones used in the present study[31]. Still, the CatWalk XT is an attractive option due to its objectiveness in processing variables automatically as opposed to manual scoring as done with fine motor skill tests.

So far, one study on TBI in rats reported behavioral deficits using the CatWalk XT, but also failed to provide evidence that this test is suitable to measure long-term impairment after the lesion[21]. In this study TBI was induced in rats using a different model, penetrating ballistic-like brain injury, which created a considerable lesion at the level of the striatum while leaving the majority of the motor cortex intact. Using this particular model of TBI, the authors were able to measure effects in the same parameters as presented here for up to two weeks post-surgery. After one month, forelimb deficits seemed to be resolved when using the Catwalk XT whereas a significant impairment was still measurable using a manual scoring method, the 12-point neuroscore[21]. To date, our study is the first to investigate whether long-term deficits are measurable by the

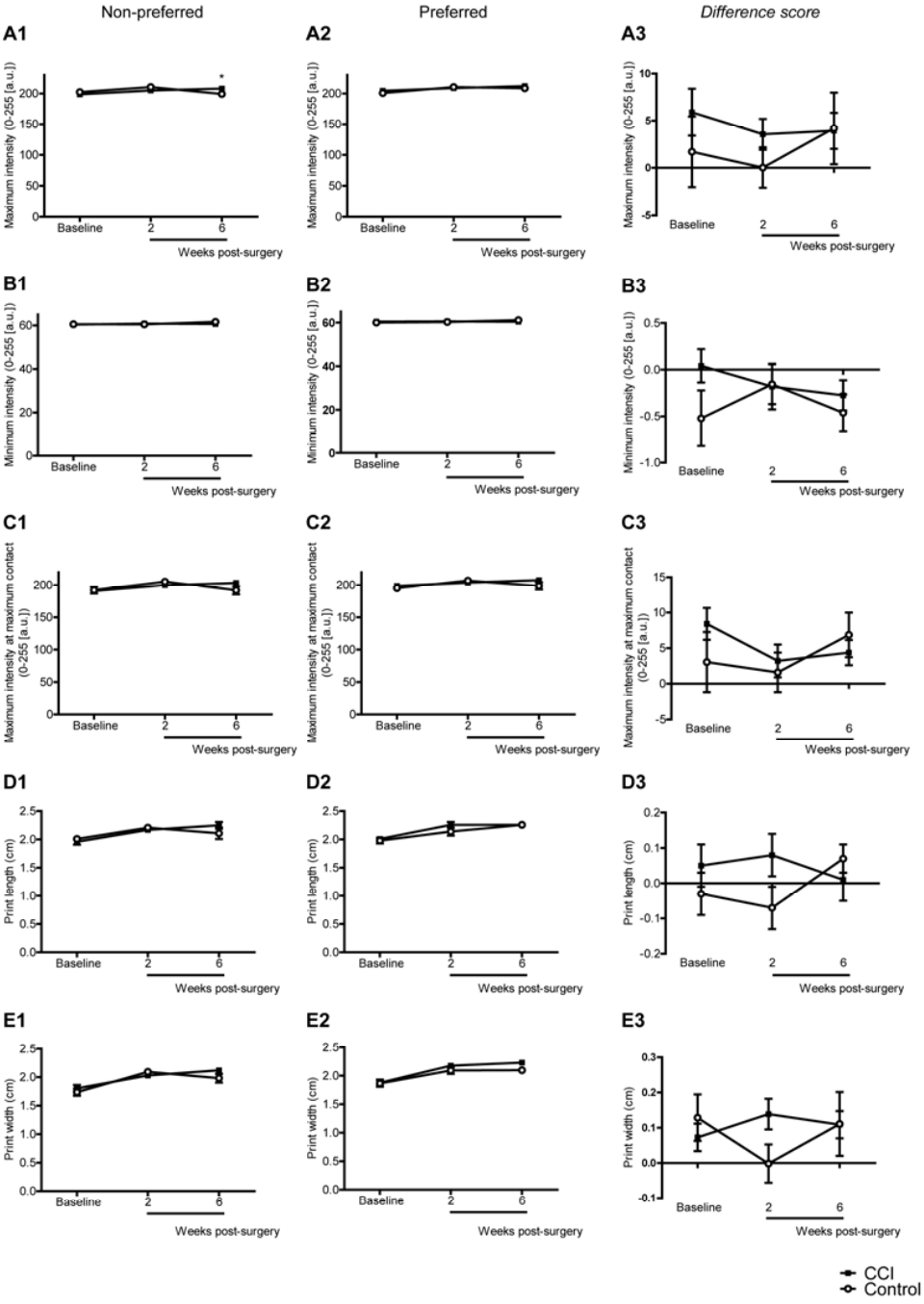
CatWalk XT in comparison with fine motor skill tests during a period of six weeks after CCI in rats. Severe CCI on the motor cortex caused robust histological lesions and persistent fine motor skill impairment in a number of specified motor tests, however, no gait-related abnormalities were detected using the CatWalk XT. These findings are in striking contrast to previous results obtained after CCI in mice[12-14]. In mice it was possible to detect functional impairments after CCI up to 31 days post-surgery using the CatWalk XT[12-14].

Our findings are an important indication that automated gait analysis systems such as the CatWalk XT may not be suitable in rat models of TBI in order to analyze the long-term effects of potential treatments on motor recovery. On the contrary, fine motor skill tests like the adhesive removal test, the cylinder test and the Montoya staircase test appear to be reliable and sensitive testing methods. Ideally, a combination of those fine motor skill tests should be used to measure long-lasting motor impairments.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from Hasselt University (BOF11603BOFMO) to SH and a grant from the Hersenstichting Nederland to YT. We are grateful for a substantial contribution in conducting the experiment by a number of external specialists. We thank Dr. René Bernard, Dr. Chris Montoya and Dr. Tracy Farr for their dedicated and outstanding advice on the execution of the behavioral tests.

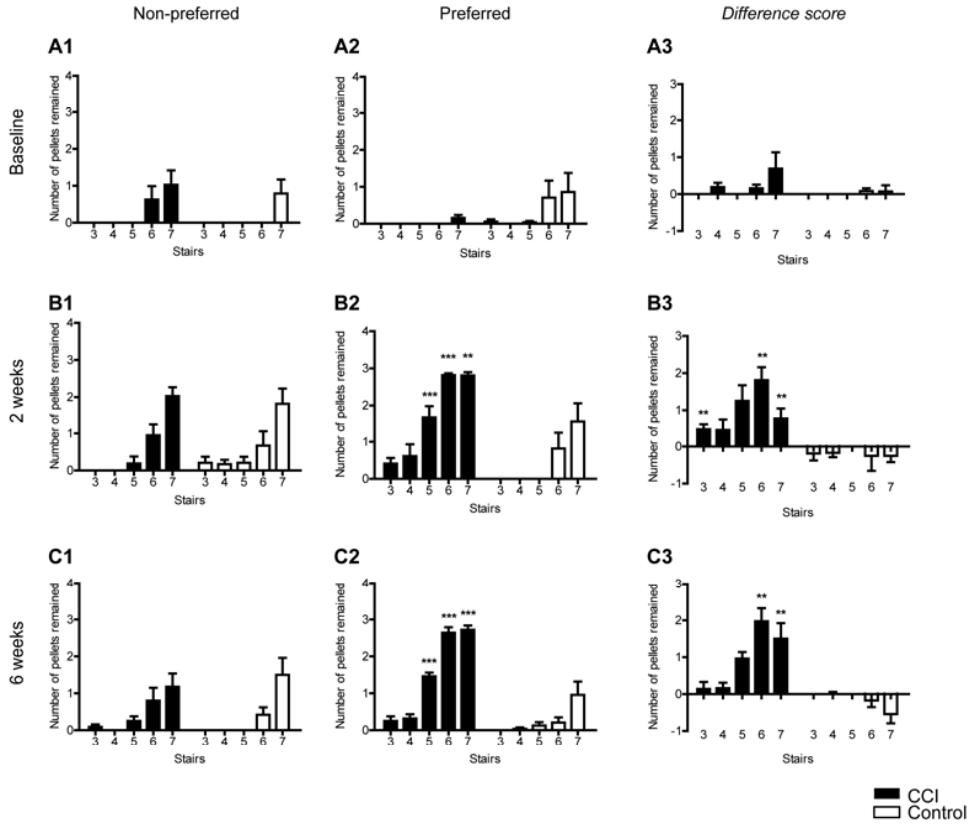
No competing financial interests exist.



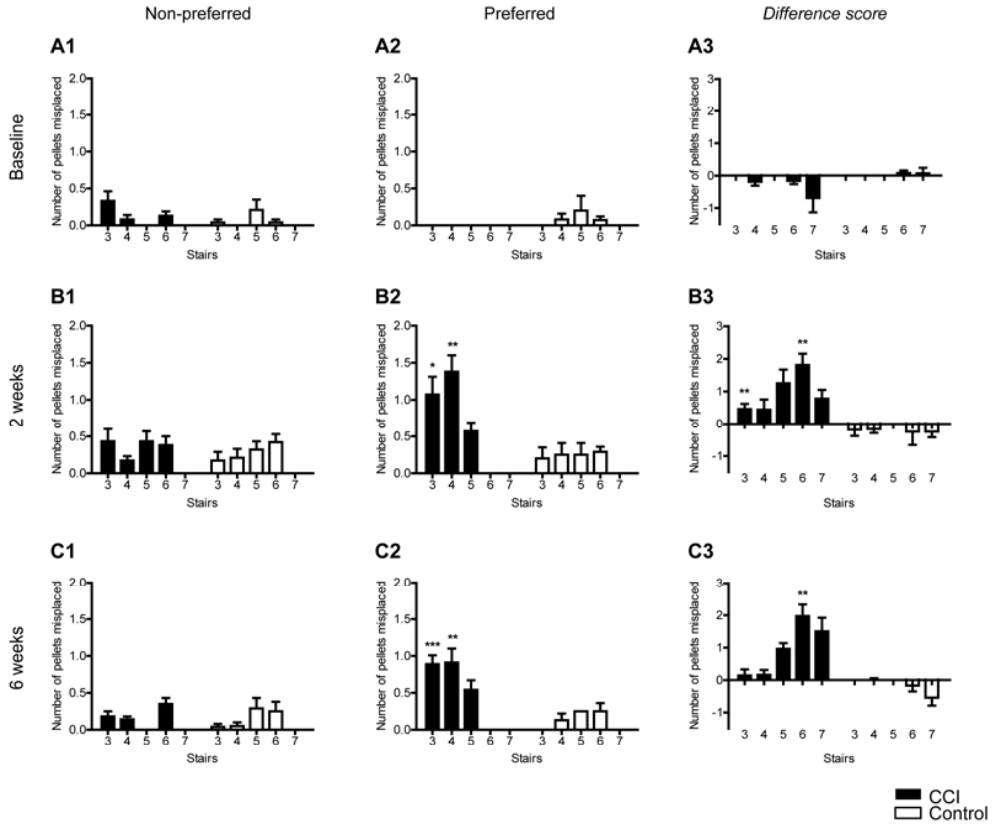
Supplementary Figure 1: CatWalk XT analysis did not reveal any impairment in the preferred forelimb

Supplementary Figure 1: CatWalk XT analysis did not reveal any impairment in the preferred forelimb

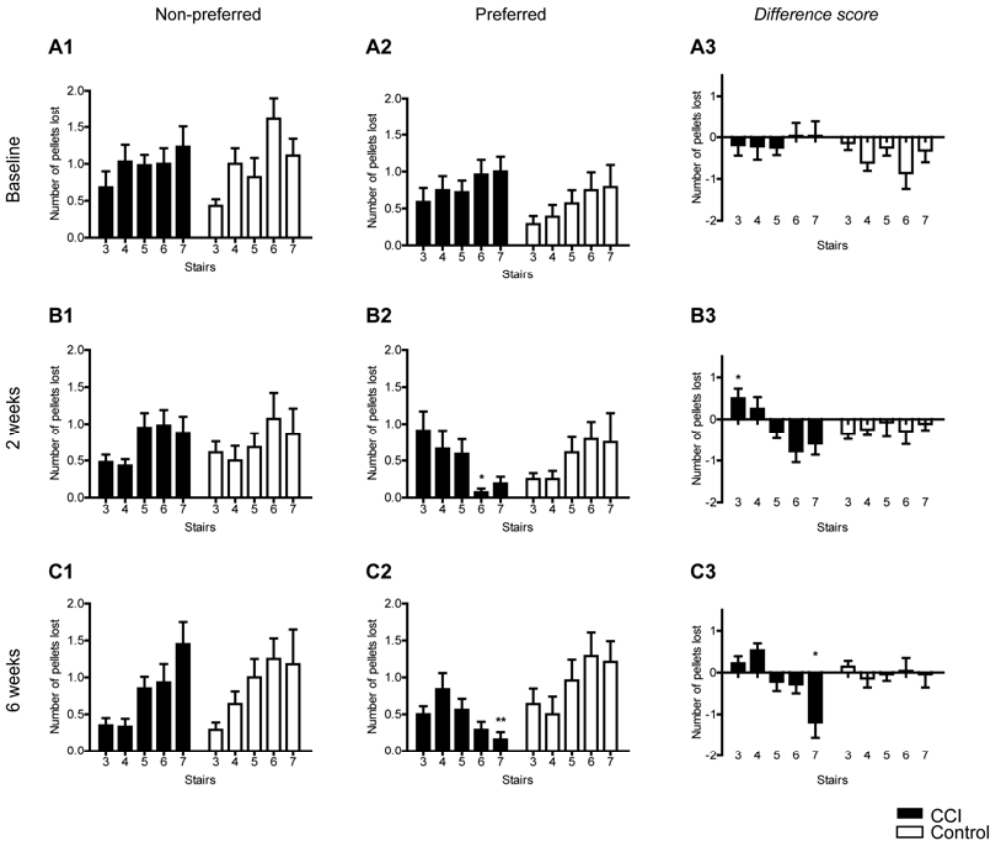
The five remaining parameters (additionally to the eight parameters shown in Fig. 3) describing individual paw functioning are shown (A1-3: maximum intensity, B1-3: minimum intensity, C1-3: maximum intensity at maximum contact, D1-3: print length, E1-3: print width). One significant difference between CCI and control rats was detected in the maximum paw print intensity of the non-preferred paw six weeks after surgery (A1). Concerning all other parameters, no significant differences between groups could be detected after CCI when analyzing scores of the individual forelimbs and the difference score (* $p < 0.05$).



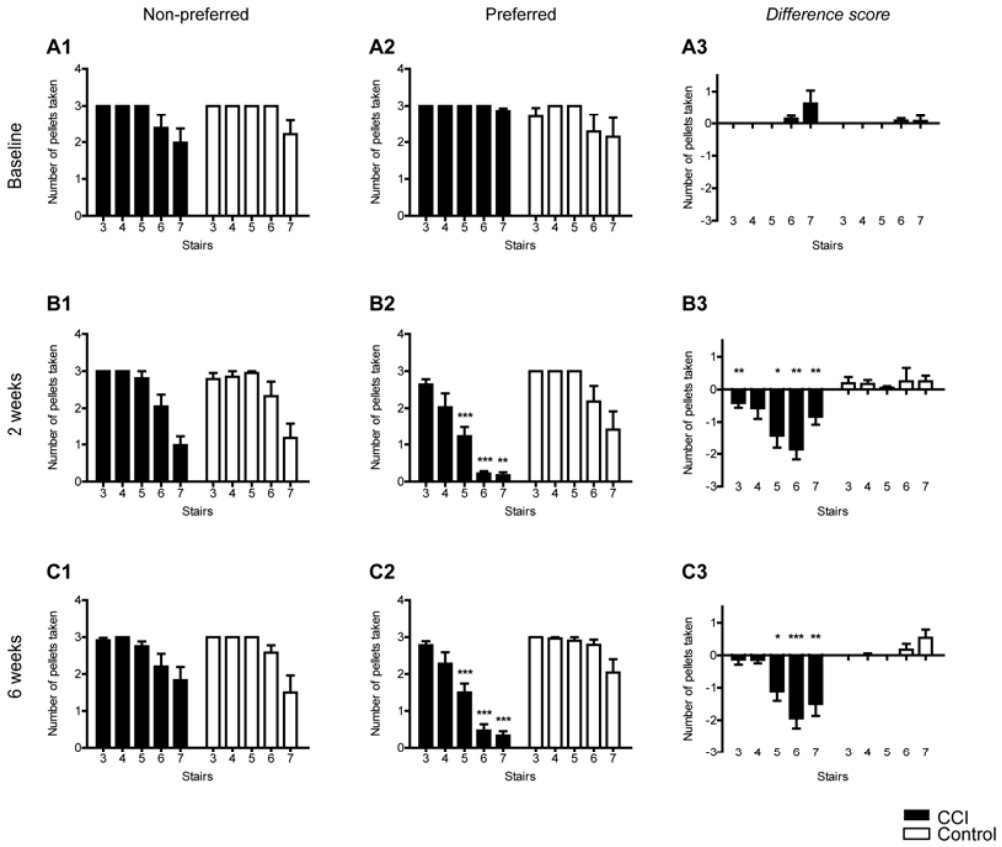
Supplementary Figure 2.1: After CCI more pellets remain at their original step



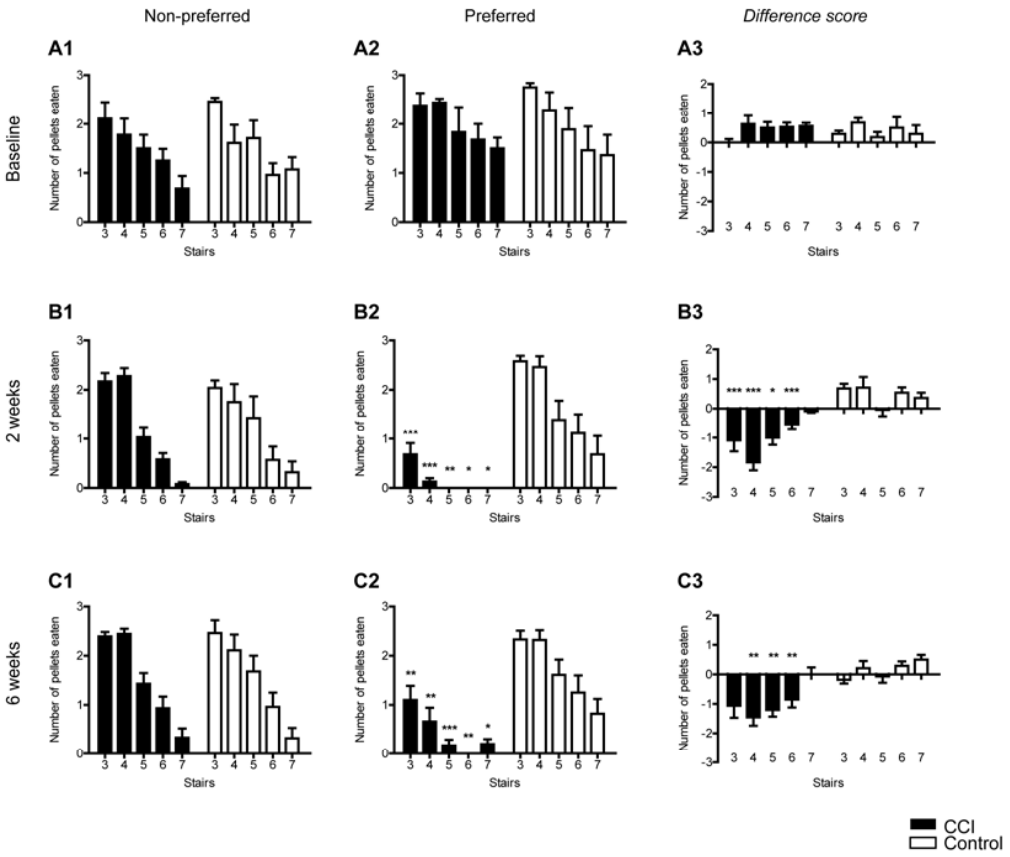
Supplementary Figure 2.2: More pellets are misplaced after CCI



Supplementary Figure 2.3: More pellets are lost when rats reached for lower steps



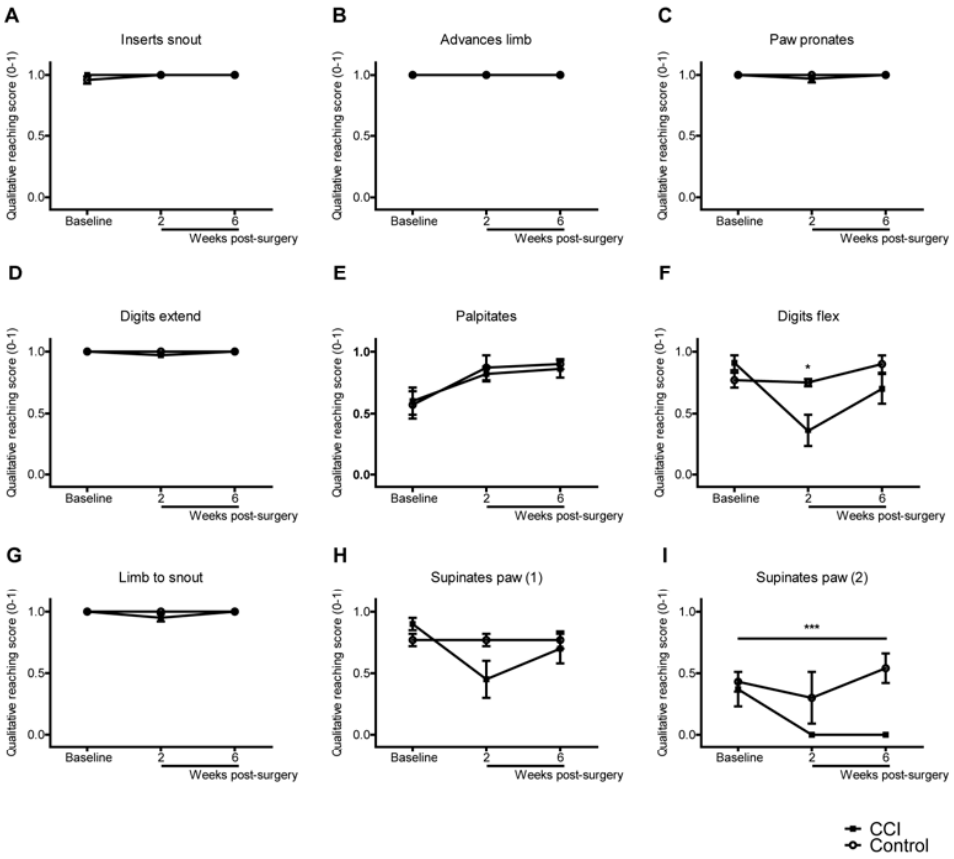
Supplementary Figure 2.4: After CCI less pellets are taken from the lower steps



Supplementary Figure 2.5: After CCI rats ate less pellets from each single step

Supplementary Figure 2: Pellet retrieval in the Montoya staircase test is significantly altered after CCI

Data of five parameters measuring pellet retrieval are shown separately for baseline, two weeks and six weeks after surgery (Supplementary Fig. 2.1 A1-3: number of pellets remained at baseline, B1-3: number of pellets remained 2 weeks after surgery, C1-3: number of pellets remained 6 weeks after surgery; Supplementary Fig. 2.2 A1-3: number of pellets misplaced at baseline, B1-3: number of pellets misplaced 2 weeks after surgery, C1-3: number of pellets misplaced 6 weeks after surgery; Supplementary Fig. 2.3 A1-3: number of pellets lost at baseline, B1-3: number of pellets lost 2 weeks after surgery, C1-3: number of pellets lost 6 weeks after surgery; Supplementary Fig. 2.4 A1-3: number of pellets taken at baseline, B1-3: number of pellets taken 2 weeks after surgery, C1-3: number of pellets taken 6 weeks after surgery; Supplementary Fig. 2.5 A1-3: number of pellets eaten at baseline, B1-3: number of pellets eaten 2 weeks after surgery, C1-3: number of pellets eaten 6 weeks after surgery). Overall, after CCI more pellets remained on the individual steps (Supplementary Fig. 2.1 B2) or were misplaced to another step (Supplementary Fig. 2.2 B2), whereas less pellets were lost (Supplementary Fig. 2.3 B2), taken (Supplementary Fig. 2.4 B2) and eaten (Supplementary Fig. 2.5 B2) at the side of the preferred paw (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).



Supplementary Figure 3: Pellet retrieval with the preferred paw is severely altered after CCI

Supplementary Figure 3: Pellet retrieval with the preferred paw is severely altered after CCI

In addition to the three parameters presented in Fig. 6, nine remaining components of a typical reaching action are shown here [A: inserts snout, B: advances limb, C: paw pronates, D: digits extend, E: palpitates, F: digits flex, G: limb to snout, H: supinates paw (1), I: supinates paw (2)]. Two weeks after CCI, rats exhibited abnormalities in flexing the digits when trying to hold onto a pellet (F) and when supinating the paw (I; * $p < 0.05$, *** $p < 0.001$).

REFERENCES

1. Bergold, P.J. (2015). Treatment of traumatic brain injury with anti-inflammatory drugs. Experimental neurology.

2. Gennai, S., Monsel, A., Hao, Q., Liu, J., Gudapati, V., Barbier, E.L. and Lee, J.W. (2015). Cell-Based therapy for traumatic brain injury. British journal of anaesthesia 115, 203-212.

3. Shin, S.S., Dixon, C.E., Okonkwo, D.O. and Richardson, R.M. (2014). Neurostimulation for traumatic brain injury. Journal of neurosurgery 121, 1219-1231.

4. Brody, D.L., Mac Donald, C., Kessens, C.C., Yuede, C., Parsadanian, M., Spinner, M., Kim, E., Schwetye, K.E., Holtzman, D.M. and Bayly, P.V. (2007). Electromagnetic controlled cortical impact device for precise, graded experimental traumatic brain injury. Journal of neurotrauma 24, 657-673.

5. Bilgen, M. (2005). A new device for experimental modeling of central nervous system injuries. Neurorehabilitation and neural repair 19, 219-226.

6. Xiong, Y., Mahmood, A. and Chopp, M. (2013). Animal models of traumatic brain injury. Nature reviews. Neuroscience 14, 128-142.

7. Zorner, B., Filli, L., Starkey, M.L., Gonzenbach, R., Kasper, H., Rothlisberger, M., Bolliger, M. and Schwab, M.E. (2010). Profiling locomotor recovery: comprehensive quantification of impairments after CNS damage in rodents. Nature methods 7, 701-708.

8. Chen, S.F., Hsu, C.W., Huang, W.H. and Wang, J.Y. (2008). Post-injury baicalein improves histological and functional outcomes and reduces inflammatory cytokines after experimental traumatic brain injury. British journal of pharmacology 155, 1279-1296.

9. d'Avila, J.C., Lam, T.I., Bingham, D., Shi, J., Won, S.J., Kauppinen, T.M., Massa, S., Liu, J. and Swanson, R.A. (2012). Microglial activation induced by brain trauma is suppressed by post-injury treatment with a PARP inhibitor. Journal of neuroinflammation 9, 31.

10. Lam, T.I., Bingham, D., Chang, T.J., Lee, C.C., Shi, J., Wang, D., Massa, S., Swanson, R.A. and Liu, J. (2013). Beneficial effects of minocycline and botulinum toxin-induced constraint physical therapy following experimental traumatic brain injury. Neurorehabilitation and neural repair 27, 889-899.

11. Harris, N.G., Mironova, Y.A., Hovda, D.A. and Sutton, R.L. (2010). Chondroitinase ABC enhances pericontusion axonal sprouting but does not confer robust improvements in behavioral recovery. Journal of neurotrauma 27, 1971-1982.

12. Neumann, M., Wang, Y., Kim, S., Hong, S.M., Jeng, L., Bilgen, M. and Liu, J. (2009). Assessing gait impairment following experimental traumatic brain injury in mice. Journal of neuroscience methods 176, 34-44.

13. Wang, Y., Neumann, M., Hansen, K., Hong, S.M., Kim, S., Noble-Haeusslein, L.J. and Liu, J. (2011). Fluoxetine increases hippocampal neurogenesis and induces epigenetic factors but does not improve functional recovery after traumatic brain injury. Journal of neurotrauma 28, 259-268.

14. Cross, D.J., Garwin, G.G., Cline, M.M., Richards, T.L., Yarnykh, V., Mourad, P.D., Ho, R.J. and Minoshima, S. (2015). Paclitaxel improves outcome from traumatic brain injury. Brain research 1618, 299-308.

15. Jahanshahi, A., Schonfeld, L., Janssen, M.L., Hescham, S., Kocabicak, E., Steinbusch, H.W., van Overbeeke, J.J. and Temel, Y. (2013). Electrical stimulation of the motor cortex enhances progenitor cell migration in the adult

rat brain. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale 231, 165-177.

16. Fonoff, E.T., Pereira, J.F., Jr., Camargo, L.V., Dale, C.S., Pagano, R.L., Ballester, G. and Teixeira, M.J. (2009). Functional mapping of the motor cortex of the rat using transdural electrical stimulation. Behavioural brain research 202, 138-141.

17. Paxinos, G. and Watson, C. (2007). The rat brain in stereotaxic coordinates, 6th edition. Elsevier: London, UK.

18. Farr, T.D. and Trueman, R.C. (2011). Functional assessment of subcortical ischemia. In: *Animal models of movement disorders*. Springer Science+Business Media, pps. 91-114.

19. Kloth, V., Klein, A., Loettrich, D. and Nikkhah, G. (2006). Colour-coded pellets increase the sensitivity of the staircase test to differentiate skilled forelimb performances of control and 6-hydroxydopamine lesioned rats. Brain research bulletin 70, 68-80.

20. Clarke, J., Ploughman, M. and Corbett, D. (2007). A qualitative and quantitative analysis of skilled forelimb reaching impairment following intracerebral hemorrhage in rats. Brain research 1145, 204-212.

21. Mountney, A., Leung, L.Y., Pedersen, R., Shear, D. and Tortella, F. (2013). Longitudinal assessment of gait abnormalities following penetrating ballistic-like brain injury in rats. Journal of neuroscience methods 212, 1-16.

22. Bondi, C.O., Semple, B.D., Noble-Haeusslein, L.J., Osier, N.D., Carlson, S.W., Dixon, C.E., Giza, C.C. and Kline, A.E. (2014). Found in translation: Understanding the biology and behavior of experimental traumatic brain injury. Neuroscience and biobehavioral reviews.

23. Starkey, M.L., Bleul, C., Zorner, B., Lindau, N.T., Mueggler, T., Rudin, M. and Schwab, M.E. (2012). Back seat driving: hindlimb corticospinal neurons assume forelimb control following ischaemic stroke. Brain : a journal of neurology 135, 3265-3281.

24. Trueman, R.C., Harrison, D.J., Dwyer, D.M., Dunnett, S.B., Hoehn, M. and Farr, T.D. (2011). A Critical Re-Examination of the Intraluminal Filament MCAO Model: Impact of External Carotid Artery Transection. Translational stroke research 2, 651-661.

25. Schallert, T., Upchurch, M., Lobaugh, N., Farrar, S.B., Spirduso, W.W., Gilliam, P., Vaughn, D. and Wilcox, R.E. (1982). Tactile extinction: distinguishing between sensorimotor and motor asymmetries in rats with unilateral nigrostriatal damage. Pharmacology, biochemistry, and behavior 16, 455-462.

26. Schallert, T., Fleming, S.M., Leasure, J.L., Tillerson, J.L. and Bland, S.T. (2000). CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology 39, 777-787.

27. Montoya, C.P., Campbell-Hope, L.J., Pemberton, K.D. and Dunnett, S.B. (1991). The "staircase test": a measure of independent forelimb reaching and grasping abilities in rats. Journal of neuroscience methods 36, 219-228.

28. Parkkinen, S., Ortega, F.J., Kuptsova, K., Huttunen, J., Tarkka, I. and Jolkkonen, J. (2013). Gait impairment in a rat model of focal cerebral ischemia. Stroke research and treatment 2013, 410972.

29. Simone, L., Rozzi, S., Bimbi, M. and Fogassi, L. (2015). Movement related activity during goal-directed hand actions in monkey ventrolateral prefrontal cortex. The European journal of neuroscience.

30. Grillner, S. and Wallen, P. (1985). Central pattern generators for locomotion, with special reference to vertebrates. Annual review of neuroscience 8, 233-261.

31. Schaar, K.L., Brenneman, M.M. and Savitz, S.I. (2010). Functional assessments in the rodent stroke model. Experimental & translational stroke medicine 2, 13.

CHAPTER 5

EVALUATING RODENT MOTOR FUNCTIONS FROM ALL ANGLES: WHICH TESTS TO CHOOSE

Lisa-Maria Schönfeld, Dearbhaile Dooley, Ali Jahanshahi, Yasin Temel[#] and Sven Hendrix[#]

[#] Yasin Temel and Sven Hendrix contributed equally to this review

To be submitted

ABSTRACT

Damage to the motor cortex induced by stroke or traumatic brain injury (TBI) can result in chronic motor deficits. For the development of effective therapies to restore motor functioning, animal models are used, which show symptoms that reflect the clinical population. However, the use of experimental animals raises valid ethical and methodological concerns. To decrease discomfort caused by experimental procedures and to increase the quality of the results, non-invasive behavioral testing methods are needed, which are sensitive enough to measure a specific motor behavior.

A broad variety of behavioral tests are available to determine deficits in rodent motor functions after stroke or TBI. The current review describes and evaluates tests that are based on three categories: Tests to evaluate fine motor skills and grip strength, tests for gait and inter-limb coordination and neurological deficit scores. Furthermore, we share our thoughts on how to standardize behavioral scoring and presentation of test results to increase data comparability between studies. To conclude, we present a set of recommendations on how to choose the best behavioral test for a new research line.

INTRODUCTION

Insults to the central nervous system, caused by stroke or traumatic brain injury (TBI), can lead to devastating impairments in many facets of the patients' daily life. Depending on the exact injury location, these impairments can consist of gait disturbances, sensory neglect, hemiparesis or loss of fine motor skills. TBI is prevalent in adolescents and young adults, especially males[1], since they exhibit more risk taking behavior compared to other generations. Young TBI survivors cause an even higher socioeconomic burden, which then strengthens the need for constant improvement of treatment opportunities.

To conduct preclinical research on therapies for motor dysfunction, animal models are used, which show a disease pathophysiology similar to the clinical population. Ideally non-human primates are the organisms of choice due to their great physiological similarity to humans, which increases the translational value of the outcomes. However, from an ethical perspective, the similarity of non-human primates to humans sparks the discussion *not* to use them as experimental animals, but rather choose model organisms, such as rodents, which are less alike to humans. There is a considerable debate on how to assign an ethical or moral status to different kinds of mammals and this sensitive topic has been reviewed elsewhere[2]. To circumvent some of those ethical concerns, scientists tend to use smaller animals such as rodents. Additionally, rodents are attractive models organisms due to their high reproduction rate and relatively low costs.

Experimental design according to the 3Rs

Animal research must be performed according to the '3Rs': replacement, reduction and refinement[3]. Considerable effort is invested to discover alternative

techniques and thereby successfully replacing animal testing[4, 5]. However, scientific questions concerning changes in locomotion or other forms of behavior can only be answered with the help of a living organism. Still, animal experiments can be refined substantially by choosing behavioral testing methods that are sensitive and standardized. Thereby, unnecessary discomfort or even the number of animal experiments needed, can be reduced in accordance to the 3Rs[6].

Measuring motor functions in experimental animals

Despite the obvious differences between rodents and primates, both organisms possess surprisingly similar anatomical and functional manifestations of motor behavior[7]. However, measuring neurological impairments in animals remains a challenging task regardless of the species involved, since the physiological manifestation of a certain impairment is not translated one on one from humans to animals and *vice versa*[7]. Rodents, for example, differ highly from man in the way of limb utilization by walking on four paws and using different muscle groups. Furthermore, to survive in nature, rodents show a greater robustness against central insults that compromise their mobility and several compensatory mechanisms take over in case a certain motor pathway gets damaged[8, 9]. Therefore, highly specialized and rodent-specific tests are needed to detect the specific motor dysfunction that results from an experimental intervention.

Fortunately, a broad number of rodent tests can be used to measure motor deficits, even deficits that are not visible in freely moving animals. Motor deficits usually emerge from specific damage to a limb representation on the motor cortex and/or the striatum, which both show a somatotopic organization[10]. The tests discussed in the next sections can measure a variety of motor behaviors

with a clear clinical relevance. *Fine motor skills* are vital to everyday functioning and range from brushing our teeth in the morning to setting the alarm clock at night. In addition, disturbed *gait and limb coordination* severely compromise a patient's independence and cause a reliance on walking aids of various kinds.

TESTS TO MEASURE FINE MOTOR SKILLS

Fine motor skills of humans, such as picking up a pen or closing a zipper, are vital in daily life. At first sight, these skills seem hard to replicate in animal models, since animals do not perform comparable tasks. Still, rodents inherently possess a great capacity to grasp and manipulate small objects. As fine motor skills are not necessarily observed in the homecage, one needs to design a challenging testing environment to reveal rodent fine motor skills.

In the next section of this review, the following behavioral tests will be discussed:

- Single pellet reaching test: reaching for pellets through a small opening
- Montoya staircase test: reaching for pellets that lie on a staircase
- Adhesive removal test: contacting and removing small adhesives that are placed under the front paws
- Vermicelli handling test: handling and eating of small pasta pieces
- Grid test: walking over a wire mesh
- Cylinder test: exploration of a vertical wall with the forelimbs
- Grip strength test: holding on to a bar in the presence of a pulling force

Single pellet reaching test

The 'single pellet reaching', or 'skilled reaching test', was developed to provide a view on reaching activity in rats and was adapted lateron to also allow testing of

mice[8, 11]. Animals have to reach for and grasp individual food pellets, allowing a detailed analysis of forelimb motion and potential deviations when trying to eat the pellets. The single pellet reaching test has been used to detect long-term deficits of forelimb function in rat and mouse models of ischemic stroke or TBI[8, 12-17].

Testing environment

Animals are placed in a testing chamber, which consists of a Plexiglas box mounted on a pedestal. A corridor of two Plexiglas walls leads to a slid in the front wall through which the animals can stick their forelimb and reach for food pellets located in a shelf outside. More specifically, the food pellets are placed in small indentations on the left and right edge of the shelf to encourage reaching with the paw contralateral to the desired pellet. Pellet reaching is videotaped from the front, bottom and/or side to quantify forelimb motion frame by frame.

Testing and analysis of performance

Standard testing procedure

Animals are food deprived to reach 90% of their free feeding weight prior to training and testing to increase their motivation to reach for food. During the first week of training, daily sessions last between 30 minutes to one hour and rats learn to reach for pellets either directly in the reaching chambers or in a slightly modified training environment, the so-called 'food tray task'[8]. In the food tray task, animals are transferred to boxes with metal bars in the front and can reach through the bars into a bigger tray containing food pellets outside the training boxes. As soon as animals learn how to reach for pellets through the bars, training is continued in the testing box, which is expected to happen after

one week of training. In the testing box, food pellets are placed contralaterally to the preferred limb, being replaced either when the pellet was successfully grasped or knocked off the platform. Each animal received 20 pellets per day during training. After successful pellet retrieval, a pellet is dropped in the back of the box to distract rats from the slit and allow a repositioning for their next reach[8]. Testing takes place by presenting 20 pellets, sometimes preceded by five practice pellets[18], to the animal.

Behavior related to grasping can be scored by observing the animal during the testing session; however, the gold standard is filming the animal from several angles, to achieve a three-dimensional view of its behavior and allow offline analysis. Different strategies exist to evaluate the grasping performance of an animal. In the original version of the test a 'reach' occurs, when the paw is advanced through the slit. If a rat manages to grasp a pellet and eat it from its paw, the behavior is scored as a 'hit'[8]. However, when the pellet is knocked off the platform, the reach has failed. The number of hits can be expressed as a percentage of the total amount of reaches[13-15]. Using a more detailed scoring method, a score of '1' is assigned when the animal reaches through the slit and eventually eats the pellet, even if this takes several attempts (given that the paw is not retracted back inside the cage). In case the pellet is grasped but dropped inside the box, a score of '0.5' is given, whereas a score of '0' is given when the pellet is knocked off the platform[18]. Also, a success rate can be calculated as the total numeric score divided by the total number of reaches[18]. One might consider excluding animals, which do not fulfill a baseline success rate of 60% or higher[15, 18] to reduce variation of scores within the groups caused by inter-individual differences in grasping performance.

Qualitative assessment

Changes in grasping behavior or forelimb position relative to overall body posture can be assessed with different qualitative rating scales[8, 12-14]. Pellet retrieval consists of a series of single movement components and through qualitative analyses deviant movement components can be identified. Recently a semi-automated method to analyze reaching movements was proposed, which could detect behavioral deficits after stroke in mice without the need for a time consuming frame-by-frame inspection[11].

Pro and Con

By using the single pellet reaching test, the experimenter is enabled to take a detailed look at reaching and grasping behavior of each forelimb on its own. At the same time, this extensive and subjective analysis might be a big disadvantage. Analysis has to occur for each reaching movement separately, especially when more details of forelimb movement are assessed. In addition, a variation in scores can be caused when ratings are done by different observers. Automated test versions, which process behavioral data based on an algorithm[11], are being developed to reduce the time needed for analyzing the reaching patterns and minimize variations between observers. Unfortunately, all automated systems are rather expensive and therefore not widely distributed across labs, which makes data comparison difficult.

Setup dimensions might not allow testing of multiple animals in parallel, which further increases the time needed for test trials. Furthermore, different terminology used across studies makes it hard to compare behavioral outcomes, since the meaning of the term 'reach' ranges from sticking the paw through the opening to successful pellet retrieval. Lastly, repeated movement of an impaired

forelimb might result in a training effect and improve limb function over time, which could influence results. This might be seen as an unwanted side effect; on the contrary, behavioral improvement through repeated limb use, or in other words 'physical rehabilitation therapy', has a high clinical relevance, since physical rehabilitation therapy is the method of choice for treating motor impairments in humans.

Montoya staircase test

The 'Montoya staircase test' was developed as a method to objectively investigate fine motor skills such as reaching and grasping of food pellets[19]. In contrast to the single pellet reaching test, with the Montoya staircase test performance of both forelimbs separately can be measured within the same test session. The staircase test was developed while searching for a quantitative readout of the individual forelimbs in parallel, which neither required restrain of the contralateral limb nor time-intensive scoring of each reaching movement by an observer[19]. The staircase has been used to assess unilateral forelimb deficits in several animal models of cortical damage, such as stroke caused by MCAo, local ischemia or intracerebral hemorrhage, unilateral 6-OHDA lesions, TBI and peripheral nerve lesions[20-24].

Testing environment

The original apparatus consists of a Plexiglas box with a platform extending over two thirds of the length of the box. On the left and the right hand side of the platform a staircase is located with indentations on each step to hold the food pellets (Figure 1). The increasing depth of the staircase makes it more difficult to reach for the lower steps. An additional indentation is made on the floor of

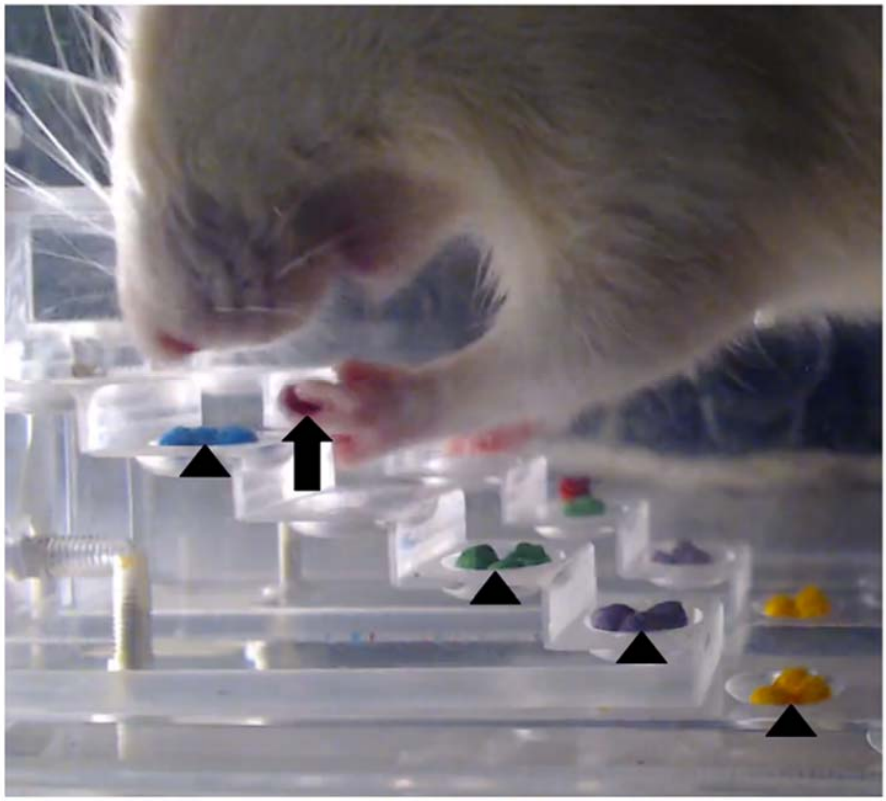


Figure 1: A rat is shown inside of the Montoya staircase testing box, reaching towards the staircase and grasping a red sucrose pellet (arrow). Sucrose pellets lying on each step were color-coded in blue, red, green, purple and yellow (arrowheads) to allow the collection of additional information on grasping success. Note that the upper two steps were left empty; from these steps, the rats could retrieve pellets by mouth, which prevents grasping behavior.

the box resulting in several levels of the steps and each step can be baited with sucrose pellets. While the size of the initial staircase box was tailored to adult rats, an smaller version of the testing boxes is also available for mice[25].

Testing and analysis of performance

Animals undergo food deprivation to reach a steady weight of 90% of their free-feeding state to increase their motivation to reach for the food pellets. Although Montoya et al. reported immediate reaching behavior of rats during the first habituation session to the box[19], most studies rely on a training period for the animals in order to achieve a steady baseline reaching performance. Animal training lasts between 10 to 21 days and preferably takes place twice daily[26-28]. The conditions wherein the animals are habituated to the staircase apparatus resemble the regular testing conditions, e.g. baiting all steps with a fixed number of sucrose pellets and leaving the animals in the testing boxes for 15 minutes; however, some studies report a more refined habituation approach, where the time to expose the animals to the closed box is gradually prolonged[29] and the number of pellets per stair is increased initially to attract the animal's attention to the steps[30]. The rats need to obtain more than 55% of the presented pellets with their preferred limb before reaching performance can be reliably measured[21, 26, 27]. For the final testing conditions, the reaching performance assessed during the last 3-5 days of training[22, 30] or during two separate days, where the animals are tested twice daily, is averaged[23, 26, 27, 31].

Standard testing procedure

In the standard testing procedure both sides are baited with two or three food pellets per stair, followed by placing the animal in the box for 15 min and counting the number of remaining pellets after the trial is finished. Additionally, the side where the animals consume the most pellets can be designated as the preferred side.

Speed test

Both sides are baited and reaching performance is assessed during the first 15 seconds to measure the initial preference for one paw over the other[19].

Side test or forced paw use

Both sides are baited separately (e.g. in the first test round pellets are placed only on the left staircase and *vice versa* in the second test round) and the animals remain in the testing box for 5 minutes. This modified paradigm aims to clarify whether decreased pellet consumption on one side might be due to a lack of attention spend on that specific side or a unilateral motor disability[19, 30].

Color-coding of pellets

To increase the sensitivity of the staircase test, sucrose pellets can be colored separately for each step using odorless food coloring. After a test session, colored pellets will allow to identify if the pellets on a certain step were simply untouched or had been knocked off from a higher stair. The following parameters can be assessed: (1) Number of 'remaining pellets' for pellets still in place at their original location, (2) number of 'eaten pellets', for the number of pellets of a certain color, gone at the end of a testing session, (3) number of 'pellets misplaced' for the pellets, which are found back at a stair other than their original location and (4) number of 'pellets lost', for pellets displaced in a way making them impossible to retrieve, which is usually on the ground in the back of the testing box[20, 26, 32].

Qualitative motor assessment

Pellet retrieval requires the ability to extend the forelimb, spread the digits, hold the pellet and bring the pellet to the mouth. To unravel deficits in one part of the sequence of movements, a qualitative evaluation of the reaching actions can be performed. While being tested in the standard paradigm, the animals are filmed and performance is scored offline. Then, the absence or presence of each component of a typical reach is being scored. For more details, see Clarke et al.[31]

Pro and Con

With the staircase test, fine motor skills of both forelimbs can be investigated separately in the same testing session, without the need to restrain the contralateral limb. The resulting scores are quantitative readouts of forelimb function that can be obtained fast. Due to the small size of the staircase boxes and the fact that functional assessment does not require observation of the animals inside the boxes, several animals can be tested in parallel. Different testing paradigms are available to increase sensitivity of the test and provide the experimenter with an additional set of data. Also postural deficits do not interfere with reaching ability, since lying on the platform stabilizes the trunk of the animals[31].

Although the testing sessions are fast, it takes several weeks of daily training before the animals learn to locate and grasp the pellets from the steps. Smaller animals can turn around on the platform and retrieve pellets with the opposite forelimb, concealing a unilateral deficit. Pellet retrieval by mouth from the upper steps is frequently observed during training sessions, which can prevent animals from learning the essential grasping motion. Leaving the respective steps empty

during training and testing sessions can easily solve this problem. Lastly, to allow qualitative assessment of reaching actions, each testing box needs to be filmed from two sides, which places a high technical and economic strain on the research group.

Adhesive removal test

The 'adhesive removal test' is used to measure unilateral tactile extinction and motor deficits. Unlike the previously discussed grasping tests, no food reward is offered in the adhesive removal test; instead small adhesives are placed on the distal-radial portion of the forelimbs (Figure 2), which the animals want to remove as quickly as possible. The time to contact and time to remove the adhesives is recorded[33]. The adhesive removal test is used in mouse and rat models of ischemic stroke or TBI to measure sensorimotor impairments[22, 28, 34, 35].

Testing environment

Testing takes place either in the homecage or in a Plexiglas box; the latter allows filming rats from both front and bottom, but it also requires a habituation period to reduce their anxiety to this novel surrounding.

Testing and analysis of performance

Rats are picked up and the adhesives are attached underneath the left and right paw in a random order. In case of a unilateral lesion, the person attaching the adhesives should be blind to which limb is affected and apply equal pressure to both paws when attaching the adhesives. The rat is placed into the testing environment and will notice (usually by sniffing or shaking the paw) and remove

each sticker, one at a time[33]. A training period before testing is frequently included to reach a stable baseline performance[22]; based on our experience

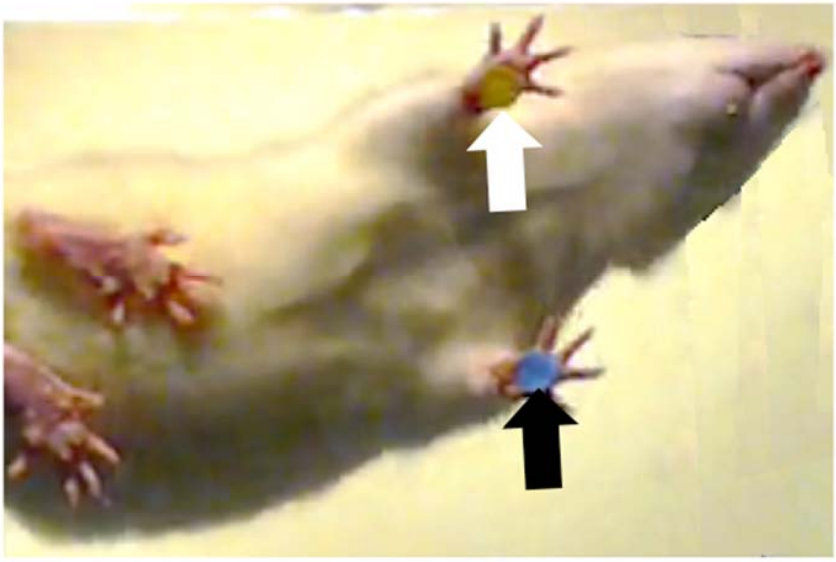


Figure 2: The ability to feel and remove adhesives from the paws is tested with the adhesive removal test. Here, a blue sticker dot was placed underneath the left front paw (black arrow) and a yellow sticker dot was placed underneath the right front paw (white arrow). The animal's reaction towards these stickers is filmed inside a Perspex cage.

healthy untrained rats manage to remove the adhesives in less than a minute. Testing occurs over three to five trials with an interval of minimal five minutes between trials whereas one trial is finished either after removal of both adhesives or after exceeding a pre-defined time interval[22, 34, 35]. For each limb the time is taken until the sticker is contacted and removed. The latency to contact and remove is afterwards reported for the impaired forelimb only or for both forelimbs separately.

Standard testing procedure

Adhesives of an equal size are used for both the impaired limb and the healthy limb and adhesive size stays constant throughout all testing trials.

Somatosensory neutralization test

An additional variant of the adhesive removal test consists of first recognizing the impaired side after a unilateral lesion and then increasing adhesive size underneath the impaired limb while decreasing adhesive size underneath the healthy limb. This change of adhesive size is continued until the animal shows an equally fast response for the impaired and healthy forelimb while the ratio of adhesive sizes indicates the severity of the functional damage[33].

Pro and Con

The adhesive removal test can be used to discriminate motor behavior into two distinct components: the sensing of a foreign object underneath the paw and its removal. Unfortunately, several studies do not distinguish between both variables, but report the latency to remove as the only outcome variable. We discovered that rats with a unilateral lesion mainly show deficits in feeling the adhesive under the impaired paw[29] and a strong sensory deficit might be mistaken as motor impairment in case both variables are separated. Furthermore, adhesive size is not standardized between studies and can vary substantially in diameter; however, no study has been published that describes a non-linear relationship between adhesive size and response time[34-36].

Vermicelli handling test

The 'vermicelli handling test' in rodents assesses loss of function in fine motor skills of the forepaws comparable to fine motor skill impairments observed frequently in the hands of hemiparetic patients. While the animals try to eat food with an unusual shape, goal-directed manipulation of the food with both forelimbs can be observed. Precise rating scales differ between studies, but in general the vermicelli handling test is used to measure deficits in skilled forelimb function in rodent models of TBI, Parkinson's disease and stroke[37-40].

Testing environment

Animals are provided with pieces of uncooked vermicelli and their behavior while eating the pasta pieces is observed. Animals might be food restricted prior to testing to increase their motivation for immediate pasta consumption[34]. Also, prior to the test sessions, animals should be familiarized with pasta pieces in their homecage to decrease their fear of novel food[37]. The absence (score '0') or presence (score '1') of pasta holding and consumption with each paw together with the animal's posture is scored, either during the test or offline in case the session was filmed[37]. Additionally, the number of times an animal drops the pasta piece, the duration of eating and the total number of paw adjustments are quantified[34, 38, 39].

Pro and Con

Observing vermicelli handling based on a predefined rating scheme is another method to assess forelimb usage and fine motor skills. By choosing different rating scales, the focus can be placed on either coordination of both forelimbs or on paw properties such as digit flexion. However, besides the reliance on

observer-based scores, a disadvantage of the vermicelli handling test is that it does not distinguish between motor and sensory disabilities[37]. In addition, care needs to be taken to stick to the same brand of pasta and keep an equal length of the vermicelli pieces, since rats break down longer pieces prior to handling, which confounds the purpose of the test[37].

Grid test

The grid test assesses both walking accuracy and grasping with fore- and hindlimbs, while the animals traverse an unusual surface. By walking over a grid, animals need to coordinate paw placement carefully and also grasp the grid wires in order not to slip through the holes. The grid test is frequently used to measure motor impairment in animals before and after TBI[14, 24, 41].

Testing environment

The animals are placed on a grid surface elevated above the ground, with different sizes of grid openings depending on the species tested[36, 42, 43]. While walking over the grid, the paws occasionally slip off the grid and fall through the wire. In healthy animals those 'foot faults' happen equally often with the left and right limb, whereas animals with a unilateral lesion show more foot faults with the contralateral limb[33].

Testing and analysis of performance

Healthy animals need less than two minutes to get habituated to walking over the grid. The test situation consists of counting the number of total steps and foot faults during a period between 2.5 and 5 minutes, either directly during the test or based on video footage[24, 42, 43]. Healthy animals quickly walk over the

grid but animals with motor deficits might move slower and pause in between, which results in less total steps during a time-restricted test session. A sufficient amount of steps per session can be achieved by recording the walking time instead of sticking to a fixed time frame during a test session[43]. Data of the contralateral limb is usually shown as a percentage of foot faults for that limb relative to all steps.

Pro and Con

Since no extensive training other than a period of habituation to the grid surface is necessary and testing sessions are relatively short, the grid test can be conducted fast. Filming the foot faults by placing a camera at an angle underneath the grid will increase reliability of the scoring, since more than one observer can score the animals. However, since rats need to be watched continuously to prevent escaping, the test cannot be performed with several animals at the same time. A general problem with tests that rely on gait is, that some animals are not willing to move. In this case their motivation for walking attempts can be increased by offering food rewards.

Cylinder test

The 'cylinder test' or 'paw asymmetry test' measures forelimb use during exploration of a vertical surface. The cylinder test is based on the inherent curiosity of rodents to explore a new environment. It has been used to assess long-term deficits in rats and mice with TBI and ischemic or hemorrhagic stroke[23, 31, 44, 45].

Testing environment

Animals are placed in a translucent cylinder with a diameter that is smaller than the total length of the animal including its tail, and a height that prevents escaping (Figure 3). Animals are filmed either from the top, bottom or front,

Figure 3: The cylinder test measures exploration of vertical surfaces. The rat shown in the picture rears and leans towards the cylinder wall using the forelimbs (red circles). Counting the number of wall contact allows detection of a potential asymmetry between the forelimbs.

whereas in the latter case a mirror is placed opposite to the camera to film wall contacts at the back of the cylinder.

Testing and analysis of performance

Explorative activity, which consists of rearing and leaning towards the cylinder wall, is filmed during a period between 5 and 10 minutes. The main behavioral readout consists of counting the weight-bearing wall contacts with both forelimbs simultaneously ('both') and the left and right forelimb separately ('left' or 'right'). A wall contact is counted as 'left' or 'right' when previously both paws had left the cylinder wall or after the rat initiates a new rear[46, 47]. Either all wall contacts or a predefined number of contacts can be scored and data can be expressed as the percentage of wall contacts with one specific limb relative to the total number of wall contacts[48]. In addition, the limb used to land after rearing can be noted[13].

The cylinder test does not require training to reach a certain performance; on the contrary, exposing the animals to the cylinders too frequently is discouraged since it might decrease the novelty of the environment and therefore explorative behavior. Since the cylinder test should not be repeated within a short time frame, performance analysis has to be based on a single test session.

Pro and Con

The cylinder test is used to assess asymmetry between the forelimbs in a relatively short test session and, if the trials are filmed, several animals can be tested in parallel. Furthermore, in healthy animals it is possible to assess forelimb preference based on the number of individual wall contacts.

Repeated performance in the cylinder test does not require an identical testing environment, since novelty stimulates exploration. Overexposure to the cylinders by repeated testing should be prevented. Based on our experience, an

interval of two weeks between trials does not decrease vertical exploration behavior.

Grip strength test

The 'grip strength test' measures grasping skills and forelimb muscle strength by the animal holding onto a bar and being pulled away from it with increasing force. Using this test, deficits resulting from insults like stroke or TBI have been measured in rats and mice[49-52].

Testing equipment

The main equipment needed for the grip strength test is a grasping handle, usually a bar or a ring, connected to a load cell, which measures the force of the handle when being pulled.

Behavioral testing and analysis of performance

Animals grasp the handle of the device and are horizontally pulled away until they loose grip. The force needed to break the grip is recorded over 3 to 10 trials giving an indication of forelimb muscle strength.

Pro and Con

Measuring grip strength is a fast method to determine changes of grasping ability and muscle strength without requiring any specific training of either the animal or the experimenter. Animals should be handled with particular care to avoid that they let go of the grip out of stress or distraction.

Maurissen et al. pointed out that it is unclear what is measured exactly by the grip strength test, since results can be heavily confounded by several factors,

such as the sampling rate of the different devices, the angle from which the tail of the animal is being pulled away and body weight, which tends to differ between lesioned animals and healthy controls[49].

CONCLUDING REMARKS: TESTS TO MEASURE FINE MOTOR SKILLS

Numerous tests are available, which can be used to measure fine motor skill impairments. The tests presented here rely on scoring by an experimenter, which makes them vulnerable for subjective influences. However, quantitative scoring of the single pellet reaching test and the Montoya staircase test can be considered robust against a biased observer, since counting the number of eaten sucrose pellets is a clearly identifiable readout parameter. When using a quantitative assessment (e.g. counting the number of pellets), the Montoya staircase test is superior to the single pellet reaching test in the number of readout parameters that can be measured. A disadvantage of both tests is the amount of training needed for the animals in order to learn how to perform the reaching tasks. Tests that require little to no training of the animals, but can still be used to assess fine motor skills, are the adhesive removal test, the vermicelli handling test, the grid test, the cylinder test and the grip strength test. The testing sessions are conducted relatively fast and the tests are attractive because they can be performed using equipment available in every lab. However, in each of these tests additional types of motor behavior are measured: in the adhesive removal test and vermicelli handling test sensory functions co-occur with forelimb movement, in the grid test, intact gait is a prerequisite and in the cylinder test, the animals need to keep balance to be able to rear and stand on the hindlimbs. The grip strength test is used to measure muscle strength in addition to grasping and holding on to a bar. Thus,

impairments of sensory functions, balance and gait have to be excluded before interpreting the results of the reviewed tests.

TESTS FOR GAIT AND INTER-LIMB COORDINATION

The ability to move in space is vital for all living organisms and gets severely affected in case gait is impaired. Gait is the synchronized movement of all limbs in order to move forward and in rodents gait belongs to the most overt behaviors observed in the home cage. Although gait is easily observable and therefore, in contrast to fine motor skills, does not require highly specialized tests, different testing setups are available that range from very simplistic ones requiring only a flat surface to complex automated systems. In this section the following tests will be reviewed:

- BBB/BMS: limb placement, joint movements
- CatWalk XT: gait-related limb functions
- Rotarod: walking on a rotating rod
- Beam walking test: crossing an elevated, narrow beam

Basso, Beattie and Bresnahan (BBB) and Basso Mouse Scale (BMS) locomotion rating

Developed as a method to evaluate locomotion after spinal cord injury (SCI), the 'Basso, Beattie and Bresnahan' (BBB) scale for rats and the 'Basso mouse scale' (BMS) for mice rely on observer ratings while the animals walk around in an open field[53, 54]. Originally the BBB was used for both rats and mice, but since mice differ from rats in their locomotor behavior and recovery from SCI, the BMS was developed as a specific version for mice. The BBB consists of a 21-

point rating scale, whereas the BMS is reduced to 9 points. Still, both scales focus on joint movement and coordinated placement of the limbs.

Both BBB and BMS are used to assess functional impairment in rodent models of SCI. When the BBB has been tested in TBI rats that received a controlled cortical impact (CCI) to the motor cortex, no functional impairments were detected, although rats in that respective study showed deficits in paw placement in the cylinder test[55]. These findings suggest that the BBB is not suitable to test motor impairments after TBI.

Testing environment

Any flat surface can serve as a basis for scoring with the BBB or BMS. Before locomotion can be assessed in its full potential, animals must be habituated to the arena, since open spaces elicit anxiety and, in case of repeated testing, the environment must stay constant[54].

Testing and analysis of performance

After being familiarized with the new environment, animals are left to move around freely for 4 minutes and behavior of the individual limbs is scored directly by two blinded observers or it is filmed for offline analysis[54]. Data is usually shown as the total score obtained at one timepoint.

Pro and Con

Both BBB and BMS are widely used to assess locomotor deficits in animal models of SCI, which is a substantial advantage when comparing outcomes between different studies. Furthermore, the testing sessions are conducted quickly with minimal equipment. Very subtle changes in limb movements are

described in the rating scales; therefore scoring requires experience. To get experienced, observers need to be trained for a considerable amount of time before they can reliably score the animals using either the BMS or the BBB scale. Since ratings are done subjectively, at least two blinded observers should score the animals, which is made possible by analysis based on video footage.

CatWalk XT

The 'CatWalk XT' is a fully automated analysis system used to assess a large number of parameters associated with gait-related abnormalities while requiring minimal involvement of the experimenter. It is used in rodent models of SCI, muscle denervation, Parkinson's disease and less frequently for stroke or TBI[56-61]. Since its development, the CatWalk focuses on gait, i.e. providing data about the spatiotemporal relation between the four paws. Recently, the system became more refined to measure functional deficits for each paw separately. Still the applicability for animal models with unilateral lesions remains debatable and published results are sparse. Using the CatWalk XT, some deficits were detectable in individual limb functioning at one month after stroke[62, 63], whereas for rat models of TBI convincing prove of long-term deficits measureable by the CatWalk is lacking[29]. Mountney et al. showed reductions in paw pressure after TBI in rats only at two weeks after lesion, whereas assessment with a 12-point neuroscore scale still showed deficits after four weeks[61]. In striking contrast, deviations in a broad number of parameters were detectable after TBI in mice for up to one month after the lesion[60, 64, 65].

Testing environment

The apparatus consists of a narrow corridor on a glass walkway, which the animal crosses (Figure 4 A-B). The glass plate is indirectly illuminated in green and lights up upon paw contact. Body contours of the animal are visualized by a

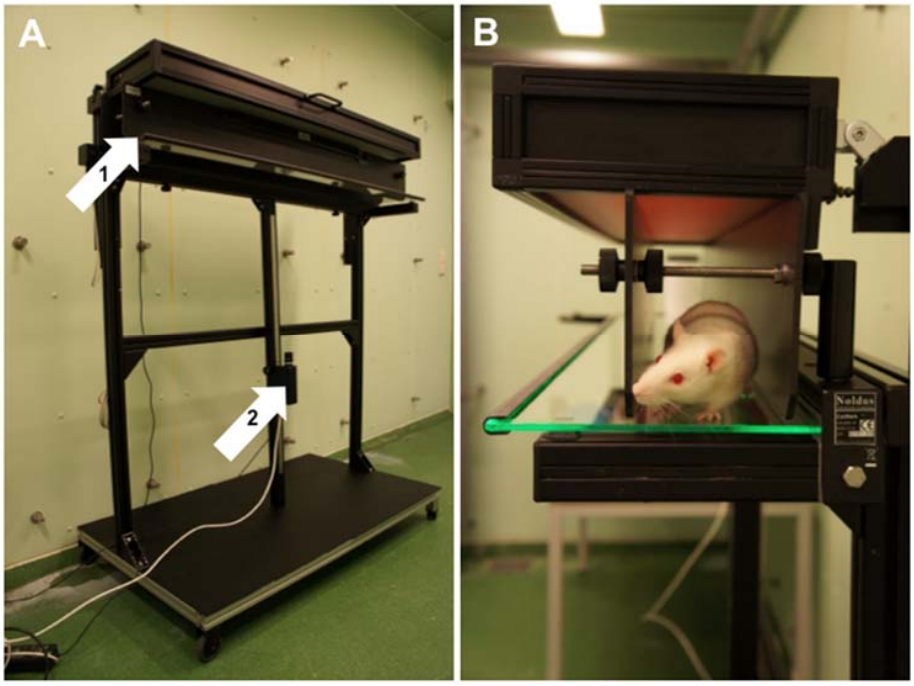


Figure 4: The CatWalk XT is an automated gait analysis system consisting of a walkway (arrow 1) situated above a camera (arrow 2; **A**). The rat has crossed the glass plate at the bottom of the walkway from one end to the other and waits for a reward provided by the experimenter (**B**).

red backlight. A camera underneath the walkway films the traversing animal and the accompanying software automatically detects footprints and calculates a large row of parameters.

Testing and analysis of performance

Prior to testing, animals have to be habituated to the walkway and learn to cross it from end to end, which can be stimulated either by food rewards or the prospect of a dark goal box that provides shelter[63, 66]. When choosing food as reward strategy animals might be food deprived during training and testing sessions to further increase their motivation to obtain the reward.

Each time the animal has crossed the walkway, the software indicates, which runs to keep and which runs to discard according to pre-defined criteria, such as a minimum and maximum run duration and a variation of the walking speed, which should not exceed 30%[61]. Furthermore, crossings of the walkway need to be fluent without any interruptions. A minimum of three runs per testing session should be obtained and averaged by the experimenter after the gait parameters of the individual runs have been extracted.

Based on the filmed runs, the software can assign labels to the four paws (left front, right front, left hind, right hind); however, it is strongly recommended for the experimenter to check the choices made by the software, as they are error-prone. The software might rate runs as suitable for analysis, even if the animal turns in the middle of the walkway and runs back to the direction it originally started. Also labels of ipsilateral front- and hindpaws might be mixed up in case of spatial overlap.

Pro and Con

When used for rodent models with overt locomotor impairments, such as models of SCI or Parkinson's disease, the CatWalk XT is unrivalled in its originality and amount of output it provides. However, the CatWalk XT should not be the only

behavioral test in animal models with a unilateral brain lesion, especially those models lacking visible locomotor impairments.

Most parameters are influenced by changes in animal weight and running speed, which complicates longitudinal studies assessing the change of motor deficits over time[63]. Despite being an automated test, based on our own experience we know that the involvement of the experimenter is still high, since animals have to be trained and motivated to properly cross the walkway. When analyzing the filmed runs, the choices made by the software should be treated with caution and checked by the experimenter, which adds to the amount of time invested in a testing session. As a last note, the CatWalk XT system is an expensive one, which makes it unattainable for small research groups with limited funding resources.

Rotarod test

The 'rotarod' is used to measure the time an animal can stay on a horizontal, rotating rod. Using this test, it cannot be distinguished between different gait-related behaviors such as coordination, balance, strength and endurance, which all influence the latency to stay on the rod. In rat and mouse models of TBI, stroke and SCI, animals tend to fall from the rotarod earlier compared to healthy controls[67-71].

Testing environment

The rotarod test consists of an elevated rod available in different diameters, depending on the animal species being tested; in general the diameter should be smaller than the length of the tested animal. The rod rotates automatically

with various velocities and it can be subdivided into several compartments by plastic walls, which allows testing of multiple animals at once (Figure 5).

Testing and analysis of performance

Standard testing procedure

Animals are placed on the slowly rotating rod and speed is increased steadily until the animals fall onto the landing plate (Figure 5). Animals can be habituated to the still rod first and are then trained on the accelerating rod for a

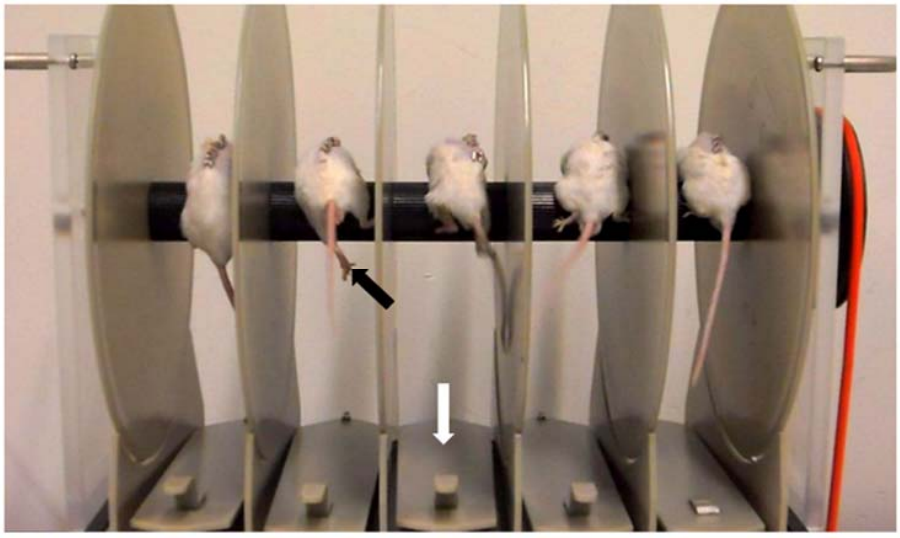


Figure 5: The rotarod measures the latency of an animal to stay on a rotating rod. Several mice can walk on the rod in parallel and the rotating velocity is increased until the animals slip (black arrow) and fall down from the rod on the landing plate (white arrow). Upon contact with the landing plate, the latency to stay on the rod is stopped automatically for each animal.

few minutes. Testing session consist of two to four trials using conditions similar to the training sessions[67, 68]. Data is presented as the latency to fall in seconds or as percentages relative to baseline.

Constant speed

In order to correct for motor abnormalities not directly related to the lesion, latency to fall can be measured at a constant speed[67]. However, if the constant speed is too slow, differences between injured and healthy animals are not detectable, so it is highly advised to choose a rotation speed that is challenging enough for injured animals[43].

Pro and Con

Since the rotarod is used to measure gait in a time-dependent manner, it does not require a detailed evaluation other than keeping track of the latency to fall from the rod, which makes the test robust against subjective influences. Furthermore, it is possible to test multiple animals at the same time. Unfortunately the rotarod is a relatively costly device, considering that only a single readout parameter is produced. Also, a decrease in latency to fall can have numerous causes, like coordination deficits or muscle weakness, which cannot be further specified with this testing setup. Based on our own experience we know that mice, unwilling to walk on the rotarod, can develop the habit of actively jumping off the rod straight away. This creates an artificially short latency to fall, which is unrelated to the severity of their functional impairment but merely depending on their motivation to do the test. On the contrary, severely impaired mice can develop compensatory strategies to prolong the latency to fall, like tightly holding on to the rod and spinning around with it or using the forelimbs only when walking on the rod. Therefore additional observation of the animals' behavior while doing the rotarod test is required to exclude animals that show such unwanted behavior.

Beam walking test

The 'beam walking test' or 'balance beam test' is used to analyze gait in rodents in a testing environment that challenges their ability to keep balance. In this testing environment, locomotion is made difficult by requiring the animal to cross an elevated beam with a narrow diameter. The beam walking test has been used in rodents to measure impairments after both TBI and stroke[72-74].

Testing environment

Depending on the species, animals have to walk over a narrow beam of 1-3 cm width that is stretched between a pole and either their homecage or a dark compartment[35, 75]. The composition of the beam can be smooth, like plastic or wood, but alternatively a rope might be used, which allows additional grip by using the claws.

Testing and analysis of performance

Standard testing conditions

Training the animals to cross the beam can be useful to achieve a stable baseline measurement[75]. The latency until an animal traverses the beam is taken and hindlimb performance can be rated on a 7-point scale[35]. If animals hesitate to cross the beam, walking can be stimulated by tapping on the tail[75]. However, this must be performed in all animals to keep testing sessions comparable.

Supporting ledge to prevent compensatory strategies

A ledge is installed next to the narrow beam, which can be used as a crutch to prevent the animals from falling[76]. Healthy animals manage to balance over the

beam without additional support, whereas animals with impaired fore- or hindlimb function frequently use the ledge as a support to cross the beam. Using the ledge as a support prevents the development of compensatory strategies, which might conceal a true impairment. As a readout parameter, the number of slips with each limb is scored.

Pro and Con

The beam walking test requires minimal equipment and can be conducted fast, since one run should not exceed 60 seconds[56]. Scoring takes place during testing, which make results prone to subjective influences by the observer. These influences can be minimized by filming the test sessions and having two blinded observers score the animals.

CONCLUDING REMARKS: TESTS FOR GAIT AND INTER-LIMB COORDINATION

Several tests are available to quantify gait-related behavior and deviations in limb coordination. As gait is such an overt behavior, it allows the possibility of automated analysis, which makes the reliance on observer-based scores unnecessary. An example of automated gait analysis is the CatWalk XT, where, in theory, a great amount of data collection and processing is done by the accompanying software. In practice, the experimenter still needs to invest a considerable amount of time in training the animal and controlling the quality of the acquired data. In addition, automated tests are very expensive, which makes them unattainable for small labs. Cheap alternatives are observer-based rating scales such as the BMS and BBB, that, based on our experience, have a sensitivity comparable to the CatWalk XT. In contrast to fully automated

systems, the BMS and BBB rely on subjective ratings by an observer, which may be a confounding factor that influences the results. However, using at least two well-trained and blinded observers will reduce these unwanted influences to a minimum.

Still, both the CatWalk XT and the BMS and BBB are time-consuming, because they require training of either the animals or the experimenter himself. The rotarod is an alternative test to measure gait, which neither requires extensive training of the animals (except for a short habituation period), nor training of the experimenter on how to perform the test. Unfortunately with the rotarod, gait is only measured with a single readout parameter that reflects a combination of several motor behaviors. Therefore, using the rotarod does not allow discrimination between different types of gait impairment. This is in contrast to the BMS and BBB, which have a more complex scoring scale, but also result in one outcome parameter, and the CatWalk XT that produces a wide array of gait-related outcome parameters. However, the biological relevance of each CatWalk XT parameter may not be clear to every investigator.

Similar to the rotarod, the single outcome parameter obtained with the balance beam test does not only represent gait, but also other, related behaviors, such as keeping balance and fine motor skills. However, an advantage of the balance beam test over the rotarod might be its potential availability in all labs, since the testing environment can be easily build from readily available equipment.

Although it is easy to measure gait in rodents, it remains challenging to model gait impairments with a similar disease course as observed in the clinical population. Rodents walk on four limbs and recover spontaneously from impairments in gross locomotion in developing compensatory strategies or increased central nervous system plasticity. Therefore, findings, which rely on

the gait tests reviewed here, should be treated with caution and can never be translated directly to human patients.

NEUROLOGICAL DEFICIT SCORES

Neurological deficit scores are used to measure the impairment of several types of motor behavior. In this section, the following scales used for a rodent neurological examination will be reviewed:

- Bederson scale: paw asymmetry and balance, general mobility
- Garcia scale: general mobility, paw asymmetry, grip strength, reaction to sensory stimuli
- Modified neurological severity score: general mobility, reaction to sensory stimuli, paw asymmetry and balance

The 'Bederson scale', originating in 1986, is one of the oldest rating scales to describe the neurological status of an animal after stroke[77]. A more detailed assessment method is the 'Garcia scale', where neurological deficits are evaluated based on a number of tests that measure different areas of functioning[78]. The 'modified Neurological Severity Score' (mNSS) is an 18-point examination schedule that contains tests for sensory reactions, gait, balance and reflexes[79].

Both the Bederson and the Garcia scale are used to detect neurological differences in animals as early as 1-2 days after stroke and the resulting values correlate with the infarct size[77, 78]. Also, the Bederson scale and the mNSS have been used to assess long-term impairment after TBI[35, 61, 80].

Testing and analysis of performance

The equipment needed for the different neurological examinations depends on the type of tests the animal will be subjected to. Concerning the Bederson scale, the necessary equipment is readily available in a lab whereas the Garcia scale requires some simple tools such as a wire mesh, which the rat can climb up, or a blunt stick such as a cotton swab.

Bederson scale

Different tests reflecting different types of neurological functions are rated by an observer on a scale from 0-3 during 3-5 minutes. In case an animal does not show abnormalities in one of the tests, it receives a final score and will not proceed to the following test. First, animals are held at their tails to observe forelimb flexion as a measure of paw asymmetry. Healthy animals extend both forelimbs towards the ground, receiving a score of '0', whereas stroke animals tend to flex the limb contralateral to infarction receiving a score of '1'. Second, animals are placed on a plastic-coated paper sheet, gently grabbed by the experimenter and pushed to the side to measure their ability to keep balance. Healthy animals will resist the lateral push whereas impaired animals show less resistance on the contralateral side (score '2'). Lastly, general mobility, in particular circling behavior, is observed in an open space. Impaired animals show circling towards the paretic side and receive a score of '3'.

Garcia scale

The Garcia scale is subdivided into six tests, which all have to be completed by the animal regardless of the severity of the lesion[78]. Impairment is rated using scores, which range from 0-3. In test 1, general mobility of the animal in its

homecage is observed, with special attention to the ability and willingness to approach the cage walls. In test 2, the animal is held by the tail and symmetry of movement between all four limbs is rated. In test 3, forepaw outstretching as an additional measure of asymmetry is rated. By pulling up the tail, the hindlimbs of the animal are lifted upwards and walking occurs on forelimbs only. In test 4, vertical climbing on a wire mesh is evaluated together with the grip strength when being pulled away from the wire surface. In tests 5 and 6 the animal's reaction to a sensory stimulus, here touch at the side of the body and at the whiskers, is evaluated.

Modified Neurological Severity Score (mNSS)

The mNSS evaluates neurological functions based on four tests that are comparable to the Garcia scale. In test 1, general mobility is evaluated by raising the animal by its tail and observing locomotion on a flat surface. In test 2, the reaction to sensory stimuli is evaluated by providing visual and tactile sensations and stimulating the hindlimb muscles. In test 3, animals have to keep balance to walk over a beam, similar to the beam walking test reviewed earlier, and in the last tests, asymmetry between the limbs assessed by the presence or absence of reflexes is tested. For a description of the scoring criteria belonging to the individual tests, we refer to Chen et al.[79]

Pro and Con

Rating neurological deficits on an ordinal scale is a traditional method to define functional deficits in animal models. Unfortunately, fine graduations in disability could be overlooked by using an ordinal rating scale[81]. Finally, as with all non-

automated tests, rating is based on the observation by an experimenter, who can bias the scores.

CONCLUSION: NEUROLOGICAL DEFICIT SCORES

The different forms of neurological examination are attractive methods to screen for the presence of a number of behavioral deficits. They are conducted fast, without costly equipment, but require at least two well-trained and blinded observers to reduce subjective influences during data collection. The different scales used for a neurological examination complicate data comparison between research groups, but within the same research group neurological examinations might be useful to get a quick indication about the neurological status of an animal. Although the scales are complex and measure a broad variety of rodent behaviors, only one outcome parameter is provided that does not specify the exact type of impairment.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

A wide array of tests are available to measure deficits in skilled limb use resulting from stroke, TBI or neurodegenerative diseases (Table 1). In addition to a thorough description of the tests and listing a number of their advantages and disadvantages, we want to provide general recommendations on how to choose the best behavioral test for a specific scientific question (Table 2). First, complex tests are superior to simple tests in the number of aspects being investigated that belong to one type of behavior, for example gait. The BBB assesses gait through rating various individual behaviors, such as hip movements, ankle movements and stepping, whereas the rotarod measures gait only by the time spend on the rotating rod. Simple tests can be conducted fast

and are therefore appealing to do a screening for motor impairment; however, even for screening purposes we prefer to choose complex tests, provided that they are already established in the lab. Second, tests that measure multiple readout parameters generate a large amount of data within a single session. The CatWalk XT, where animals have to walk through a corridor, provides a

TESTS TO MEASURE FINE MOTOR SKILLS				
Test	**Animal models**	**Testing paradigms**	**Readouts**	**Data shown**
Single pellet reaching test	TBI Stroke	Pellet presentation to impaired side Qualitative: Limb movement, body position	Grasped and eaten Grasped but dropped	Success rate: Retrieval/ trials of both limbs or impaired limb only Qualitative Various rating scales
Staircase test	TBI Stroke Parkinson's disease Peripheral nerve lesion	Both sides baited Side test Speed test Color coding Qualitative rating	Total eaten pellets per side Pellets remaining, misplaced, lost, taken and eaten Pellets retrieved, dropped, left Qualitative scores of reaching action	Number of pellets at side of contra-lesional limb or both sides Qualitative Scores 0-1 for 13 typical reaching actions

Adhesive removal test	TBI Stroke	Fixed adhesive size Somatosensory neutralization test	Time to contact Time to remove	Time in seconds for both limbs or impaired limb only

Vermicelli handling	TBI Stroke Parkinson's disease	n/a	Usage of paws, mouth, dropping pasta, head position Duration of eating Number of pauses	Scores 0-1 for pasta holding and eating with contra-lesional limb Duration in seconds
Adhesive removal test	TBI Stroke	Fixed adhesive size Somatosensory neutralization test	Time to contact Time to remove	Time in seconds for both limbs or impaired limb only
Vermicelli handling	TBI Stroke Parkinson's disease	n/a	Usage of paws, mouth, dropping pasta, head position Duration of eating Number of pauses	Scores 0-1 for pasta holding and eating with contralesio nal limb Duration in seconds
Grid test	TBI	n/a	Number of foot faults Number of slips	Foot faults with both forelimbs Number of foot faults relative to walking time
Cylinder test	TBI Stroke	n/a	Wall contacts with individual forelimbs and both forelimbs Paw used for landing	Contralesio nal forelimb contacts relative to all contacts A-symmetry scores (com-parison of

				contra-lesional and ipsilesional forelimb)
Grip strength test	TBI Stroke	n/a	Force in grams	Grams for both forelimbs or hindlimbs
TESTS FOR GAIT AND INTER-LIMB COORDINATION				
Test	**Animal models**	**Testing paradigms**	**Readout**	**Data shown**
Basso, Beattie and Bresnahan (BBB) scale	SCI (rats)	21-point rating scale	Joint movement Limb placement	Score between 0-21
Basso mouse scale (BMS)	SCI (mice)	9-point rating scale	Paw position Limb placement	Score between 0-9
Rotarod	TBI Stroke SCI	Accelerated speed Constant speed	Latency to fall off the rod	Latency in seconds
CatWalk XT	TBI Stroke SCI Parkinson's disease Muscle denervation	n/a	Gait-related parameters	Scores for each paw separately Scores for all four paws
Beam walking	TBI Stroke	With or without supporting ledge	7 point scale to evaluate walking Time to cross beam	Score between 0-7 Time in seconds

NEUROLOGICAL DEFICIT SCORES				
Test	**Animal models**	**Testing paradigms**	**Readout**	**Data shown**
Bederson scale	Stroke TBI	n/a	Paw asymmetry and balance, general mobility	3 tests, 0-3 points each
Garcia scale	Stroke	n/a	Paw asymmetry, general mobility, grip strength, reaction to sensory stimuli	6 tests, 0-3 points each
Modified neurological severity score (mNSS)	TBI	n/a	Paw asymmetry and balance, general mobility, reaction to sensory stimuli	5 tests, 0-6 points each

Table 1: An overview of the reviewed tests including the respective animal models, the different testing paradigms, the readout parameters and the way of data presentation. The mentioned animal models refer to both rats and mice if not stated otherwise. TBI: traumatic brain injury; SCI: spinal cord injury; n/a: not available.

large amount of gait-related readout parameters related to all limbs, whereas the grid test is used to measure gait and fine motor skills only by counting the number of foot faults for each limb. Third, multiple tests that measure the same underlying behavior, for example fine motor skills of the forelimbs, should be avoided since every test can increase the amount of stress and discomfort experienced by the animals. Fourth, automated tests are attractive because they do not rely on subjective, observer-based scores; however, we are aware of their high price that may be disadvantageous for small research groups with limited funding. Scoring of neurological deficits needs to take place by at least

two blinded and well-trained observers, which makes the testing procedure time consuming, involving several members of a research group. Tests such as the CatWalk XT and the rotarod require little to no involvement of the experimenter while the animals perform the test and therefore, the scores are relatively objective and do no require the involvement of more than one experimenter. However, the automated tests reviewed here are only used to measure gait-related behaviors and cannot measure fine motor skills such as grasping or movement of the wrists. Therefore, we suggest combining an automated test with a fine motor skill test that, if possible, is complex and generates multiple readouts, such as the staircase test or the adhesive removal test. Lastly, it is important to choose behavioral tests that are the 'gold standard', especially for investigators who start a new research line. Gold standard tests are most regularly published for a specific animal model, even if these tests do not fulfill all of the criteria mentioned above. Usage of the same test across different research groups increases the comparability of results and decreases the number of unnecessary animal experiments.

ADVICE	**THIS MEANS**	**EXAMPLE**
Complex tests are better than simple tests	Complex tests measure *multiple aspects of one type of behavior*, e.g. gait	Complex test: BBB/BMS Simple test: Rotarod
Multiple readouts are better than a single readout	Multiple readouts lead to more than one *outcome variable*	Multiple readouts: CatWalk XT (*mean intensity*, *print area*, *stride length*, amongst others) Single readout: Grid test (*foot faults*)
Avoid multiple tests	Every additional test may *stress* the animals and	Choose one fine motor skill test instead of combining the single

	takes time	pellet reaching test, the staircase test and the adhesive removal test
Automated tests are better than observer-based scoring	Automated tests are *objective* and do not require two or more blinded observers	Automated test: CatWalk XT Observer-based test: BBB/BMS
Combine an automated gait test with a fine motor skill test	After *TBI or stroke*, gait may be intact whereas fine motor skills are impaired	CatWalk XT + staircase test Rotarod + adhesive removal test
Choose the 'gold standard test'	'Gold standard tests' are *regularly published* for a specific animal model	Stroke: Single pellet reaching test SCI: BBB/BMS

Table 2: Six recommendations are shown that should assist in the selection of the best behavioral test. BBB: Basso, Beattie and Bresnahan rating scale; BMS: Basso mouse scale; SCI: spinal cord injury.

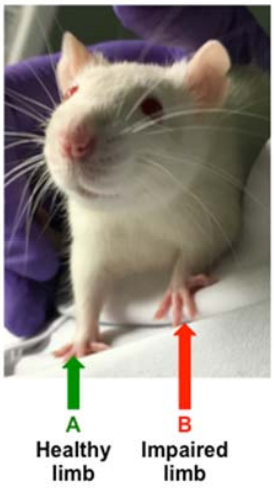
The majority of reviewed behavioral tests is suitable to measure motor deficits in rodent models of stroke and TBI. However, comparing the severity of a given impairment between two independent research groups may remain difficult, since timepoints of assessment, scoring schedule and data presentation can vary greatly (Table 1). Additional complications might be created by ordinal rating scales that are used in complex tests such as the BMS, the BBB or the neurological deficit scores. When using an ordinal rating scale, a two-fold increase in scores does not mean that one animal performs twice as good as another animal, since the individual tests of each rating scale measure separate functions, but the final performance is summarized with one score.

Overall, there is a strong need for consensus about how to perform animal experiments and presenting data in a uniform way [82]. In case of measuring unilateral functional impairments after a cortical lesion, data are not always

presented as functional scores of the impaired limb, but sometimes differences or ratios between the impaired and the healthy limb are calculated and shown instead. To make data presentation more comparable, a combination of results might be shown: One graph consisting of data of the healthy forelimb, one graph consisting of data of the impaired forelimb and additionally a 'difference score' calculated by subtracting the score of the healthy limb from the score of the impaired limb as a direct measure of limb asymmetry (Figure 6).

The effects of behavioral tests on animals: Stress or a stimulating variety?

Planning and performing animal experiments must be done according to the 3Rs: replacement, reduction and refinement[83]. To answer scientific questions related to locomotion or specific motor skills, animal models are used and alternative methods are still not available, yet. Nevertheless, the experimental paradigm can be refined and the number of animals in an experiment can be reduced by carefully choosing the right behavioral tests. Table 2 summarizes criteria to choose the best behavioral test for a new research line.



Healthy limb	Impaired limb	Difference score
A	B	B - A

Figure 6: To increase comparability of results between studies, the following way of standardized data presentation is proposed: One graph showing the functional score of the healthy limb ('A'), ipsilateral to the lesion, and one graph showing the functional score of the impaired limb ('B'), contralateral to the lesion. In addition, the so-called 'difference score' is calculated by subtracting the score of the healthy limb from the score of the impaired limb ('B-A').

It is obvious that execution of behavioral tests forms an interruption of an animal's daily life in a cage; the question remains whether this interruption is stressful or stimulating. Handling by the experimenter and the introduction to a foreign environment can elicit stress on its own. Furthermore, the specific design of some testing setups can trigger anxiety or exacerbate pre-existing pain. Examples include placing animals in a large open field[84] to allow manual scoring of motor behavior using the BBB or BMS or introducing the animals to small testing boxes (e.g. Montoya staircase test). Schallert et al. suggest that the animals get habituated to the experimenter and the testing paradigms after gentle and repeated exposure to the new sensations[85]. Habituation may decrease the experienced stress levels drastically; however, this needs to be

supported by analyses of physiological stress parameters, such as blood corticosterone levels.

Some testing paradigms require additional procedures such as food restriction, which creates a stressful component that does not decrease after repeated exposure and may have a negative impact on the results. Therefore it is a justified debate if testing paradigms that require food restriction are acceptable or should be replaced by paradigms that measure comparable behavioral functions without the need to interfere with the feeding behavior of the animal.

The amount of discomfort caused by behavioral tests needs to be kept as low as possible to comply with the 3Rs and performing experiments according to the 3Rs increases the quality of the behavioral data.

Sensory deficits and pain might worsen motor impairment

Due to a close spatial proximity between the motor cortex and the somatosensory cortex in rodents[86] and a high amount of connectivity between both regions[87], lesions in the motor cortex may spread towards surrounding cortical areas and cause a significant dysfunction in sensory regions. Furthermore, chronic pain can occur after lesions to the central nervous system and is a well-known complication after SCI or stroke in humans[88, 89]. Both sensory deficits and pain might negatively influence motor behavior in rodents; for example, an animal may not grasp a food pellet because it does not feel the pellet or the movement creates pain, although the locomotor functions are intact. The presence of sensory impairments in addition to motor deficits can be assessed with tests such as the 'corner test' or the 'vibrissae-elicited forelimb placing test'[22, 36]. To test the presence of pain, a number of behavioral tests are reviewed elsewhere[90].

Concluding remarks

Measuring functional impairment in rodent models of stroke or TBI is crucial to understand the course of the disease and the efficacy of novel treatments. The chosen tests should be sensitive enough to detect subtle behavioral impairments over an extended period of time and document various characteristics of the deficit, while causing minimal discomfort. The current review describes a number of tests that fulfill those criteria (Table 1) and should assist researchers to select the ideal tests for measuring skilled limb use in animal models with unilateral motor deficits (Table 2).

REFERENCES

1. Cassidy, J.D., Carroll, L.J., Peloso, P.M., Borg, J., von Holst, H., Holm, L., Kraus, J., Coronado, V.G. and Injury, W.H.O.C.C.T.F.o.M.T.B. (2004). Incidence, risk factors and prevention of mild traumatic brain injury: results of the WHO Collaborating Centre Task Force on Mild Traumatic Brain Injury. Journal of rehabilitation medicine, 28-60.

2. Prescott, M.J. (2010). Ethics of primate use. Advances in Science & Research 5, 11-22.

3. Russel, W.M.S. and Burch, R.L. (1959). The Principles of Humane Experimental Technique. Methuen: London.

4. Kim, J., Lee, H., Selimovic, S., Gauvin, R. and Bae, H. (2015). Organ-on-a-chip: development and clinical prospects toward toxicity assessment with an emphasis on bone marrow. Drug safety 38, 409-418.

5. Giri, S. and Bader, A. (2015). A low-cost, high-quality new drug discovery process using patient-derived induced pluripotent stem cells. Drug discovery today 20, 37-49.

6. Tannenbaum, J. and Bennett, B.T. (2015). Russell and Burch's 3Rs then and now: the need for clarity in definition and purpose. Journal of the American Association for Laboratory Animal Science : JAALAS 54, 120-132.

7. Cenci, M.A., Whishaw, I.Q. and Schallert, T. (2002). Animal models of neurological deficits: how relevant is the rat? Nature reviews. Neuroscience 3, 574-579.

8. Whishaw, I.Q. (2000). Loss of the innate cortical engram for action patterns used in skilled reaching and the development of behavioral compensation following motor cortex lesions in the rat. Neuropharmacology 39, 788-805.

9. Carmichael, S.T., Wei, L., Rovainen, C.M. and Woolsey, T.A. (2001). New patterns of intracortical projections after focal cortical stroke. Neurobiology of disease 8, 910-922.

10. Carelli, R.M. and West, M.O. (1991). Representation of the body by single neurons in the dorsolateral striatum of the awake, unrestrained rat. The Journal of comparative neurology 309, 231-249.

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

12. Gilmour, G., Iversen, S.D., O'Neill, M.F. and Bannerman, D.M. (2004). The effects of intracortical endothelin-1 injections on skilled forelimb use: implications for modelling recovery of function after stroke. Behavioural brain research 150, 171-183.

13. Metz, G.A., Antonow-Schlorke, I. and Witte, O.W. (2005). Motor improvements after focal cortical ischemia in adult rats are mediated by compensatory mechanisms. Behavioural brain research 162, 71-82.

14. Adkins, D.L., Ferguson, L., Lance, S., Pevtsov, A., McDonough, K., Stamschror, J., Jones, T.A. and Kozlowski, D.A. (2015). Combining Multiple Types of Motor Rehabilitation Enhances Skilled Forelimb Use Following Experimental Traumatic Brain Injury in Rats. Neurorehabilitation and neural repair.

15. Vaagenes, I.C., Tsai, S.Y., Ton, S.T., Husak, V.A., McGuire, S.O., O'Brien, T.E. and Kartje, G.L. (2015). Binge ethanol prior to traumatic brain injury worsens sensorimotor functional recovery in rats. PLoS One 10, e0120356.

16. Liu, Z., Li, Y., Cui, Y., Roberts, C., Lu, M., Wilhelmsson, U., Pekny, M. and Chopp, M. (2014). Beneficial effects of gfap/vimentin reactive astrocytes for axonal remodeling and motor behavioral recovery in mice after stroke. Glia 62, 2022-2033.

17. Nakagawa, H., Ueno, M., Itokazu, T. and Yamashita, T. (2013). Bilateral movement training promotes axonal remodeling of the corticospinal tract and recovery of motor function following traumatic brain injury in mice. Cell death & disease 4, e534.

18. Starkey, M.L., Bleul, C., Zorner, B., Lindau, N.T., Mueggler, T., Rudin, M. and Schwab, M.E. (2012). Back seat driving: hindlimb corticospinal neurons assume forelimb control following ischaemic stroke. Brain : a journal of neurology 135, 3265-3281.

19. Montoya, C.P., Campbell-Hope, L.J., Pemberton, K.D. and Dunnett, S.B. (1991). The "staircase test": a measure of independent forelimb reaching and grasping abilities in rats. Journal of neuroscience methods 36, 219-228.

20. Kloth, V., Klein, A., Loettrich, D. and Nikkhah, G. (2006). Colour-coded pellets increase the sensitivity of the staircase test to differentiate skilled forelimb performances of control and 6-hydroxydopamine lesioned rats. Brain research bulletin 70, 68-80.

21. Pagnussat Ade, S., Michaelsen, S.M., Achaval, M. and Netto, C.A. (2009). Skilled forelimb reaching in Wistar rats: evaluation by means of Montoya staircase test. Journal of neuroscience methods 177, 115-121.

22. Bouet, V., Freret, T., Toutain, J., Divoux, D., Boulouard, M. and Schumann-Bard, P. (2007). Sensorimotor and cognitive deficits after transient middle cerebral artery occlusion in the mouse. Experimental neurology 203, 555-567.

23. MacLellan, C.L., Langdon, K.D., Botsford, A., Butt, S. and Corbett, D. (2013). A model of persistent learned nonuse following focal ischemia in rats. Neurorehabilitation and neural repair 27, 900-907.

24. Harris, N.G., Mironova, Y.A., Hovda, D.A. and Sutton, R.L. (2010). Chondroitinase ABC enhances pericontusion axonal sprouting but does not confer robust improvements in behavioral recovery. Journal of neurotrauma 27, 1971-1982.

25. Baird, A.L., Meldrum, A. and Dunnett, S.B. (2001). The staircase test of skilled reaching in mice. Brain research bulletin 54, 243-250.

26. Santos, M.V., Pagnussat, A.S., Mestriner, R.G. and Netto, C.A. (2013). Motor Skill Training Promotes Sensorimotor Recovery and Increases Microtubule-Associated Protein-2 (MAP-2) Immunoreactivity in the Motor Cortex after Intracerebral Hemorrhage in the Rat. ISRN neurology 2013, 159184.

27. Windle, V., Szymanska, A., Granter-Button, S., White, C., Buist, R., Peeling, J. and Corbett, D. (2006). An analysis of four different methods of producing focal cerebral ischemia with endothelin-1 in the rat. Experimental neurology 201, 324-334.

28. Trueman, R.C., Harrison, D.J., Dwyer, D.M., Dunnett, S.B., Hoehn, M. and Farr, T.D. (2011). A Critical Re-Examination of the Intraluminal Filament MCAO Model: Impact of External Carotid Artery Transection. Translational stroke research 2, 651-661.

29. Schönfeld, L.M., Jahanshahi, A., Lemmens, E., Schipper, S., Dooley, D., Joosten, E.A., Temel, Y. and Hendrix, S. (2016). Long-term motor deficits after controlled cortical impact in rats can be detected by fine motor skill tests but not by automated gait analysis (under revision).

30. Abrous, D.N. and Dunnett, S.B. (1994). Paw reaching in rats: the staircase test. Neuroscience Protocols 3, 1-11.

31. Clarke, J., Ploughman, M. and Corbett, D. (2007). A qualitative and quantitative analysis of skilled forelimb reaching impairment following intracerebral hemorrhage in rats. Brain research 1145, 204-212.

32. Beaudin, S.A., Nisam, S. and Smith, D.R. (2013). Early life versus lifelong oral manganese exposure differently impairs skilled forelimb performance in adult rats. Neurotoxicology and teratology 38, 36-45.

33. Barth, T.M., Jones, T.A. and Schallert, T. (1990). Functional subdivisions of the rat somatic sensorimotor cortex. Behavioural brain research 39, 73-95.

34. d'Avila, J.C., Lam, T.I., Bingham, D., Shi, J., Won, S.J., Kauppinen, T.M., Massa, S., Liu, J. and Swanson, R.A. (2012). Microglial activation induced by brain trauma is suppressed by post-injury treatment with a PARP inhibitor. Journal of neuroinflammation 9, 31.

35. Chen, S.F., Hsu, C.W., Huang, W.H. and Wang, J.Y. (2008). Post-injury baicalein improves histological and functional outcomes and reduces inflammatory cytokines after experimental traumatic brain injury. British journal of pharmacology 155, 1279-1296.

36. Kuypers, N.J. and Hoane, M.R. (2010). Pyridoxine administration improves behavioral and anatomical outcome after unilateral contusion injury in the rat. Journal of neurotrauma 27, 1275-1282.

37. Allred, R.P., Adkins, D.L., Woodlee, M.T., Husbands, L.C., Maldonado, M.A., Kane, J.R., Schallert, T. and Jones, T.A. (2008). The vermicelli handling test: a simple quantitative measure of dexterous forepaw function in rats. Journal of neuroscience methods 170, 229-244.

38. Jacqmain, J., Nudi, E.T., Fluharty, S. and Smith, J.S. (2014). Pre and post-injury environmental enrichment effects functional recovery following medial frontal cortical contusion injury in rats. Behavioural brain research 275, 201-211.

39. Dunkerson, J., Moritz, K.E., Young, J., Pionk, T., Fink, K., Rossignol, J., Dunbar, G. and Smith, J.S. (2014). Combining enriched environment and induced pluripotent stem cell therapy results in improved cognitive and motor function following traumatic brain injury. Restorative neurology and neuroscience 32, 675-687.

40. Plowman, E.K., Maling, N., Rivera, B.J., Larson, K., Thomas, N.J., Fowler, S.C., Manfredsson, F.P., Shrivastav, R. and Kleim, J.A. (2013). Differential sensitivity of cranial and limb motor function to nigrostriatal dopamine depletion. Behavioural brain research 237, 157-163.

41. Ajao, D.O., Pop, V., Kamper, J.E., Adami, A., Rudobeck, E., Huang, L., Vlkolinsky, R., Hartman, R.E., Ashwal, S., Obenaus, A. and Badaut, J. (2012). Traumatic brain injury in young rats leads to progressive behavioral deficits coincident with altered tissue properties in adulthood. Journal of neurotrauma 29, 2060-2074.

42. Goffus, A.M., Anderson, G.D. and Hoane, M. (2010). Sustained delivery of nicotinamide limits cortical injury and improves functional recovery following traumatic brain injury. Oxidative medicine and cellular longevity 3, 145-152.

43. Onyszchuk, G., Al-Hafez, B., He, Y.Y., Bilgen, M., Berman, N.E. and Brooks, W.M. (2007). A mouse model of sensorimotor controlled cortical impact: characterization using longitudinal magnetic resonance imaging, behavioral assessments and histology. Journal of neuroscience methods 160, 187-196.

44. Lam, T.I., Bingham, D., Chang, T.J., Lee, C.C., Shi, J., Wang, D., Massa, S., Swanson, R.A. and Liu, J. (2013). Beneficial effects of minocycline and botulinum toxin-induced constraint physical therapy following experimental traumatic brain injury. Neurorehabilitation and neural repair 27, 889-899.

45. Donega, V., Nijboer, C.H., van Velthoven, C.T., Youssef, S.A., de Bruin, A., van Bel, F., Kavelaars, A. and Heijnen, C.J. (2015). Assessment of long-term safety and efficacy of intranasal mesenchymal stem cell treatment for neonatal brain injury in the mouse. Pediatric research 78, 520-526.

46. Freret, T., Bouet, V., Leconte, C., Roussel, S., Chazalviel, L., Divoux, D., Schumann-Bard, P. and Boulouard, M. (2009). Behavioral deficits after distal focal cerebral ischemia in mice: Usefulness of adhesive removal test. Behavioral neuroscience 123, 224-230.

47. Farr, T.D. and Trueman, R.C. (2011). Functional assessment of subcortical ischemia. In: *Animal models of movement disorders*. Springer Science+Business Media, pps. 91-114.

48. Schallert, T., Fleming, S.M., Leasure, J.L., Tillerson, J.L. and Bland, S.T. (2000). CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology 39, 777-787.

49. Maurissen, J.P., Marable, B.R., Andrus, A.K. and Stebbins, K.E. (2003). Factors affecting grip strength testing. Neurotoxicology and teratology 25, 543-553.

50. Madinier, A., Wieloch, T., Olsson, R. and Ruscher, K. (2014). Impact of estrogen receptor beta activation on functional recovery after experimental stroke. Behavioural brain research 261, 282-288.

51. Linden, J., Fassotte, L., Tirelli, E., Plumier, J.C. and Ferrara, A. (2014). Assessment of behavioral flexibility after middle cerebral artery occlusion in mice. Behavioural brain research 258, 127-137.

52. Evans, T.M., Jaramillo, C.A., Sataranatarajan, K., Watts, L., Sabia, M., Qi, W. and Van Remmen, H. (2015). The effect of mild traumatic brain injury on peripheral nervous system pathology in wild-type mice and the G93A mutant mouse model of motor neuron disease. Neuroscience 298, 410-423.

53. Basso, D.M., Fisher, L.C., Anderson, A.J., Jakeman, L.B., McTigue, D.M. and Popovich, P.G. (2006). Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. Journal of neurotrauma 23, 635-659.

54. Basso, D.M., Beattie, M.S. and Bresnahan, J.C. (1996). Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection. Experimental neurology 139, 244-256.

55. Inoue, T., Lin, A., Ma, X., McKenna, S.L., Creasey, G.H., Manley, G.T., Ferguson, A.R., Bresnahan, J.C. and Beattie, M.S. (2013). Combined SCI and TBI: recovery of forelimb function after unilateral cervical spinal cord injury (SCI) is retarded by contralateral traumatic brain injury (TBI), and ipsilateral TBI balances the effects of SCI on paw placement. Experimental neurology 248, 136-147.

56. Chen, C.J., Cheng, F.C., Su, H.L., Sheu, M.L., Lu, Z.H., Chiang, C.Y., Yang, D.Y., Sheehan, J. and Pan, H.C. (2015). Improved neurological outcome by intramuscular injection of human amniotic fluid derived stem cells in a muscle denervation model. PLoS One 10, e0124624.

57. Mondello, S.E., Sunshine, M.D., Fischedick, A.E., Moritz, C.T. and Horner, P.J. (2015). A cervical hemi-contusion spinal cord injury model for the

investigation of novel therapeutics targeting proximal and distal forelimb functional recovery. Journal of neurotrauma.

58. Li, S., Shi, Z., Zhang, H., Liu, X., Chen, S., Jin, J., Wang, Y., Jia, W. and Li, H. (2013). Assessing gait impairment after permanent middle cerebral artery occlusion in rats using an automated computer-aided control system. Behavioural brain research 250, 174-191.

59. Westin, J.E., Janssen, M.L., Sager, T.N. and Temel, Y. (2012). Automated gait analysis in bilateral parkinsonian rats and the role of L-DOPA therapy. Behavioural brain research 226, 519-528.

60. Wang, Y., Neumann, M., Hansen, K., Hong, S.M., Kim, S., Noble-Haeusslein, L.J. and Liu, J. (2011). Fluoxetine increases hippocampal neurogenesis and induces epigenetic factors but does not improve functional recovery after traumatic brain injury. Journal of neurotrauma 28, 259-268.

61. Mountney, A., Leung, L.Y., Pedersen, R., Shear, D. and Tortella, F. (2013). Longitudinal assessment of gait abnormalities following penetrating ballistic-like brain injury in rats. Journal of neuroscience methods 212, 1-16.

62. Liu, Y., Ao, L.J., Lu, G., Leong, E., Liu, Q., Wang, X.H., Zhu, X.L., Sun, T.F., Fei, Z., Jiu, T., Hu, X. and Poon, W.S. (2013). Quantitative gait analysis of long-term locomotion deficits in classical unilateral striatal intracerebral hemorrhage rat model. Behavioural brain research 257, 166-177.

63. Parkkinen, S., Ortega, F.J., Kuptsova, K., Huttunen, J., Tarkka, I. and Jolkkonen, J. (2013). Gait impairment in a rat model of focal cerebral ischemia. Stroke research and treatment 2013, 410972.

64. Neumann, M., Wang, Y., Kim, S., Hong, S.M., Jeng, L., Bilgen, M. and Liu, J. (2009). Assessing gait impairment following experimental traumatic brain injury in mice. Journal of neuroscience methods 176, 34-44.

65. Cross, D.J., Garwin, G.G., Cline, M.M., Richards, T.L., Yarnykh, V., Mourad, P.D., Ho, R.J. and Minoshima, S. (2015). Paclitaxel improves outcome from traumatic brain injury. Brain research 1618, 299-308.

66. Koopmans, G.C., Deumens, R., Honig, W.M., Hamers, F.P., Steinbusch, H.W. and Joosten, E.A. (2005). The assessment of locomotor function in spinal cord injured rats: the importance of objective analysis of coordination. Journal of neurotrauma 22, 214-225.

67. Brody, D.L., Mac Donald, C., Kessens, C.C., Yuede, C., Parsadanian, M., Spinner, M., Kim, E., Schwetye, K.E., Holtzman, D.M. and Bayly, P.V. (2007). Electromagnetic controlled cortical impact device for precise, graded experimental traumatic brain injury. Journal of neurotrauma 24, 657-673.

68. Vonder Haar, C., Emery, M.A. and Hoane, M.R. (2012). Chronic folic acid administration confers no treatment effects in either a high or low dose following unilateral controlled cortical impact injury in the rat. Restorative neurology and neuroscience 30, 291-302.

69. Doll, D.N., Engler-Chiurazzi, E.B., Lewis, S.E., Hu, H., Kerr, A.E., Ren, X. and Simpkins, J.W. (2015). Lipopolysaccharide exacerbates infarct size and results in worsened post-stroke behavioral outcomes. Behavioral and brain functions : BBF 11, 32.

70. Kim, K., Park, H.W., Moon, H.E., Kim, J.W., Bae, S., Chang, J.W., Oh, W., Yang, Y.S. and Paek, S.H. (2015). The Effect of Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells in a Collagenase-Induced Intracerebral Hemorrhage Rat Model. Experimental neurobiology 24, 146-155.

71. Vidal, P.M., Lemmens, E., Geboes, L., Vangansewinkel, T., Nelissen, S. and Hendrix, S. (2013). Late blocking of peripheral TNF-alpha is ineffective after spinal cord injury in mice. Immunobiology 218, 281-284.

72. Qu, C., Lu, D., Goussev, A., Schallert, T., Mahmood, A. and Chopp, M. (2005). Effect of atorvastatin on spatial memory, neuronal survival, and vascular density in female rats after traumatic brain injury. Journal of neurosurgery 103, 695-701.

73. Gao, Y., Xu, S., Cui, Z., Zhang, M., Lin, Y., Cai, L., Wang, Z., Luo, X., Zheng, Y., Wang, Y., Luo, Q., Jiang, J., Neale, J.H. and Zhong, C. (2015). Mice lacking glutamate carboxypeptidase II develop normally, but are less susceptible to traumatic brain injury. Journal of neurochemistry.

74. Qu, H.L., Zhao, M., Zhao, S.S., Xiao, T., Song, C.G., Cao, Y.P., Jolkkonen, J. and Zhao, C.S. (2015). Forced limb-use enhanced neurogenesis and behavioral recovery after stroke in the aged rats. Neuroscience 286, 316-324.

75. Shijo, K., Ghavim, S., Harris, N.G., Hovda, D.A. and Sutton, R.L. (2015). Glucose administration after traumatic brain injury exerts some benefits and no adverse effects on behavioral and histological outcomes. Brain research 1614, 94-104.

76. Schallert, T. (2006). Behavioral tests for preclinical intervention assessment. NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics 3, 497-504.

77. Bederson, J.B., Pitts, L.H., Tsuji, M., Nishimura, M.C., Davis, R.L. and Bartkowski, H. (1986). Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. Stroke; a journal of cerebral circulation 17, 472-476.

78. Garcia, J.H., Wagner, S., Liu, K.F. and Hu, X.J. (1995). Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. Stroke; a journal of cerebral circulation 26, 627-634; discussion 635.

79. Chen, J., Li, Y., Wang, L., Zhang, Z., Lu, D., Lu, M. and Chopp, M. (2001). Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. Stroke; a journal of cerebral circulation 32, 1005-1011.

80. Wang, S., Chopp, M., Nazem-Zadeh, M.R., Ding, G., Nejad-Davarani, S.P., Qu, C., Lu, M., Li, L., Davoodi-Bojd, E., Hu, J., Li, Q., Mahmood, A. and Jiang, Q. (2013). Comparison of neurite density measured by MRI and histology after TBI. PLoS One 8, e63511.

81. Desland, F.A., Afzal, A., Warraich, Z. and Mocco, J. (2014). Manual versus Automated Rodent Behavioral Assessment: Comparing Efficacy and Ease of Bederson and Garcia Neurological Deficit Scores to an Open Field Video-Tracking System. Journal of central nervous system disease 6, 7-14.

82. Lemmon, V.P., Ferguson, A.R., Popovich, P.G., Xu, X.M., Snow, D.M., Igarashi, M., Beattie, C.E., Bixby, J.L. and Consortium, M. (2014). Minimum information about a spinal cord injury experiment: a proposed reporting standard for spinal cord injury experiments. Journal of neurotrauma 31, 1354-1361.

83. Manciocco, A., Chiarotti, F., Vitale, A., Calamandrei, G., Laviola, G. and Alleva, E. (2009). The application of Russell and Burch 3R principle in rodent models of neurodegenerative disease: the case of Parkinson's disease. Neuroscience and biobehavioral reviews 33, 18-32.

84. Prut, L. and Belzung, C. (2003). The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. European journal of pharmacology 463, 3-33.

85. Schallert, T., Woodlee, M.T. and Fleming, S.M. (2003). Experimental focal ischemic injury: behavior-brain interactions and issues of animal handling and

housing. ILAR journal / National Research Council, Institute of Laboratory Animal Resources 44, 130-143.

86. Benali, A., Weiler, E., Benali, Y., Dinse, H.R. and Eysel, U.T. (2008). Excitation and inhibition jointly regulate cortical reorganization in adult rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 28, 12284-12293.

87. Farkas, T., Kis, Z., Toldi, J. and Wolff, J.R. (1999). Activation of the primary motor cortex by somatosensory stimulation in adult rats is mediated mainly by associational connections from the somatosensory cortex. Neuroscience 90, 353-361.

88. Mulla, S.M., Wang, L., Khokhar, R., Izhar, Z., Agarwal, A., Couban, R., Buckley, D.N., Moulin, D.E., Panju, A., Makosso-Kallyth, S., Turan, A., Montori, V.M., Sessler, D.I., Thabane, L., Guyatt, G.H. and Busse, J.W. (2015). Management of Central Poststroke Pain: Systematic Review of Randomized Controlled Trials. Stroke; a journal of cerebral circulation 46, 2853-2860.

89. Balazy, T.E. (1992). Clinical management of chronic pain in spinal cord injury. The Clinical journal of pain 8, 102-110.

90. Barrot, M. (2012). Tests and models of nociception and pain in rodents. Neuroscience 211, 39-50.

CHAPTER 6

MOTOR CORTEX SIMULATION DOES NOT LEAD TO FUNCTIONAL RECOVERY AFTER EXPERIMENTAL CORTICAL INJURY IN RATS

Lisa-Maria Schönfeld, Ali Jahanshahi, Evi Lemmens, Matthias Bauwens, Sarah-Anna Hescham, Sandra Schipper, Melanie Lagiére, Sven Hendrix[#] and Yasin Temel[#]

[#] Sven Hendrix and Yasin Temel contributed equally to this article

To be submitted

ABSTRACT

Motor impairments are among the major complications that develop after cortical damage caused by either stroke or traumatic brain injury (TBI). Motor cortex stimulation (MCS) can improve motor functions in animal models of stroke by inducing neuroplasticity. In the current study, the therapeutic effect of chronic MCS was assessed in a rat model of severe cortical damage. A controlled cortical impact (CCI) was applied to the forelimb area of the motor cortex followed by implantation of flat electrodes covering the lesion area. Forelimb function was assessed using the Montoya staircase test and the cylinder test before and after a period of chronic MCS. Furthermore, the effect of MCS on tissue metabolism and lesion size was measured using [^{18}F]-fluorodesoxyglucose (FDG) µPET scanning. CCI caused a considerable lesion at the level of the motor cortex and dorsal striatum, together with a long-lasting behavioral phenotype of forelimb impairment. However, MCS applied to the CCI lesion did not lead to any improvement in limb functioning when compared to non-stimulated control rats. Also, MCS neither changed lesion size nor distribution of FDG. The current study questions the utility of MCS as a standalone treatment in a rat model of severe cortical damage.

INTRODUCTION

Cortical damage due to traumatic brain injury (TBI) or stroke often leads to persistent functional impairments if the motor cortex is part of the traumatized or infarcted brain tissue. The resulting motor impairments are disabling and form a major socio-economic burden[1, 2]. Thus far, the only clinically proven therapy for patients with motor deficits is physical rehabilitation therapy; still, many patients do not achieve a complete recovery. To enhance the efficacy of physical rehabilitation therapy, motor cortex stimulation (MCS) has been proposed as a potential therapeutic approach[3, 4]. Recently, the results of a multicenter study got published, where stroke patients suffering from hemiplegia received six weeks of MCS via implanted epidural electrodes, which co-occured with physical rehabilitation therapy[5]. The authors reported a promising recovery course of the patients receiving MCS together with physical therapy that was still present six months after cessation of the therapy[5]. MCS has also been applied in rodent and non-human primate models of an ischemic infarct and resulted in improved limb function, again when being combined with physical rehabilitation therapy[6-8]. However, limited data are available investigating the effect of MCS on its own without an additional intervention.

One of the mechanisms explaining the therapeutic effect of MCS on motor recovery is neuroplasticity. In a previous study, we found that MCS applied to naïve rats increased cell proliferation in the subventricular zone (SVZ) compared to non-stimulated controls[9]. Furthermore, a higher number of neural stem and progenitor cells (NSPCs) and mature neurons were detected in the motor cortex underlying the electrode[9]. This finding could be explained by a process called electrotaxis, where cells migrate along a current gradient towards the stimulation electrode[10]. In other studies using different forms of electrical

stimulation, this increase of NSPCs at the side of stimulation was further confirmed, strengthening the hypothesis of electrotaxis *in vivo*[11, 12].

Animal models can be used to mimic clinical symptoms in a standardized way. With a controlled cortical impact (CCI) a cortical lesion can be created in rats that results in long-lasting functional deficits[13]. Similar to humans, rats possess a topographic organization of the motor cortex, where distinct cortical areas control the function of specific body parts[14, 15] and a CCI lesion in the forelimb area of the motor cortex can cause deficits in motor functions specific to the contralateral forelimb[13].

In the present study, we tested whether MCS as a standalone treatment is able to achieve functional recovery in a rat model of severe CCI in the forelimb area of the motor cortex. To document functional recovery, we measured the effect of MCS on forelimb function and metabolic brain activity.

MATERIALS & METHODS

Subjects

All animal experiments were conducted according to the directive 2010/63/EU on the protection of animals used for scientific purposes and had been approved by the local ethical committee for animal experiments at Maastricht University. Forty male Sprague-Dawley rats (Charles River, France), weighing around 400 g at the time of surgery, were housed in pairs under a reversed 12 h light/dark cycle. Housing and testing facilities were kept at a constant temperature of 22° C and a humidity of 40-60%. Animals received standard laboratory chow (Sniff, Germany) and acidified water (pH 2.3-2.7) *ad libitum*. All behavioral assessments took place during the dark phase of the reversed night-day cycle (between 7 am and 7 pm), which is the active period of the rats.

CCI induction and electrode implantation

Induction of CCI was performed as previously described in detail[13]. Shortly, a craniotomy was made above the forelimb area of the motor cortex (coordinates AP 0-3.5 mm, ML 0.5-4 mm relative to bregma) contralateral to the dominant paw, as determined by baseline performance in the Montoya staircase test. All rats received a CCI using an electromagnetically driven impactor device (Leica Impact One, Leica Biosystems, USA) with an impactor tip of 3 mm diameter, an impact depth of 5 mm and a velocity of 3 m/s. Polyurethane-isolated flat electrodes (3.4x3 mm; Medtronic, USA) with six exposed monopolar platinum/iridium contact points, were positioned on top of the CCI lesion (Fig. 1a-b) and a reference wire was anchored in the contralateral skull (coordinates AP 1.75 mm, ML 2.25 mm relative to bregma). Both the electrode and the reference wire were fixed with dental cement (Paladur; Heraeus, Germany) exposing two electrode contact pins (Multi-Contact, Switzerland) on the animal's head to allow connection to the electrical stimulator. Animals from the control group received non-functional dummy electrodes of the same size and material. After surgery, rats were left to recover for two weeks.

Motor cortex stimulation

MCS was applied daily for 2 h during a period of 31 consecutive days. Stimulation parameters were chosen based on previous studies[6, 7, 16] and consisted of a frequency of 30 Hz, 1 ms pulse width and biphasic constant current set at 50% of the current that evoked a motor threshold. MCS was delivered through a digital stimulator (DS8000, World Precision Instruments, Germany) while rats stayed individually in stimulation chambers (width 28 cm, depth 50 cm, height 47.8 cm) under conditions similar to their homecage.

Control animals underwent the same procedure without any current being delivered.

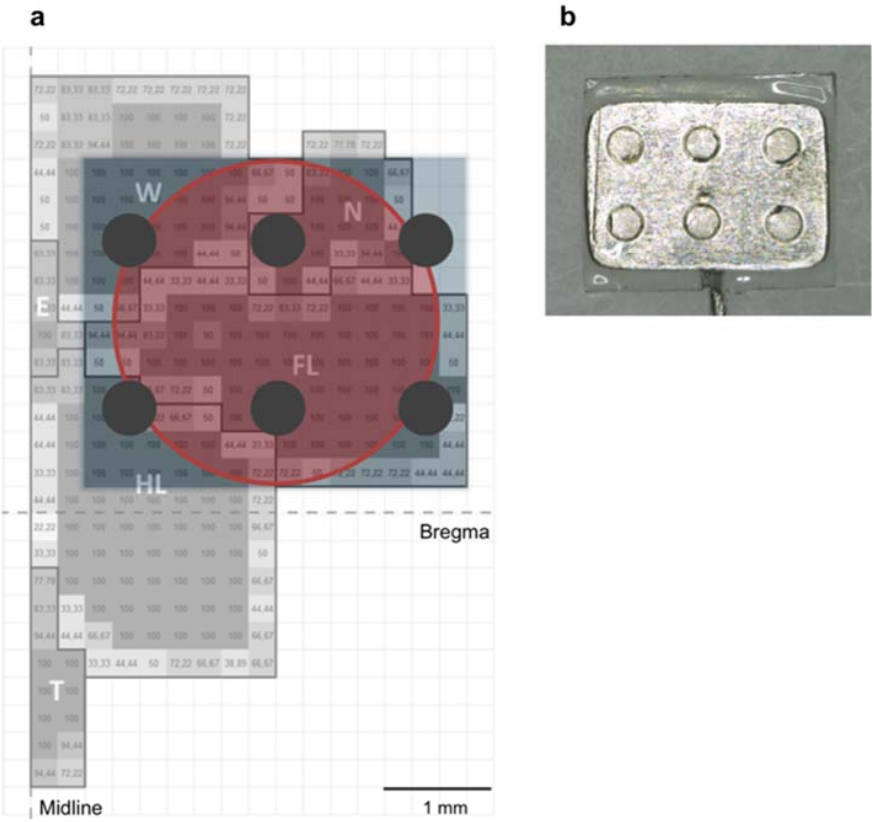


Figure 1: Location of the CCI and electrode placement. A schematic representation of the somatotopic organization of the rat motor cortex is shown, modified from a previous publication[42] (**a**). The CCI targeted large parts of the forelimb area (FL; CCI area represented by a red circle), followed by electrode placement on top of the lesion (blue square). Dark circles represent the monopolar electrode contacts that delivered the current. On the right, a scaled-down picture of the electrode lead is shown (real size 3.4x3 mm; **b**).

Behavioral testing

Montoya staircase test

Reaching and grasping abilities of both forelimbs were assessed with the Montoya staircase test. In short, rats have to retrieve sucrose pellets (Test Diet, USA) lying on each step of a staircase located on the left and on the right hand side of a platform inside a narrow translucent box[17]. All steps, except the highest one, were baited with three sucrose pellets. Rats were habituated to the staircase boxes and then trained daily until they retrieved a minimum of 55% of the pellets from at least one of the staircases[18]. Testing sessions lasted 15 minutes and took place on two consecutive days, twice daily with a minimal inter-trial interval of three hours resulting in a total of four testing sessions per timepoint of behavioral assessment. During the training and testing period, rats were food deprived to 85-90% of their free-feeding weight to increase their motivation for pellet reaching. Data are presented as the total number of eaten pellets for the impaired and healthy forelimb separately, as well as using a *difference score* defined as the score of the healthy forelimb subtracted from the score of the impaired forelimb. Behavioral testing in the Montoya staircase test was performed at three timepoints: Before CCI, two weeks after CCI and after the four weeks of (sham) MCS.

Cylinder test

Paw use during vertical exploration was measured by the cylinder test as described previously[13]. Rats were transferred to Perspex cylinders on an illuminated platform and recorded from above during 10 minutes (GoPro Hero 4, GoPro, USA), while they explored the cylinder by rearing and leaning against the wall. Based on the video footage, twenty wall contacts were scored and used for

analysis. Wall contacts occurred by the individual paws ('impaired', 'healthy') or by both paws simultaneously ('both'). Data are presented as the percentage of the wall contacts with either the impaired or the healthy forelimb relative to the total twenty wall contacts. In addition, the *difference score* of the percentages is shown to visualize asymmetry between both forelimbs. The difference score was calculated by setting the number of wall contacts of the individual forelimbs after CCI relative to their corresponding number of wall contacts at baseline, followed by subtracting the score of the healthy limb from the score of impaired limb[19]. Behavioral testing using the cylinder test was performed at four timepoints: Before CCI, two weeks after CCI, two weeks after initiation of (sham) MCS and lastly after four weeks of (sham) MCS.

Functional imaging

Distribution of glucose as an indirect indicator of metabolic central activity was visualized *in vivo* using a μPET scanner (μPET Focus, Siemens, the Netherlands). Rats were anesthetized with Isoflurane and received 10-20 mBq ^{18}F-fluorodesoxyglucose (FDG; GE Healthcare, the Netherlands) intravenously, immediately followed by scanning the entire region of the brain during 30 minutes. A static image was reconstructed using OSEM2D. μPET scanning took place twice per animal; on the first day rats from the MCS group were scanned with stimulation off, whereas stimulation was switched on 10 minutes before scanning and during the 30 minutes of scanning on the second day to visualize potential acute effects of MCS with subsequent autoradiography. Control animals were scanned twice under the same conditions without any stimulation being delivered. The lesion volume was calculated by delineation of the virtual

CCI area in horizontal brain slices of the static image using pmod image analysis software (pmod Technologies, Switzerland).

Autoradiography was performed after the second μPET scan to visualize FDG distribution at a higher spatial resolution. After transcardial perfusion with 4% paraformaldehyde, brains were frozen and cut into 50 μm thick sections. Autoradiography phosphor plates (GE Healthcare, the Netherlands) were exposed to the frozen brain sections during approximately 2 hours and read for each animal with a Typhoon FLA7000IP scanner (GE Healthcare, the Netherlands). For each animal, the injured and healthy hemispheres of three sections at a comparable bregma level were delineated and intensity of FDG signal was measured using ImageQuant TL software (GE Healthcare, the Netherlands). Signal intensity (arbitrary units, a.u.) was corrected for the injected amount of MBq, animal weight, the time from injections until exposure to the autoradiography plate and the duration of exposure on the autoradiography plate. To correct for inter-individual fluctuations, intensity values were expressed by dividing the radioactive counts measured within the lesioned hemisphere by the radioactive counts measured within the healthy hemisphere for each individual animal. In addition, intensity values for each hemisphere are shown separately.

Statistical analysis

Data are presented as mean ± standard error of the mean and were analyzed with repeated-measures ANOVA (SPSS 20, IBM, US) with time (baseline, post CCI and post stimulation) as within-subjects factor and group (MCS and control) as between-subjects factor. Imaging data were analyzed with one-way ANOVA and p-values below 0.05 were considered significant. Outliers identified by SPSS

as “extreme values” were excluded. Furthermore, animals that lost their electrodes during the course of the experiment and therefore could not undergo stimulation during 31 days were excluded from the analysis.

RESULTS

Chronic motor cortex stimulation failed to recover grasping skills and paw asymmetry during vertical exploration behavior

The Montoya staircase test was used to assess the recovery of reaching and grasping skills that were impaired by severe CCI. Repeated measures ANOVA revealed that the number of eaten pellets with the healthy paw increased after CCI for both non-stimulated rats and rats that received MCS [$F(2, 44) = 14.27$, $p < 0.000$; Fig. 2a] without any significant difference between rats that received MCS and rats that received sham stimulation. The number of eaten pellets with the impaired paw decreased after CCI [$F(2, 46) = 85.18$, $p < 0.000$; Fig. 2b] with a significant difference between the groups at all timepoints [$F(1, 23) = 6,18$, $p < 0.05$]. However, this difference between groups remained equal at all timepoints (2.41, 2.45 and 2.87 pellets), which indicates a lack of functional improvement caused by MCS. The *difference score* was calculated by subtracting the number of pellets eaten with the healthy paw from the number of pellets eaten with the impaired paw. Using this score, a decline over time was shown implying worse pellet retrieval with the impaired paw [$F(2, 46) = 71.44$, $p < 0.000$; Fig. 2c]. Again, MCS treatment did not affect the number of pellets eaten after CCI. These results indicate that MCS did not have any effect on the improvement of grasping and reaching behavior after CCI.

The cylinder test was performed to measure vertical exploration behavior with the individual forelimbs. After CCI, all animals showed an increased reliance on

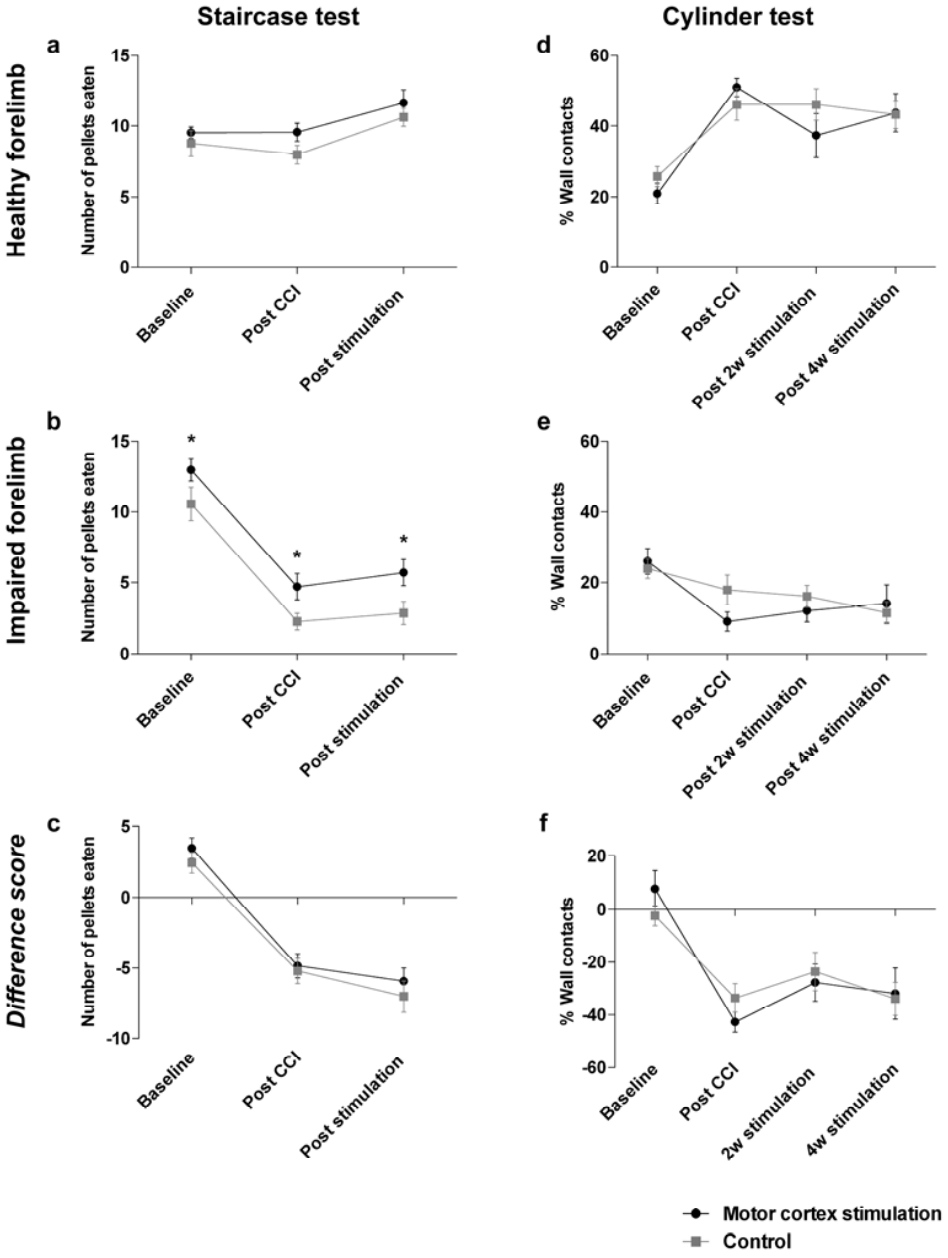


Figure 2: Chronic motor cortex stimulation after a unilateral CCI neither recovered reaching and grasping skills nor paw use during vertical exploration. CCI to the forelimb area of the motor cortex did not affect pellet retrieval with the healthy paw (**a**) whereas it resulted in significantly less pellet retrieval with the impaired paw (**b**), as measured in the Montoya staircase test.

This behavioral impairment was unaltered by MCS. Concerning the impaired paw, an overall significant difference between groups at all time points was detected, which was unrelated to the application of MCS. The *difference score* also reflected the tendency of less pellets eaten with the impaired paw (**c**).
Use of the cylinder test showed that after a unilateral CCI rats increased the use of their ipsilateral paw to lean against the cylinder walls (**d**), while neglecting the paw contralateral to the CCI lesion (**e**). The *difference score* also show a decreased use of the impaired forelimb to lean against the cylinder wall (**f**). This effect of CCI on motor impairment was not restored by MCS.

the healthy paw to lean against the cylinder wall [$F(3, 69) = 19.30$, $p < 0.000$; Fig. 2d] at the cost of using their impaired paw [$F(3, 66) = 6.77$, $p < 0.000$; Fig. 2e]. However, MCS treatment did not restore usage of the impaired paw. Analysis of the *difference score* showed a stronger asymmetry in paw use after CCI [$F(1.8, 66) = 19.73$, $p < 0.000$; Fig. 2f]. Still, treatment with MCS could not resolve this asymmetry in wall contacts between both forelimbs. Taken together, the results obtained with the cylinder test indicated that treatment with MCS could not restore forelimb use for vertical exploration behavior.

Lesion volume and glucose metabolism in the lesioned hemisphere did not change despite motor cortex stimulation

Delineation of the lesion area in the reconstructed µPET images was performed to estimate of the amount of histological damage that was still present after chronic application of MCS (Fig. 3a). The lesion size of animals that received chronic MCS was not significantly different from the lesion size of non-stimulated controls [$F(1,8) = 2.98$, $p > 0.05$; Fig. 3b], which indicated a lack in overt tissue recovery.

Imaging the distribution of FDG in brain slices was performed to measure functional recovery that may have occurred directly at the tissue level (Fig. 3c1-

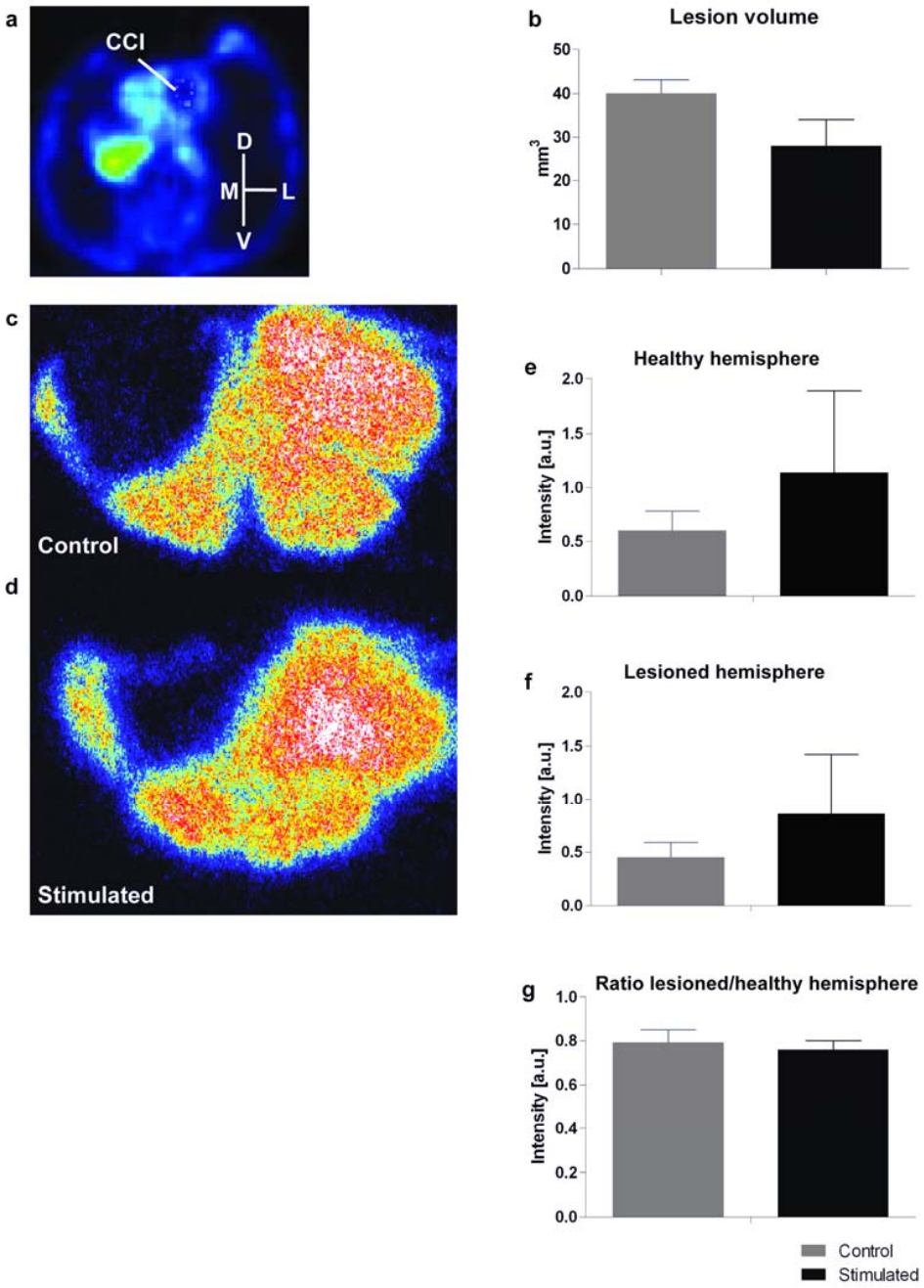


Figure 3: Neither lesion volume nor glucose distribution were affected by chronic motor cortex stimulation. A representative μPET image is shown in a horizontal plane, used to delineate the lesion area (white arrow, **a**). Chronic

MCS of the lesion area did not significantly change the size of the CCI lesion (**b**). Representative autoradiography images are shown of a control rat (**c1**) and a rat that received MCS (**c2**). The ratio of radioactive signal intensity (arbitrary units, a.u.) within the lesioned hemisphere relative to the healthy hemisphere did not differ between control rats and rats that received MCS (**d**), similar to the individual intensities for the lesioned and healthy hemisphere separately (**e-f**). M = medial, L = lateral, D = dorsal, V = ventral, MCS = motor cortex stimulation.

2). The ratio of intensity values between the healthy and the lesioned hemisphere did not differ between the two groups [$F(1,7) = 0.12$, $p > .05$; Fig. 3d]. When comparing the amount of radioactive counts for each hemisphere separately, no difference was detected between the groups, either [lesioned hemisphere: $F(1,7) = 0.64$, $p > .05$; healthy hemisphere: $F(1,7) = 0.61$, $p > .05$; Fig. 3e-f], meaning that MCS treatment did not influence FDG distribution.

DISCUSSION

Motor impairments are among the most debilitating consequences of stroke or TBI and have a strong impact on a patient's activities of daily living. Electrical stimulation of the motor cortex did cause functional improvements in animal models of stroke and the ability of MCS to improve motor recovery in humans is currently under investigation[5, 20]. In rodent models of stroke, functional recovery was measurable as improved limb placement in response to a sensory cue, grasping or balance[21, 22]. An increased formation of new blood vessels and dendritic sprouting has been found in addition to more NeuN-positive cells in the ischemic cortex[21, 23]. Although stroke and TBI have a different cause, both result in strikingly similar effects on the cellular level, such as excitotoxicity, oxidative stress and inflammatory responses[24]; therefore treatments effective in animal models of stroke might also be applicable to animal models of TBI.

In the current study the potential of chronic MCS to achieve functional recovery after a cortical lesion was assessed. To induce the cortical lesion, rats received a CCI on the forelimb area of the motor cortex contralateral to the preferred limb. MCS was applied to freely moving rats during a period of 31 consecutive days and different aspects of forelimb function were assessed before and after the stimulation period. We measured fine motor skills with the Montoya staircase test and the cylinder test and both tests are sensitive ways to detect asymmetrical paw use after a cortical lesion[13, 18, 25]. Furthermore, comparable tests were used in studies where a regenerative effect of MCS was detected[12, 26]. In addition, we visualized the distribution of a radioactive glucose analog, FDG, to detect potential changes in brain metabolism after chronic MCS.

In line with previous research, we found that severe CCI created long-lasting motor impairments specific to the contralateral forelimb[13], which could be detected for up to eight weeks after the insult. Motor impairments observed in patients with a cortical lesion usually have a chronic course; therefore an animal model, which retains a motor impairment over time, is essential.

In the current study, we wanted to test the therapeutic potential of MCS as a standalone treatment administered in a homecage-environment. This procedure was different compared to previous research, where MCS was always administered together with physical rehabilitation training. In the current study, MCS as an independent treatment did not cause any improvement of motor impairments. After CCI, reaching and grasping skills with the impaired limb were equally affected in rats that received MCS compared to non-stimulated control rats. Also, after CCI rats predominantly used their healthy forelimb during vertical exploration and usage of the other, impaired, forelimb was not restored after chronic MCS.

In line with these findings, no changes in either lesion size or FDG distribution were detected after the application of MCS. An increase in FDG distribution after MCS might have been an indirect measure of restored tissue functionality, whereas the absence of any MCS-induced change indicates a lack of treatment effect on energy metabolism.

A few studies on TBI in rats have reported a therapeutic effect of MCS[27-29]. In those studies, MCS was co-administered with behavioral rehabilitation training during a period between two[27, 28] and nine weeks[29]. In all studies, an improvement of forelimb function at the end of the stimulation period was reported[27-29], additionally to an increase of the cortical area for wrist movement representation[29]. However, the TBI lesions created in these studies were either considerably smaller in size or more diffuse compared to our lesions, which may increase the likelihood of regeneration. Also, in these studies the implanted electrodes did not only cover the damaged cortex, but also stimulated spared cortical regions, which could facilitate re-mapping of lost functions onto the surrounding cortex. Lastly, MCS was always administered together with daily behavioral training, which represents the clinical situation, but does not provide any information about the therapeutic effect of MCS on its own.

Three factors might explain why MCS did not induce functional regeneration in our study. First, the our CCI lesion was very severe and damaged the corpus callosum and parts of the striatum in addition to the entire forelimb area of the motor cortex. We chose for that specific set of parameters to induce a CCI since a previous study by our group confirmed the presence of long-lasting behavioral impairments with this specific type of CCI[13]. In studies using milder CCI lesions in the motor cortex spontaneous recovery of motor functions was measured, starting already at 2 weeks after the lesion[14, 30, 31]. Taken together, behavioral

improvement might be more likely after a milder lesion since the area to regenerate is smaller and surrounding cortical regions are spared which might allow functional re-mapping of the lost area.

Second, numerous studies, where MCS caused functional improvements, chose to not stimulate the damaged brain area directly, but positioned the electrodes in the lesion penumbra[6, 8, 22, 26, 29, 32, 33]. Electrical stimulation of the spared surrounding cortex may allow plasticity processes in contrast to the stimulation of a damaged brain region that develops into a large morphological cavity[13]. In a number of studies the therapeutic effect of MCS was not explained by tissue restoration at the lesion side, but by remapping of the lost functions onto the spared cortex around the lesion[29, 32, 34]. In the current study, the lesion area was stimulated directly to clarify whether the influence of MCS on NSPCs found in an earlier experiment[16] could rescue damaged cortical tissue. In a previous study we showed an increased amount of NSPCs at the stimulated cortex, which could have been migrated from the SVZ[16]. Under *in vitro* conditions, electrical fields can induce migration of NSPCs towards the current source, a process known as electrotaxis[35-39], and *in vitro* electrotaxis is a widely proven phenomenon for different cells types, including NSPCs [37-39]. Also, stimulation of the striatal penumbra after ischemic stroke in rats has been shown to increase the number of proliferating cells in the vicinity of the electrode and this finding co-occurred with a decreased lesion size and behavioral improvement[12]. However, another study using a non-invasive form of cortical stimulation applied in healthy rats did not observe any directional migration of labeled neural stem cells in response to an electrical field[40]. The authors concluded that accumulation of neural stem cells at the stimulated cortical area is rather due to local cell proliferation and not to cell migration from neurogenic regions[40]. These contrary

findings indicate, that *in vivo* electrotaxis first needs to be reliably demonstrated, before its role in stimulation-induced motor recovery can be investigated.

Third, in previous studies MCS has been delivered while the animals underwent rehabilitative therapy in the form of repetitive reaching with the impaired forelimb[29, 32, 34]. Probably an additional behavioral stimulus, that is stronger and more specific to the impaired forelimb than mere locomotion in a homecage, is necessary to cause improvement through MCS. Pairing of MCS with rehabilitation therapy may result in a synergistic effect and there are few reports showing a therapeutic effect of MCS without any additional intervention[6, 26, 41].

In this study, MCS as a standalone treatment did not cause functional recovery after severe cortical damage in rats. Future studies should clarify the effect of MCS at the single cell level and refine the animal model and stimulation paradigm accordingly.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from Hasselt University (BOF11603BOFMO) to SH and a grant from the Hersenstichting Nederland and Medtronic, US, (A1196117) to YT.

No competing financial interests exist.

REFERENCES

1. Walker, W.C. and Pickett, T.C. (2007). Motor impairment after severe traumatic brain injury: A longitudinal multicenter study. Journal of rehabilitation research and development 44, 975-982.

2. Parker, V.M., Wade, D.T. and Langton Hewer, R. (1986). Loss of arm function after stroke: measurement, frequency, and recovery. International rehabilitation medicine 8, 69-73.

3. Brown, J.A., Lutsep, H.L., Weinand, M. and Cramer, S.C. (2006). Motor cortex stimulation for the enhancement of recovery from stroke: a prospective, multicenter safety study. Neurosurgery 58, 464-473.

4. Levy, R., Ruland, S., Weinand, M., Lowry, D., Dafer, R. and Bakay, R. (2008). Cortical stimulation for the rehabilitation of patients with hemiparetic stroke: a multicenter feasibility study of safety and efficacy. Journal of neurosurgery 108, 707-714.

5. Levy, R.M., Harvey, R.L., Kissela, B.M., Winstein, C.J., Lutsep, H.L., Parrish, T.B., Cramer, S.C. and Venkatesan, L. (2015). Epidural Electrical Stimulation for Stroke Rehabilitation: Results of the Prospective, Multicenter, Randomized, Single-Blinded Everest Trial. Neurorehabilitation and neural repair.

6. Adkins, D.L., Campos, P., Quach, D., Borromeo, M., Schallert, K. and Jones, T.A. (2006). Epidural cortical stimulation enhances motor function after sensorimotor cortical infarcts in rats. Experimental neurology 200, 356-370.

7. Baba, T., Kameda, M., Yasuhara, T., Morimoto, T., Kondo, A., Shingo, T., Tajiri, N., Wang, F., Miyoshi, Y., Borlongan, C.V., Matsumae, M. and Date, I. (2009). Electrical stimulation of the cerebral cortex exerts antiapoptotic, angiogenic, and anti-inflammatory effects in ischemic stroke rats through

phosphoinositide 3-kinase/Akt signaling pathway. Stroke; a journal of cerebral circulation 40, e598-605.

8. Plautz, E.J., Barbay, S., Frost, S.B., Friel, K.M., Dancause, N., Zoubina, E.V., Stowe, A.M., Quaney, B.M. and Nudo, R.J. (2003). Post-infarct cortical plasticity and behavioral recovery using concurrent cortical stimulation and rehabilitative training: a feasibility study in primates. Neurological research 25, 801-810.

9. Jahanshahi, A., Schonfeld, L., Janssen, M.L., Hescham, S., Kocabicak, E., Steinbusch, H.W., van Overbeeke, J.J. and Temel, Y. (2013). Electrical stimulation of the motor cortex enhances progenitor cell migration in the adult rat brain. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale.

10. Jahanshahi, A., Schonfeld, L.M., Lemmens, E., Hendrix, S. and Temel, Y. (2013). In Vitro and In Vivo Neuronal Electrotaxis: A Potential Mechanism for Restoration? Molecular neurobiology.

11. Rueger, M.A., Keuters, M.H., Walberer, M., Braun, R., Klein, R., Sparing, R., Fink, G.R., Graf, R. and Schroeter, M. (2012). Multi-session transcranial direct current stimulation (tDCS) elicits inflammatory and regenerative processes in the rat brain. PLoS One 7, e43776.

12. Morimoto, T., Yasuhara, T., Kameda, M., Baba, T., Kuramoto, S., Kondo, A., Takahashi, K., Tajiri, N., Wang, F., Meng, J., Ji, Y.W., Kadota, T., Maruo, T., Kinugasa, K., Miyoshi, Y., Shingo, T., Borlongan, C.V. and Date, I. (2011). Striatal stimulation nurtures endogenous neurogenesis and angiogenesis in chronic-phase ischemic stroke rats. Cell Transplant 20, 1049-1064.

13. Schönfeld, L.M., Jahanshahi, A., Lemmens, E., Schipper, S., Dooley, D., Joosten, E.A., Temel, Y. and Hendrix, S. (2016). Long-term motor deficits after

controlled cortical impact in rats can be detected by fine motor skill tests but not by automated gait analysis (under revision).

14. Nishibe, M., Barbay, S., Guggenmos, D. and Nudo, R.J. (2010). Reorganization of motor cortex after controlled cortical impact in rats and implications for functional recovery. Journal of neurotrauma 27, 2221-2232.

15. Starkey, M.L., Bleul, C., Zorner, B., Lindau, N.T., Mueggler, T., Rudin, M. and Schwab, M.E. (2012). Back seat driving: hindlimb corticospinal neurons assume forelimb control following ischaemic stroke. Brain : a journal of neurology 135, 3265-3281.

16. Jahanshahi, A., Schonfeld, L., Janssen, M.L., Hescham, S., Kocabicak, E., Steinbusch, H.W., van Overbeeke, J.J. and Temel, Y. (2013). Electrical stimulation of the motor cortex enhances progenitor cell migration in the adult rat brain. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale 231, 165-177.

17. Montoya, C.P., Campbell-Hope, L.J., Pemberton, K.D. and Dunnett, S.B. (1991). The "staircase test": a measure of independent forelimb reaching and grasping abilities in rats. Journal of neuroscience methods 36, 219-228.

18. Windle, V., Szymanska, A., Granter-Button, S., White, C., Buist, R., Peeling, J. and Corbett, D. (2006). An analysis of four different methods of producing focal cerebral ischemia with endothelin-1 in the rat. Experimental neurology 201, 324-334.

19. Harris, N.G., Mironova, Y.A., Hovda, D.A. and Sutton, R.L. (2010). Chondroitinase ABC enhances pericontusion axonal sprouting but does not confer robust improvements in behavioral recovery. Journal of neurotrauma 27, 1971-1982.

20. Shin, S.S., Dixon, C.E., Okonkwo, D.O. and Richardson, R.M. (2014). Neurostimulation for traumatic brain injury. Journal of neurosurgery 121, 1219-1231.

21. Cheng, X., Li, T., Zhou, H., Zhang, Q., Tan, J., Gao, W., Wang, J., Li, C. and Duan, Y.Y. (2012). Cortical electrical stimulation with varied low frequencies promotes functional recovery and brain remodeling in a rat model of ischemia. Brain research bulletin 89, 124-132.

22. Moon, S.K., Shin, Y.I., Kim, H.I., Kim, H., Lee, J.O. and Lee, M.C. (2009). Effect of prolonged cortical stimulation differs with size of infarct after sensorimotor cortical lesions in rats. Neuroscience letters 460, 152-155.

23. Kang, C., Yang, C.Y., Kim, J.H., Moon, S.K., Lee, S., Park, S.A., Han, E.H. and Zhang, L.Q. (2013). The effect of continuous epidural electrical stimulation on neuronal proliferation in cerebral ischemic rats. Annals of rehabilitation medicine 37, 301-310.

24. Lopez, M.S., Dempsey, R.J. and Vemuganti, R. (2015). Resveratrol neuroprotection in stroke and traumatic CNS injury. Neurochemistry international 89, 75-82.

25. MacLellan, C.L., Langdon, K.D., Botsford, A., Butt, S. and Corbett, D. (2013). A model of persistent learned nonuse following focal ischemia in rats. Neurorehabilitation and neural repair 27, 900-907.

26. Zhou, Q., Zhang, Q., Zhao, X., Duan, Y.Y., Lu, Y., Li, C. and Li, T. (2010). Cortical electrical stimulation alone enhances functional recovery and dendritic structures after focal cerebral ischemia in rats. Brain research 1311, 148-157.

27. Yoon, Y.S., Yu, K.P., Kim, H., Kim, H.I., Kwak, S.H. and Kim, B.O. (2012). The effect of electric cortical stimulation after focal traumatic brain injury in rats. Annals of rehabilitation medicine 36, 596-608.

28. Yoon, Y.S., Cho, K.H., Kim, E.S., Lee, M.S. and Lee, K.J. (2015). Effect of Epidural Electrical Stimulation and Repetitive Transcranial Magnetic Stimulation in Rats With Diffuse Traumatic Brain Injury. Annals of rehabilitation medicine 39, 416-424.

29. Jefferson, S.C., Clayton, E.R., Donlan, N.A., Kozlowski, D.A., Jones, T.A. and Adkins, D.L. (2015). Cortical Stimulation Concurrent With Skilled Motor Training Improves Forelimb Function and Enhances Motor Cortical Reorganization Following Controlled Cortical Impact. Neurorehabilitation and neural repair.

30. Goffus, A.M., Anderson, G.D. and Hoane, M. (2010). Sustained delivery of nicotinamide limits cortical injury and improves functional recovery following traumatic brain injury. Oxidative medicine and cellular longevity 3, 145-152.

31. Shijo, K., Ghavim, S., Harris, N.G., Hovda, D.A. and Sutton, R.L. (2015). Glucose administration after traumatic brain injury exerts some benefits and no adverse effects on behavioral and histological outcomes. Brain research 1614, 94-104.

32. Boychuk, J.A., Adkins, D.L. and Kleim, J.A. (2011). Distributed versus focal cortical stimulation to enhance motor function and motor map plasticity in a rodent model of ischemia. Neurorehabilitation and neural repair 25, 88-97.

33. O'Bryant, A.J., Adkins, D.L., Sitko, A.A., Combs, H.L., Nordquist, S.K. and Jones, T.A. (2014). Enduring Poststroke Motor Functional Improvements by a Well-Timed Combination of Motor Rehabilitative Training and Cortical Stimulation in Rats. Neurorehabilitation and neural repair.

34. Teskey, G.C., Flynn, C., Goertzen, C.D., Monfils, M.H. and Young, N.A. (2003). Cortical stimulation improves skilled forelimb use following a focal ischemic infarct in the rat. Neurological research 25, 794-800.

35. McCaig, C.D., Rajnicek, A.M., Song, B. and Zhao, M. (2005). Controlling cell behavior electrically: current views and future potential. Physiological reviews 85, 943-978.

36. Babona-Pilipos, R., Droujinine, I.A., Popovic, M.R. and Morshead, C.M. (2011). Adult subependymal neural precursors, but not differentiated cells, undergo rapid cathodal migration in the presence of direct current electric fields. PLoS One 6, e23808.

37. Babona-Pilipos, R., Pritchard-Oh, A., Popovic, M.R. and Morshead, C.M. (2015). Biphasic monopolar electrical stimulation induces rapid and directed galvanotaxis in adult subependymal neural precursors. Stem cell research & therapy 6, 67.

38. Feng, J.F., Liu, J., Zhang, X.Z., Zhang, L., Jiang, J.Y., Nolta, J. and Zhao, M. (2012). Guided migration of neural stem cells derived from human embryonic stem cells by an electric field. Stem cells 30, 349-355.

39. Liu, J., Zhu, B., Zhang, G., Wang, J., Tian, W., Ju, G., Wei, X. and Song, B. (2015). Electric signals regulate directional migration of ventral midbrain derived dopaminergic neural progenitor cells via Wnt/GSK3beta signaling. Experimental neurology 263, 113-121.

40. Keuters, M.H., Aswendt, M., Tennstaedt, A., Wiedermann, D., Pikhovych, A., Rotthues, S., Fink, G.R., Schroeter, M., Hoehn, M. and Rueger, M.A. (2015). Transcranial direct current stimulation promotes the mobility of engrafted NSCs in the rat brain. NMR in biomedicine 28, 231-239.

41. Adkins-Muir, D.L. and Jones, T.A. (2003). Cortical electrical stimulation combined with rehabilitative training: enhanced functional recovery and dendritic plasticity following focal cortical ischemia in rats. Neurological research 25, 780-788.

42. Fonoff, E.T., Pereira, J.F., Jr., Camargo, L.V., Dale, C.S., Pagano, R.L., Ballester, G. and Teixeira, M.J. (2009). Functional mapping of the motor cortex of the rat using transdural electrical stimulation. Behavioural brain research 202, 138-141.

CHAPTER 7

GENERAL CONCLUSIONS

Motor cortex stimulation has a profound effect on neuronal cells

Electrical fields can induce a directed migration of various types of cells, which is called electrotaxis[1, 2]. Electrotaxis may occur through activation of various pathways that eventually lead to the reorganization of the cytoskeleton and cell migration. In Chapter 2, these pathways are reviewed in depth and consist for example of an asymmetric relocation of membrane-bound receptors, changes in intracellular ion concentration and pH, enzyme activation and polarization of the Golgi apparatus[3].

The presence of electrotaxis *in vitro* raised our interest to investigate whether electrotaxis was also inducible *in vivo,* in the central nervous system. Even if electrical fields *in vivo* would activate the same pathways that are involved in electrotaxis *in vitro*, the environment in which cells would migrate is very different *in vivo*. The migrating cells would be embedded in brain tissue that consists of complex cellular networks and the presence of surrounding cells and the extracellular matrix might either facilitate or complicate electrotaxis. To clarify the effects of electrical stimulation *in vivo*, we investigated in Chapter 3 whether electrotaxis could occur in the brain of healthy adult rats[4]. After a chronic period of motor cortex stimulation (MCS), we found an increase in cell proliferation in the subventricular zone (SVZ) that co-occurred with more neural stem and progenitor cells (NSPCs) as well as newborn neurons in the cortex underlying the MCS electrodes. Based on these results we suggested a migration of NSPCs from the SVZ towards the electrode, thus a potential existence of electrotaxis *in vivo*[5]. However, we could not selectively label NSPCs in the SVZ, but rather used the systemically applied proliferation marker bromodeoxyuridine (BrdU). Therefore, it was not possible to visually prove NSPCs migration from the SVZ towards the MCS electrode. Since BrdU labels

proliferating cells throughout the entire brain, we cannot rule out that the NSPCs found underneath the electrode, had already been present in the cortex and proliferated locally upon stimulation. In a recent study labeled neural stem cells were transplanted in the vicinity of the SVZ in healthy rats followed by repeated non-invasive cortical stimulation[6]. Stimulation increased the overall migration of the transplanted cells, but did not direct them towards the stimulation electrode[6]. The latter findings stress the need for additional studies to clarify the effect of electrical stimulation on the directed migration of endogenous (as opposed to transplanted) neural stem cells and thereby confirm or rule out the presence of electrotaxis *in vivo*. Still, regardless of their source, an increased number of NSPCs at the stimulation side has a promising therapeutic potential.

Rodents can model human pathology and choosing the right tests is essential

To develop novel therapies, animal models are frequently used with functional impairments that resemble neurological symptoms observed in humans. In Chapter 4, we used a standardized rat model of TBI that resulted in long-lasting unilateral forelimb impairment, comparable to a hemiparesis observed in patients with a cortical damage. The unilateral forelimb impairment was induced by mechanical damage to the forelimb area of the rat motor cortex using a controlled cortical impact (CCI). To describe the resulting impairment, we chose different behavioral tests that measure rodent motor functions. CCI to the motor cortex resulted in severe tissue damage and deficits in different aspects of forelimb function, like fine motor skills, keeping balance and tactile recognition, whereas gait-related functions remained intact[7].

If one chooses to conduct animal experiments using rodents, selecting the right behavioral testing methods is of uttermost importance in terms of ethical reasons and the quality of the resulting data. In Chapter 5, we reviewed and critically evaluated a large number of behavioral tests that are used to assess motor functions in rodent models of stroke and TBI. In addition, we suggest approaches how to standardize data presentation, in order to make results comparable between different research groups, and we present recommendations, that should help to choose the right behavioral tests when designing an animal experiment.

Can MCS induce recovery after severe TBI?

In Chapter 6, we tested the potential of MCS as a standalone treatment to achieve functional recovery after cortical damage. Again, CCI to the forelimb area of the motor cortex was induced in rats, followed by chronic MCS of the damaged cortex. We applied the same stimulation paradigm as in our previous study, which resulted in an increased number of neuronal cells in the stimulated cortex[4]. More neuronal cells at the stimulated lesion side might either cause tissue restoration or aid in the protection of the surrounding cortical tissue from further secondary damage. CCI induced severe morphological damage that resulted in fine motor skill deficits of the contralateral forelimb, which stayed constant throughout the entire duration of the study. However, MCS treatment neither caused measurable improvements of forelimb function, nor any functional recovery at the tissue level. In Chapter 6, we discussed potential components, why MCS might not have been effective to restore functions after a severe CCI, focusing on lesion size, electrode location and the presence of additional therapies. A recent study that did report MCS-induced behavioral

recovery after CCI in rats[8] differed from our study in these latter three components. In that study, the CCI lesion was milder, stimulation electrodes were placed onto spared cortical tissue and MCS treatment was combined with an additional therapy. Future experiments should be conducted to refine the stimulation paradigm and severity of the cortical lesion, but our findings question the effectiveness of MCS to restore function after severe TBI.

Concluding remarks

A number of conclusions can be drawn from the data presented in this thesis.

First, MCS applied in healthy adult rats significantly enhances NSPC proliferation in a neurogenic area close to the stimulation electrode, and increases the number of NSPCs together with their differentiation in the stimulated cortex. Given the increased number of NSPCs found underneath the stimulation electrode, it may be suggested that the electrical field had induced a directed migration of NSPCs from their side of origin towards the cortex. These cellular effects induced by MCS may be relevant in achieving functional recovery after brain damage.

Second, by creating a severe CCI lesion in the forelimb area of the motor cortex, we could induce a long-lasting motor impairment in rats that mimics hemiparesis, which is often observed after cortical damage in patients. This unilateral motor impairment was documented by using specific rodent motor tests. Both modeling and measuring motor impairments in rodents are essential processes towards the development of new and clinically relevant therapies that restore lost functions.

Third, applying MCS to the damaged motor cortex did not lead to functional recovery after severe CCI in rats. We had shown previously that MCS in healthy

rats could influence NSPC proliferation, differentiation and potentially migration, which fueled our interest to investigate the role of these processes in a rat model of cortical damage. Although we did not detect any functional improvement in our last study, the experimental design should be refined before the therapeutic role of MCS in TBI can be ruled out. Therefore, future experiments are needed to determine under which conditions and for which patient population MCS may be a successful therapeutic approach. With this thesis, we established a framework of what is feasible – and what is not.

REFERENCES

1. McCaig, C.D., Rajnicek, A.M., Song, B. and Zhao, M. (2005). Controlling cell behavior electrically: current views and future potential. Physiological reviews 85, 943-978.

2. Cortese, B., Palama, I.E., D'Amone, S. and Gigli, G. (2014). Influence of electrotaxis on cell behaviour. Integrative biology : quantitative biosciences from nano to macro 6, 817-830.

3. Jahanshahi, A., Schonfeld, L.M., Lemmens, E., Hendrix, S. and Temel, Y. (2013). In Vitro and In Vivo Neuronal Electrotaxis: A Potential Mechanism for Restoration? Molecular neurobiology.

4. Jahanshahi, A., Schonfeld, L., Janssen, M.L., Hescham, S., Kocabicak, E., Steinbusch, H.W., van Overbeeke, J.J. and Temel, Y. (2013). Electrical stimulation of the motor cortex enhances progenitor cell migration in the adult rat brain. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale.

5. Jahanshahi, A., Schonfeld, L., Janssen, M.L., Hescham, S., Kocabicak, E., Steinbusch, H.W., van Overbeeke, J.J. and Temel, Y. (2013). Electrical stimulation of the motor cortex enhances progenitor cell migration in the adult rat brain. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale 231, 165-177.

6. Keuters, M.H., Aswendt, M., Tennstaedt, A., Wiedermann, D., Pikhovych, A., Rotthues, S., Fink, G.R., Schroeter, M., Hoehn, M. and Rueger, M.A. (2015). Transcranial direct current stimulation promotes the mobility of engrafted NSCs in the rat brain. NMR in biomedicine 28, 231-239.

7. Schönfeld, L.M., Jahanshahi, A., Lemmens, E., Schipper, S., Dooley, D., Joosten, E.A., Temel, Y. and Hendrix, S. (2016). Long-term motor deficits after

controlled cortical impact in rats can be detected by fine motor skill tests but not by automated gait analysis (under revision).

8. Jefferson, S.C., Clayton, E.R., Donlan, N.A., Kozlowski, D.A., Jones, T.A. and Adkins, D.L. (2015). Cortical Stimulation Concurrent With Skilled Motor Training Improves Forelimb Function and Enhances Motor Cortical Reorganization Following Controlled Cortical Impact. Neurorehabilitation and neural repair.

VALORIZATION ADDENDUM

SOCIETAL RELEVANCE

Animal models of human pathology are still used to investigate processes impossible to be studied *in vitro*; however, the disease symptoms shown by the model organism may differ overtly from the human situation[1]. Therefore, the methods to measure functional impairment and improvement need to be sensitive and species-specific[2] to increase the translational value of the results. In other words, choosing the right behavioral tests increases the probability that preclinical results will lead to the development of new treatment opportunities. In Chapters 4 and 5, we compared and reviewed several motor tests that are well-suited to detect motor impairments in rodents.

Despite the use of prevention measures, traumatic brain injury (TBI) still affects millions of individuals worldwide leading to death or long-lasting disability in numerous cases[3]. TBI affects individuals from all age groups: young people are prone to TBI[4] due to an increased willingness of risk taking, engaging in physical activities that might lead to head injury, whereas in older people falls occur frequently[5] due to problems with balance and coordination.

Much effort is spend on improving the preventive measures to decrease the incidence of TBI; however, these measures can only decrease the number of new TBI cases, but do not help patients that already suffer from disabilities. Therefore, new treatment opportunities to restore functions after TBI are necessary. Long-lasting consequences of TBI exert a substantial strain on the patients' quality of life and take away their independence, which creates an immense socioeconomic burden[6]. Effective therapies would recover functions and independence of the patients, thereby increasing their quality of life and reducing the costs spend on patient care. Motor cortex stimulation (MCS), or in other words electrical stimulation of the motor cortex through intracranial

electrodes[7], is a more invasive intervention compared to drugs or physical rehabilitation therapy. However, if functional recovery could be achieved with MCS, it would not only benefit the present patient population, but also pave the way for research into less invasive forms of stimulation, such as transcranial direct-current stimulation (tDCS) or transcranial magnetic stimulation (TMS). In Chapter 2 and 3 we have reviewed and shown cellular changes induced by electrical stimulation, which might form a basis for MCS to be used as a therapy[8, 9].

TARGET GROUPS

The results presented in this dissertation address various target groups. First, in Chapters 4 and 5 we tested and evaluated a number of rodent motor tests based on their applicability for a certain animal model together with advantages and disadvantages. Our own experiences, described in Chapter 4, and our recommendations how to choose the best behavioral tests, described in Chapter 5, should help fellow scientists who plan research using animal models with a motor impairment and may help to reduce the number of experimental animals.

Second, research on the regenerative potential of MCS is relevant to patients suffering from motor dysfunction after cortical damage. We conducted studies using either healthy rats, to study basic mechanisms[8], or a rat model of severe TBI. The findings obtained from the latter may also be interesting to stroke research, since the lesion area and resulting symptoms are comparable between the two conditions. Third, the development of an effective treatment to improve motor functions would be a major relief to caregivers, partners or family members that are closely interacting with the patient. Finally, achieving

rehabilitation of patients and increasing their quality of life would lower the socioeconomic burden caused by healthcare and social costs.

ACTIVITIES AND PRODUCTS

We focused on two main objectives: First, the validation of a standardized rat model of TBI combined with suitable behavioral testing methods and second, elucidating the effects of MCS on the brain and testing its potential for functional regeneration after TBI. In general, a successful product is developed after positive findings have been obtained during a previous thorough testing phase. MCS as a novel approach to treat brain injury seemed promising to us after we discovered its cellular effects in healthy rats in Chapter 3. However, application of MCS in rats with severe TBI did not lead to any functional improvement in our experimental paradigm, as described in Chapter 6. We cannot exclude that the experimental design was responsible for the absence of a therapeutic effect; therefore, we cannot draw any conclusion about the use of MCS to treat brain injury at this point.

Nevertheless, we made a valuable contribution to the scientific community and lastly also to the patient population by presenting a number of rodent behavioral tests that are sensitive enough to measure motor impairment resulting from severe TBI in Chapter 4. In Chapter 5 we present numerous motor tests and we conclude with recommendations on how to choose tests to measure motor impairment in rodents. The latter should be used to refine future experimental designs to comply with the '3Rs' (replacement, reduction and refinement) of animal experiments[10] and improve the quality of the scientific outcomes. Good quality results do not only benefit the scientific community, but also society by having a high predictive validity; they may increase the likelihood that a given

therapeutic approach that has been successful in a preclinical setting, will be beneficial in the clinical population as well.

INNOVATION

Two main innovative findings resulted from the experiments conducted in the scope of this dissertation. First, we needed an animal model with a long-lasting motor impairment to test neurostimulation as a novel treatment for cortical injury. In addition, motor tests were necessary that measure the initial deficits and their improvement. In Chapter 4 we showed, which behavioral tests were sensitive enough to detect long-term forelimb motor impairment in rats and, equally important, which tests were not[11]. We detected a species-specific difference in the overt manifestation of motor impairment following TBI when using the CatWalk XT to measure gait-related deficits. Gait-related deficits after mechanical damage to the motor cortex have been reported after a corresponding lesion in mice[12, 13]. Surprisingly, we could not detect any gait-related deficits following TBI in rats, which strengthens the notion for a careful selection of the behavioral testing methods. To conclude; choosing the right behavioral testing methods will enable researchers to investigate the therapeutic potential of neurostimulation while preventing unnecessary experiments that result in the unjustifiable use of experimental animals and potentially negative data due to a suboptimal methodological design.

Second, in Chapter 3 we described the effect of MCS on cortical tissue at the cellular level[8]. In healthy rats, we found a profound effect of MCS on endogenous neural stem and progenitor cells (NSPCs) and, based on these results, we suggested a migration of these NSPCs guided by the electrical current *in vivo*. The ability of attracting endogenous stem cells to a certain area

inside the brain bears a great potential to restore damage in the central nervous system while bypassing the numerous disadvantages inherent to grafted stem cells[14].

However, we were unable to detect functional improvements caused by MCS treatment in a rat model of severe TBI, although there might be the possibility that substantial changes in the experimental setup would lead to positive results in the future. In our experiments the use of neurostimulation did not lead to functional recovery after TBI, but the profound effects of electrical current on brain cells *in vivo* as detected in Chapter 3, might not only occur when using MSC, but also in other clinically applied forms of neurostimulation, such as deep brain stimulation (DBS)[15]. With the results obtained in this dissertation we want to make researchers and clinicians aware of neuroplastic changes that may result after the delivery of electrical current to the brain. These neuroplastic changes are probably different from the intended clinical mechanism of action, but might also bear a certain therapeutic potential.

IMPLEMENTATION

From the studies described in this dissertation, we gained valuable knowledge about the methodology to conduct preclinical research on motor impairment after cortical damage. Furthermore, we investigated the mechanism of action of MCS together with its potential to restore motor impairments after cortical damage. This knowledge has and will be shared with fellow researchers, clinicians and health care organizations through presenting data at international conferences and by publishing the results in scientific journals. The findings described in this dissertation should add to the refinement of preclinical studies

on motor impairment in rodents and the potential use of neurostimulation to restore it.

REFERENCES

1. Cenci, M.A., Whishaw, I.Q. and Schallert, T. (2002). Animal models of neurological deficits: how relevant is the rat? Nature reviews. Neuroscience 3, 574-579.

2. Zorner, B., Filli, L., Starkey, M.L., Gonzenbach, R., Kasper, H., Rothlisberger, M., Bolliger, M. and Schwab, M.E. (2010). Profiling locomotor recovery: comprehensive quantification of impairments after CNS damage in rodents. Nature methods 7, 701-708.

3. Roozenbeek, B., Maas, A.I. and Menon, D.K. (2013). Changing patterns in the epidemiology of traumatic brain injury. Nature reviews. Neurology 9, 231-236.

4. Maas, A.I., Stocchetti, N. and Bullock, R. (2008). Moderate and severe traumatic brain injury in adults. Lancet neurology 7, 728-741.

5. Thompson, H.J., McCormick, W.C. and Kagan, S.H. (2006). Traumatic brain injury in older adults: epidemiology, outcomes, and future implications. Journal of the American Geriatrics Society 54, 1590-1595.

6. Ghajar, J. (2000). Traumatic brain injury. Lancet 356, 923-929.

7. Sukul, V.V. and Slavin, K.V. (2014). Deep brain and motor cortex stimulation. Current pain and headache reports 18, 427.

8. Jahanshahi, A., Schonfeld, L., Janssen, M.L., Hescham, S., Kocabicak, E., Steinbusch, H.W., van Overbeeke, J.J. and Temel, Y. (2013). Electrical stimulation of the motor cortex enhances progenitor cell migration in the adult rat brain. Experimental brain research. Experimentelle Hirnforschung. Experimentation cerebrale.

9. Jahanshahi, A., Schonfeld, L.M., Lemmens, E., Hendrix, S. and Temel, Y. (2013). In Vitro and In Vivo Neuronal Electrotaxis: A Potential Mechanism for Restoration? Molecular neurobiology.

10. Russel, W.M.S. and Burch, R.L. (1959). The Principles of Humane Experimental Technique. Methuen: London.

11. Schönfeld, L.M., Jahanshahi, A., Lemmens, E., Schipper, S., Dooley, D., Joosten, E.A., Temel, Y. and Hendrix, S. (2016). Long-term motor deficits after controlled cortical impact in rats can be detected by fine motor skill tests but not by automated gait analysis (under revision).

12. Neumann, M., Wang, Y., Kim, S., Hong, S.M., Jeng, L., Bilgen, M. and Liu, J. (2009). Assessing gait impairment following experimental traumatic brain injury in mice. Journal of neuroscience methods 176, 34-44.

13. Wang, Y., Neumann, M., Hansen, K., Hong, S.M., Kim, S., Noble-Haeusslein, L.J. and Liu, J. (2011). Fluoxetine increases hippocampal neurogenesis and induces epigenetic factors but does not improve functional recovery after traumatic brain injury. Journal of neurotrauma 28, 259-268.

14. Lindvall, O. and Kokaia, Z. (2011). Stem cell research in stroke: how far from the clinic? Stroke; a journal of cerebral circulation 42, 2369-2375.

15. Morimoto, T., Yasuhara, T., Kameda, M., Baba, T., Kuramoto, S., Kondo, A., Takahashi, K., Tajiri, N., Wang, F., Meng, J., Ji, Y.W., Kadota, T., Maruo, T., Kinugasa, K., Miyoshi, Y., Shingo, T., Borlongan, C.V. and Date, I. (2011). Striatal stimulation nurtures endogenous neurogenesis and angiogenesis in chronic-phase ischemic stroke rats. Cell Transplant 20, 1049-1064.

NEDERLANDSE SAMENVATTING

Neurostimulatie voor de behandeling van hersenletsel?

Traumatisch hersenletsel ('traumatic brain injury'; TBI) kan optreden na een slag of stoot tegen het hoofd, bijvoorbeeld tijdens een auto- of sportongeluk, maar ook na rechtstreekse schade aan de schedel. Afhankelijk van welk hersengebied de meeste schade heeft opgelopen kan TBI leiden tot een chronische verstoring van geheugen, taal of motoriek. Patienten zijn vaak niet meer in staat terug te keren naar hun voormalige dagelijkse leven en worden afhankelijk van hulpverleners. Gezien dit negatieve effect op de kwaliteit van leven is onderzoek naar nieuwe effectieve therapieën noodzakelijk. Een veelbelovende therapie is de toediening van electrische spanning op de hersenen of het ruggenmerg, oftewel neurostimulatie. In de meeste gevallen gebeurt neurostimulatie van de hersenen door het implanteren van electrodes rechtstreeks op de hersenschors of in gebieden die dieper in de hersenen liggen. De locatie van de electrodes is afhankelijk van de symptomen die behandeld moeten worden. Ondanks de regelmatige toepassing van neurostimulatie om de symptomen van bewegingsstoornissen, zoals de ziekte van Parkinson, te behandelen, zijn nog steeds niet alle effecten van electrische stimulatie op het hersenweefsel bekend. Door deze ontbrekende kennis is ook de volledige therapeutische toepasbaarheid van neurostimulatie nog niet duidelijk. Middels het onderzoek beschreven in dit proefschrift willen wij te weten komen of neurostimulatie geschikt zou kunnen zijn als een nieuwe behandeling voor TBI.

Hoofdstuk 2 bestaat uit een literatuuronderzoek om het effect van electrische velden op (hersen)cellen te beschrijven. Uit onderzoek verricht op verschillende cellijnen bleek dat elektrische stimulatie een beweging van enkele cellen richting de electrode veroorzaakt. Met behulp van het experiment zoals beschreven in **hoofdstuk 3** wilden wij onderzoeken of deze migratie van cellen naar de

electrode toe ook in een levend organisme aangetoond kan worden. Via permanent geimplanteerde electrodes werd de motorische cortex van gezonde ratten herhaaldelijk gestimuleerd ('motor cortex stimulation'; MCS). De resultaten toonden dat deze behandeling een duidelijk effect bleek te hebben op de aanmaak van nieuwe hersencellen. Een volgende stap zou zijn om te onderzoeken of het effect van MCS op de hersenen geschikt is om hersenschade te kunnen herstellen. Echter, om dit te odnerzoeken hadden we een diermodel van TBI nodig waarin we chronische afwijkingen konden meten om deze vervolgens weer te herstellen. In **hoofdstuk 4** konden wij met een standaard diermodel van TBI motorische afwijkingen meten door middel van een reeks gedragstesten. De basis voor toekomstig onderzoek naar nieuwe behandelingsvormen voor TBI werd gevormd door voorgaand diermodel te combineren met een aantal gedragstesten, welke gevoelig genoeg zijn om de motorische afwijkingen van ratten op de lange termijn te kunnen meten.

Wanneer men voor dierexperimenteel onderzoek kiest, is de selectie van gedragstesten uitermate belangrijk om zowel de kwaliteit van de resultaten te verbeteren als ook de mate van ongerief van de dieren te verminderen. **Hoofdstuk 5** bestaat uit een literatuuronderzoek naar een aantal gedragstesten die regelmatig worden gebruikt om de motoriek van knaagdieren te beoordelen. Naast een beschrijving van de verschillende gedragstesten hebben wij deze tevens kritisch beoordeeld en sluiten het hoofdstuk af met aanbevelingen over hoe men de beste gedragstest kiest.

Nadat we hadden onderzocht hoe men motorische schade in ratten opwekt en meet, konden wij in **hoofdstuk 6** testen of deze schade door middel van MCS hersteld kon worden. Helaas werden in dit experiment geen functionele verbeteringen gemeten: noch op gedrag, noch rechtstreeks in de hersenen. De

redenen waarom wij na de toepassing van MCS geen verbetering konden meten, zijn veelzijdig. We kunnen daarom niet uitsluiten dat er alsnog een positief effect van MCS op herstel na TBI zou kunnen optreden na een verandering van de experimentele setup. Momenteel kunnen we nog geen definitieve uitspraken doen over het gebruik van MCS om hersenletsel te herstellen, maar met de experimenten binnen dit proefschrift hebben we een eerste stap gezet in deze richting.

CURRICULUM VITAE

Lisa Schönfeld was born on Sunday, September 7th 1986 in Aachen, Germany. After her graduation from highschool (Heilig-Geist Gymnasium, Würselen, Germany) she started to study psychology at the University of Maastricht, the Netherlands, with the naïve aim to try understanding the human mind. In 2009 she obtained a BSc in Psychology (*cum laude*). During her Bachelor education, she was introduced to human experimental research, which fueled her curiosity to discover the unknown. Therefore, she opted for a Master education in Fundamental Neuroscience, again at the University of Maastricht, with a heavy focus on preclinical research. After spending nine months as an intern at the Center for Neurogenomics and Cognitive Research (CNCR) in Amsterdam, she obtained a MSc in Cognitive and Clinical Neuroscience in 2011.

In 2012 she started to work on a research project that was shared between the Biomedical Research Institute (BIOMED) at Hasselt University, Belgium, and the School for Mental Health and Neuroscience (MHeNS) at Maastricht University. Lisa became a PhD student both in the group of Morphology led by Prof. Sven Hendrix (Hasselt University) and the group of Experimental Neurosurgery led by Prof. Yasin Temel (Maastricht University). From 2012 until 2016 she conducted various *in vitro* and *in vivo* experiments requiring a set of advanced scientific competencies, while developing her soft skills within the Doctoral School of Medicine and Life Sciences (Hasselt University). The chapters that make up the present dissertation present the most notable findings obtained during the last four years and led to the conferment of a doctoral degree.

ACKNOWLEDGEMENTS

Prof. Sven Hendrix

Four years ago I started with my PhD project, shared between the Universities of Hasselt and Maastricht. This concept was new and challenging, for all parties involved. During the time of my PhD project, I learned and grew a lot; both scientifically and mentally. Your emphasis on quality and your critical eye for detail kept on pushing me to the next level – to become a scientist who knows what, why and how. Thank you for your loyalty.

Prof. Yasin Temel

As a student I participated in a practical, where we learned how to implant an electrode. Back then, this was by far the most fancy and complicated procedure I had ever done (or *tried*, to be precise); therefore I needed to learn more about it. I became fascinated by the use of electrical stimulation to modulate brain activity and the projects your former team members were working on. Two years later, I was in the privileged position to become one of your PhD students myself and I couldn't be prouder about the topic I was going to start with. During the last four years, you provided guidance, while at the same time giving me the freedom to follow my own ideas and to become an independent scientist. Even when times were rough, you motivated me to continue believing in my work. Thank you for your support and trust!

Dr. Ali Jahanshahi

Right from the start of my PhD project I was proud about our project and I felt honored that it was me who could continue with your previous ideas. I always admired your calmness (and patience) when guiding me through the first months of my project, together with your hands-on mentality and creativity. It

was a pleasure to have you as both my supervisor and as a colleague; I learned a lot!

Prof. Harry Steinbusch and the members of my doctoral jury, **Dr. Jerome Hendriks**, **Prof. Niels Hellings**, **Dr. Govert Hoogland**, **Prof. Ute Lindauer** and **Prof. Peter Richard Schuurman**.

Thank you Harry, for being the chairman of my doctoral jury and giving me the opportunity to conduct a large part of my education and experimental work at the School of Mental Health and Neuroscience in Maastricht and, together with **Dr. Bart Rutten**, for creating a stimulating environment for young scientists. My gratitude also goes to **Prof. Marcel Ameloot** and **Prof. Piet Stinissen** for allowing me to conduct experiments at the Biomedical Research Institute at Hasselt University.

My sincere gratefulness goes to the members of my thesis assessment committee and doctoral jury who took the time to read, evaluate and eventually approve the contents of my doctoral thesis.

Dr. Govert Hoogland

As an integral part of our team in Maastricht, you provided me with valuable input and advice, whenever necessary.

Dr. Jos Prickaerts

Dear Jos, you introduced me to Neuroscience. Thank you for believing in my capacities when you accepted me for the research master, being the only 'psychologist'. Your attitude and enthusiasm about science has both impressed

and influenced me substantially and I consider you as one of my scientific mentors.

Sandra Schipper

Liebe Sandra. Es gibt Freundschaften, bei denen profitiert einer mehr als der andere. Du hast mir so viel gegeben und nie gefragt, in vielerlei Hinsicht. Du hast mich inspiriert mit deiner Einstellung und Offenheit, beruflich und privat.
Danke für alle Abende im Keller, am Küchentisch, auf deinem Sofa, im Gästebett, in Köln, Düsseldorf (more to come) und Berlin.

Dearbhaile Dooley

Dear. I am very thankful that I have met you. You are such a wise and positive person, even when times are difficult, and I could always rely on you. I was lucky to have met you; you were an island within all surroundings.

Sarah Hescham

Mausi. Ab Tag 1 haben wir uns verstanden und du nahmst mich an die Hand, wenn ich nicht wusste wohin (sowohl experimentell, als auch in anderen dunklen Höhlen). Dass ich dadurch an manche Orte gelange, hätte ich trotzdem nie für möglich gehalten ;)! Ohne dich wären meine letzten vier Jahre deutlich weniger fröhlich und speziell gewesen.

Dr. Melanie Lagière and Dr. Céline Meriaux

Le French. Thanks to you my language skills improved dramatically; whether it was explicit French (…) or non-verbal (coffee, choreic movement or just "go"

[??]). I really enjoyed the time having you as colleagues in Maastricht and now as friends in France.

Fred, Anne, Paul and **Milaine**

My roommates, who provided special welcome ceremonies, Dutch help, interesting phone calls and lots of good talk. I wish you as the Generation Z of science all imaginable luck with your projects.

Joao, Birgit, Gusta, Lianci, Majed, Youssef and **Remco**

The second half of 'Team-el' (Remco) that consisted of a nice group of people I really appreciated as my colleagues.

Daniela, Leen, Myriam, Nathalie, Pia, Stefanie, Selien and **Tim**

Team Sven in Belgium; who always made me feel as part of the team, despite my frequent (and long) stays in Maastricht. I am happy that you were my colleagues and to me, you represented Belgium (regardless of any nationalities ;)! I wish you lots of luck and success for the future and I know, that each of you will go his/her way.

Hellen Steinbusch, Denise Hermes, Marjan Philippens, Katrien Wauterickx, Wiel Honig, Richard Frijns and **colleagues,** thank you for extraordinary support that was as flexible as scientific results are. Special thanks also to **Maurice Huinck**, the man who fixed a broken ~~heart~~ stimulator.

Vanessa, Christina and **Janina,** you are there for me whenever I needed a break from science - preferably at night. Thank you **J**, for a cherished time in Cologne, **B** for all your support in the past.

Mama und Papa

Liebe Mama, lieber Papa, danke, dass ihr immer für mich da wart und meine Entscheidungen angenommen und ohne Einschränkungen unterstützt habt. Danke für die Einstellung immer den Weg zu wählen, der am glücklichsten macht und danke für eure bedingungslose Liebe.

Oma

Meine allerliebste Oma, du hast essentiell dazu beigetragen, wer ich heute bin und ich bin glücklich über jeden (un)wichtigen Moment meines Lebens, den ich mit dir teilen kann.

•

"Merkwürdige neue Ereignisse werden zu deiner Lebensfreude beitragen". "Merkwürdig" das heißt, by chance, beyond advanced und wunderschön.