NEUROPLASTICITY INDUCED BY PERIPHERAL NERVE STIMULATION

Neuroplasticity induced by peripheral nerve stimulation

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Abstract

Non-invasive methods have been developed to induce plastic changes in the sensorimotor cortex. These rely on stimulating pairs of afferent nerves. By associative stimulation (AS) of two afferent nerves, excitability changes in the motor cortex occur as indicated by studies reporting changes in motor evoked potentials (MEPs) elicited by transcranial magnetic stimulation (TMS). Repetitive stimulation of those nerves has a potential in rehabilitation and treatment of neurological disorders like stroke or spinal cord injury. Despite promising results and applications in human subjects using these methods, little is understood about the underlying basis for the changes which are seen.

In the present study, behavioural, electrophysiological and immunohistochemical assessments were performed before and after paired associative and non-associative (NAS) median and ulnar nerve stimulation. Two macaque monkeys were trained to perform a skilled finger abduction task using refined behavioural methods. Monkeys were not able to move their thumb and index finger as selectively after one hour of paired AS as indicated by an increased number of errors and decreased performance measures. NAS however decreased error numbers and led to increased performances.

Additionally, I recorded from identified pyramidal tract neurons and unidentified cells in primary motor cortex (M1), in two macaque monkeys before and after one hour of AS (and NAS) of the median and ulnar nerve. Cell discharge was recorded in response to electrical stimulation of each nerve independently. Some cells in M1 showed changed firing rates in response to nerve stimulation after AS (and NAS).

Subsequently, structural changes in response to one week of paired AS were investigated. The laminar-specific density of parvalbumin-positive interneurons, perineuronal nets and the colocalisation of these two entities changed on the stimulated (in comparison to the non-stimulated) sensorimotor cortex.

These findings suggest that the sensorimotor cortex undergoes plastic changes in response to AS (and NAS).

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The work for the current thesis would not have been possible without the generous help and support of a number of people.

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αMN – alpha-motoneuron, *p. 2–4, 10–12*

γMN – gamma-motoneuron, *p. 13*

CSPG – chondroitin sulfate proteoglycan, *p. 183*

DAPI – 4'$,$6$-$diamidino$-$2$-$phenylindole, *p. 183–193, 195–200, 207, 208*

DNA – deoxyribonucleic acid, *p. 184*

GABA – γ aminobutyricacid, *p. 168, 173, 174, 176, 183, 212, 218, 221, 225, 226, 230–233*

GFP – green fluorescent protein, *p. 185, 186*

H_2O_2 – hydrogen peroxide, *p. 182*

cDNA – complementary deoxyribonucleic acid, *p. 184*

dH_2O – distilled water, *p. 182, 236*

ACSF – artificial cerebrospinal fluid, *p. 181*

ADL – antidromic latency, *p. 134*

ADM – abductor digiti minimi, *p. 5, 108, 159*

AP – action potential, *p. 2, 3, 10–12, 21–24, 135, 157, 221, 223, 227*

APB – abductor pollicis brevis, *p. 5, 8, 9, 16, 107, 108, 117, 119, 159, 229*

APL – abductor pollicis longus, *p. 159*

AS – associative stimulation, *p. xxi, 4–8, 17, 105, 122*

AWERB – animal welfare ethical review board, *p. 129*

BA – Brodmann area, *p. 16, 17, 165*

CaMKII – calcium calmodolin dependent protein kinase II, *p. 24*

CBC – comparative biology centre, *p. 36, 37, 57, 127*

CBT – corticobulbar tract, *p. 9, 15, 18*

CFM – centre for macaques, *p. 35*

CI – confidence interval, *p. 5, 144*

CM – corticomotoneuronal, *p. 4, 10, 18, 19, 30, 159, 170, 216*

CNS – central nervous system, *p. 12, 24*

CoG – centre of gravity, *p. 7, 107, 123, 170*

CS – conditioning stimulus, *p. 15, 17, 230*

CSP – cortical silent period, *p. xxi, 8, 9*

CST – corticospinal tract, *p. 2, 4, 5, 9, 10, 15–20*

DAB – 3,3’−−diaminobenzidine, *p. 182*

DAQ – data acquisition, *p. 111, 133*

DC – direct current, *p. 1*

dPM – dorsal premotor area, *p. 18*

ECG – electrocardiography, *p. 99*

ECR – extensor carpi radialis, *p. 5*

EDC – extensor digitorum communis, *p. xiv, xv, 26, 121, 126, 128, 131, 132, 134, 138–141, 143, 144, 149, 158–160, 163, 169, 218, 222*

EEG – electroencephalography, *p. 17, 165*

EMG – electromyography, *p. 3, 4, 51, 75–77, 95, 126, 128–130, 228*

EPSC – excitatory postsynaptic current, *p. 23*

EPSP – excitatory postsynaptic potential, *p. 3, 12*

ERS – event related synchronisation, *p. 165*

ES – electrical stimulation, *p. 2, 4, 9, 16, 19, 20, 224*

FDI – first dorsal interosseous, *p. 5, 8, 9, 16, 107, 108, 117, 119, 159, 229*

FDS – flexor digitorum superficialis, *p. 5*

FHD – focal hand dystonia, *p. 7, 31, 105, 106, 118, 119, 123, 124, 217, 219, 227–229, 234*

fMRI – functional magnetic resonance imaging, *p. 123*

GPT – grooved pegboard task, *p. 107, 119, 215, 216*

GUI – graphical user interface, *p. 60, 136*

HCl – hydrochloric acid, *p. 181*

HDF – hierarchical data format, *p. 187, 188*

HFO – high frequency oscillations, *p. 166*

HRP – horseradish peroxidase, *p. 182*

ICF – intracortical facilitation, *p. 124, 162, 230, 231*

ICMS – intra-cortical micro-stimulation, *p. 135*

IgG – immunoglobulin G, *p. 182, 184, 185*

ISI – inter-stimulus interval, *p. 17, 230*

ISIH – inter-spike interval histogram, *p. 133, 135, 136*

ITC – inter-trial coherence, *p. 165*

IZ – intermediate zone, *p. 15, 18, 19*

JTHFT – Jebson-Taylor hand function test, *p. 107*

LED – light-emitting diode, *p. 49, 51, 70*

LFP – local field potential, *p. 234*

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LoG – Laplacian of Gaussian, *p. 193, 194*

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LTP – long term potentiation, *p. 21, 23, 24, 26, 124, 125, 168, 169, 218, 219, 221, 223, 225*

M1 – primary motor cortex, *p. xiv, xv, xxii, 6, 17–19, 25–27, 30, 121, 124–129, 135, 138, 139, 141, 144, 157, 158, 160–169, 171, 175, 176, 182, 186–189, 193, 195, 197, 199, 201, 202, 204, 205, 207–210, 212, 216–225, 227–230, 232–234*

MD – musician's dystonia, *p. 31*

MEP – motor evoked potential, *p. xxi, 4–6, 8, 9, 18, 24, 25, 105, 106, 117, 123–125, 144, 158, 159, 162, 166, 167, 170, 171, 215, 218, 221, 224, 229, 230, 234*

mGluR – metabotropic glutamate receptor, *p. 21, 24, 227*

MIDI – musical instrument digital interface, *p. 108, 119*

MRC – medical research council, *p. 35*

MRI – magnetic resonance imaging, *p. 128*

MSO – maximum stimulator output, *p. 123*

MT – motor threshold, *p. 131*

MU – multi unit, *p. 121, 126, 132, 133, 138, 148–150, 160, 170*

MVC – maximum voluntary contraction, *p. 5, 6*

NAS – non-associative stimulation, *p. 4, 7, 8, 105, 123*

NMDA – N-methyl-D-aspartate, *p. 21, 23, 24, 125, 168*

NPRT – negative and positive reinforcement training, *p. 34, 72, 88, 89*

NRT – negative reinforcement training, *p. 39*

Otx2 – orthodenticle homeobox 2, *p. 212*

Parv – parvalbumin, *p. v, xvi, 26, 27, 173–176, 180–193, 195–212, 219, 225–227, 232*

PAS – paired associative stimulation, *p. 170*

PCA – principal component analysis, *p. 133*

PES – peripheral electrical stimulation, *p. 167*

PFA – paraformaldehyde, *p. 181*

PIL – Python imaging library, *p. 188, 189*

PNN – perineuronal net, *p. v, xvi, 26, 27, 173, 175, 176, 180–183, 185–212, 219, 225, 226*

PNS – peripheral nerve stimulation, *p. xvi, 25, 26, 168, 173, 176, 208–212*

PP – paired pulse (stimulation paradigm), *p. 230*

PPC – posterior parietal cortex, *p. 18*

PPI – protein phosphatase I, *p. 24*

PRT – positive reinforcement training, *p. 26, 29, 32–35, 39, 40, 58, 66, 72, 84, 85, 87, 89, 93, 94, 100, 216, 233*

PSTH – peristimulus time histogram, *p. 136–140, 142, 144*

PT – pyramidal tract, *p. 19, 129, 134*

PTN – pyramidal tract neuron, *p. 25, 125, 134, 138, 149–151, 158, 159, 162, 169, 171, 218, 219, 221, 224, 229*

RF – receptive field, *p. 162*

RFID – radio frequency identification, *p. 37*

RGB – red, green and blue, *p. 186–190, 193, 196*

RMT – resting motor threshold, *p. 178*

ROI – region of interest, *p. xv, 186–190, 192, 193, 195–197, 199, 201, 203, 207, 208, 210*

RT – reaction time, *p. 113*

S1 – primary somatosensory cortex, *p. 6, 17–19, 62, 107, 122, 144, 161, 162, 164, 165, 167, 176, 182, 186–190, 195, 197, 199, 201, 204, 208–210, 216, 224, 225, 227, 232*

SC – spinal cord, *p. 2, 18, 30*

SEM – standard error of the mean, *p. 35, 80, 81, 195, 198, 200*

SEP – somatosensory evoked potential, *p. 17, 107, 122, 144, 160, 161, 164–167, 170, 224, 234*

SICI – short-interval intracortical inhibition, *p. 230, 231*

STDP – spike-timing dependent plasticity, *p. xxi, 22, 23, 121, 126, 127, 168, 169, 221, 223, 232*

SU – single unit, *p. 121, 126, 132–136, 138–144, 147–149, 152–157, 160, 163*

TBS – tris-buffered saline, *p. 181–184*

TES – transcranial electric stimulation, *p. 162*

Texas Red – sulforhodamine 101 acid chloride, *p. 186*

TIFF – tagged image file format, *p. 186*

TMS – transcranial magnetic stimulation, *p. 1, 2, 4–6, 8, 9, 24, 25, 105, 107, 117, 123–125, 158, 162, 170, 171, 215, 218, 221, 229, 230, 233, 234*

TS – test stimulus, *p. 17, 230*

UDP – use dependent plasticity, *p. 106, 107*

UID – unidentified, *p. 134*

USB – universal serial bus, *p. 130*

VDCC – voltage dependent calcium channels, *p. 24*

VL – ventrolateral (thalamic nucleus), *p. 161*

VPL – ventral posterolateral (thalamic nucleus), *p. 161, 162*

vPM – ventral premotor area, *p. 18*

VPN – ventral posterolateral nucleus, *p. 15, 17, 18*

WC – writer's cramp, *p. 31*

WFA – Wisteria floribunda, *p. 173, 176, 182–185, 207, 211, 225, 232*

Chapter 1. General introduction

Plasticity can be described as the ability of the human brain to change in response to experience and use (Feldman, 2009). Plasticity enables the brain to extract, learn and store patterns of the sensory world. The brain can change its state by learning and experience (Honda et al., 1998; Karni and Bertini, 1997; Karni et al., 1995; Kleim et al., 1998, 2002; Pascual-Leone, 2006; Plautz et al., 2000; Robertson et al., 2004; Taubert et al., 2010), training or refinement of motor skills (Adkins et al., 2006; Xu et al., 2009; Ziemann et al., 2001), regaining motor function after cortical lesions (Emerick et al., 2003; Papadopoulos et al., 2006), thalamic lesions (Miles et al., 2005), neurological injuries (Cramer et al., 2011; Di Lazzaro et al., 2010; Fraser et al., 2002; Hallett, 2001; Roiha et al., 2011; Rosenzweig et al., 2010), amputation (Chen et al., 1998; Cohen et al., 1991; Merzenich et al., 1983) and temporary ischemic block (Ridding and Rothwell, 1995). Furthermore, neuronal circuitry of the human and non-human primate cortex can be altered by stimulating the cortex invasively with microelectrodes (Plow et al., 2009), non-invasively using transcranial magnetic stimulation (TMS), or direct current (DC) stimulation (Funke and Benali, 2011; Kujirai et al., 1993; Nitsche and Paulus, 2000). Another possibility to alter the state of the brain can be accomplished by peripheral afferent stimulation (McDonnell and Ridding, 2006; McKay et al., 2002; Pyndt and Ridding, 2004; Ridding and Uy, 2003; Ridding et al., 2001; Rosenkranz and Rothwell, 2006; Schabrun and Ridding, 2007; Stefan et al., 2000, 2002).

Peripheral afferent stimulation explicitly refers to the electrical or mechanical stimulation of peripheral nerves or motor points throughout the present dissertation. Hereby, sensory afferents (for example intrinsic hand muscles or nerves innervating those muscles) are electrically (e.g. Ridding and Uy, 2003) or mechanically (e.g. by vibration, Godde et al., 1996) stimulated for a certain amount of time.

The electrical stimulation (ES) presumably activates mostly afferents projecting to the spinal cord and furthermore to the cerebral cortex. ES probably activates in addition to afferents, efferents as well (cf. generation of M and F-waves, H-reflex later in this Chapter).

Thereby, peripheral afferent stimulation is assumed to change cortical excitability. In humans, cortical excitability is often assessed by using transcranial magnetic stimulation (TMS). TMS enables the assessment and modulation of corticofugal projections. When applied over the motor cortex, the current induced perpendicular to the magnetic field generated by the TMS coil (Figure 1.1A) activates, amongst others, neurons in deep layers of the sixth-layered neocortex directly and indirectly (Baker et al., 1994). Depending on the shape of the magnetic coil (Figure 1.1B and 1.1C), the electric field activating those neurons can be widespread or rather spatially focused (Figure 1.1D and 1.1E).

Predominantly in layer V, both the huge pyramidal neurons (Betz cells) as well as other smaller neurons (Fetz and Cheney, 1980) can produce action potentials (APs) which travel through the white matter, cross over to the contralateral side in the medulla oblongata (pyramidal decussation), continuing in the lateral corticospinal tract (CST) until they reach the spinal cord (SC). Other fibres project along the ipsilateral ventral CST. Some of the projecting neurons from the lateral CST possess monosynaptic connections with limb motoneurons, i.e. alpha-motoneurons (αMN) in the ventral (anterior) horn of the SC.

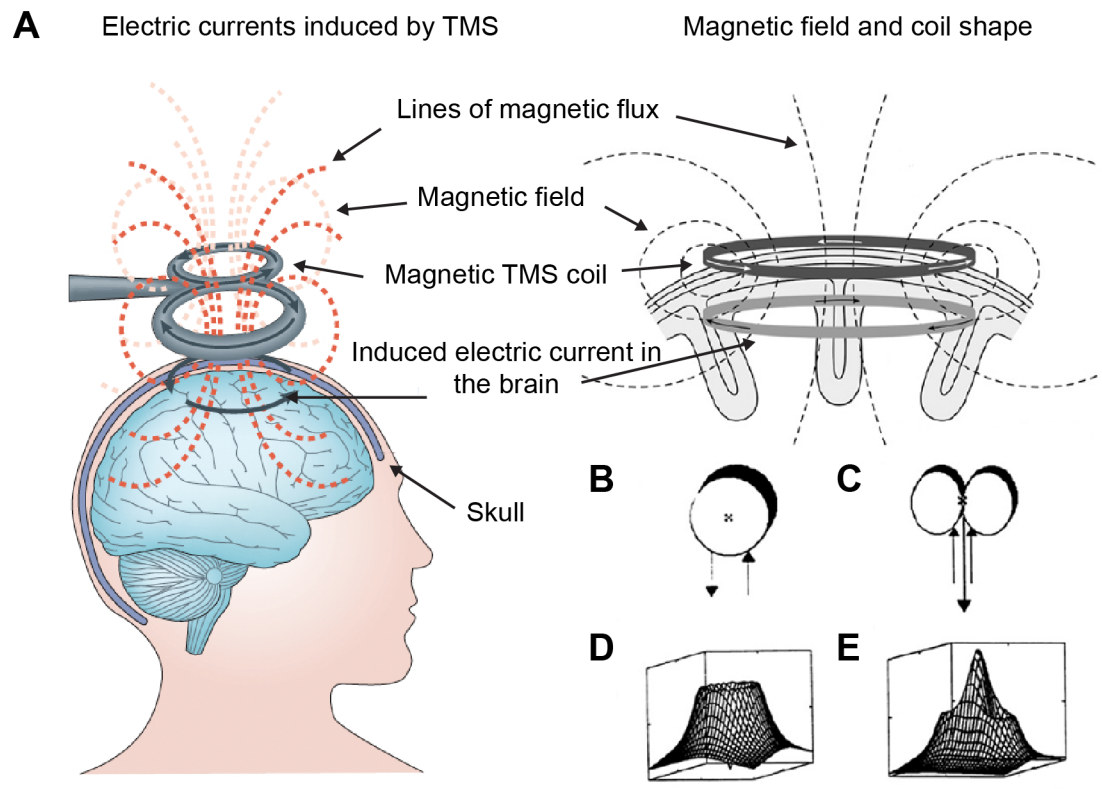

Figure 1.1: *Transcranial magnetic stimulation (TMS) induces electric currents in the brain due to electromagnetic inductions.* These currents can activate neurons in regions close to the electromagnetic stimulation site in the brain. (A) The current flowing through the magnetic coil is generating a magnetic field orthogonal to the plane of the electric current flow. Perpendicular to the magnetic field a current is induced, which can innervate groups of neurons. (B-E) Different magnetic coil shapes produce different electric fields. Figures modified from Hallett, 2007; Ridding and Rothwell, 2007.

If the αMN is depolarised sufficiently by excitatory postsynaptic potentials (EPSPs) in terms of temporal or spatial summation, these will produce APs, travelling along the axon to the neuromuscular junction. These initiate APs in the muscle surface causing contraction of the extrafusal muscle fibres.

This activation or contraction of the muscle can be measured by electromyography (EMG). In human subjects, surface EMG electrodes are used to record responses of particular muscles. Although one particular muscle is targeted, certainly activity of adjacent muscles is also picked up by the EMG electrode. Intramuscular recordings would provide a more precise measure, whereas the

procedure would be rather inconvenient for the subject. A TMS triggered event measured by EMG is called a motor evoked potential (MEP). Because the MEPs are thought to be primarily generated by monosynaptic cortico-motoneuronal (CM) connections of motor cortical neurons with αMN (and αMN constitute the MEP in the muscle), MEP size can provide a measure of CST excitability.

Repetitive peripheral nerve ES can de- or increase MEP amplitudes induced by cortical TMS (McKay et al., 2002; Ridding et al., 2000, 2001). ES has a potential in rehabilitation and treatment of neurological disorders like stroke (Dos Santos-Fontes et al., 2013; Hallett, 2001; Liao et al., 2014) or spinal cord injury (Gomes-Osman and Field-Fote, 2015; Lala et al., 2015; Ragnarsson, 2008; Roy et al., 2010; Rushton, 2003).

Effects of peripheral afferent stimulation have also been studied using paired associative stimulation (AS) protocols of two stimulation sites (Godde et al., 1996; Ridding and Uy, 2003; Schabrun and Ridding, 2007). These sites can either be muscles (motor point stimulation) or nerves. During such interventions, two muscles, muscle groups (e.g. intrinsic hand muscles) or peripheral nerves (innervating those muscles) are electrically stimulated synchronously (associative stimulation) or asynchronously (non-associative stimulation). Synchronous, associative and simultaneous stimulation are used synonymously throughout the present thesis. The same holds true for the terms asynchronous, non-associative and alternating stimulation. AS leads to an increase in MEP amplitudes (McDonnell and Ridding, 2006; McKay et al., 2002; Pyndt and Ridding, 2004; Ridding and Uy, 2003; Schabrun and Ridding, 2007), whereas paired non-associative stimulation (NAS) leads to no changes (McDonnell and Ridding, 2006; Ridding and Uy, 2003; Schabrun and Ridding, 2007). Therefore, AS is assumed to change excitability in the CST.

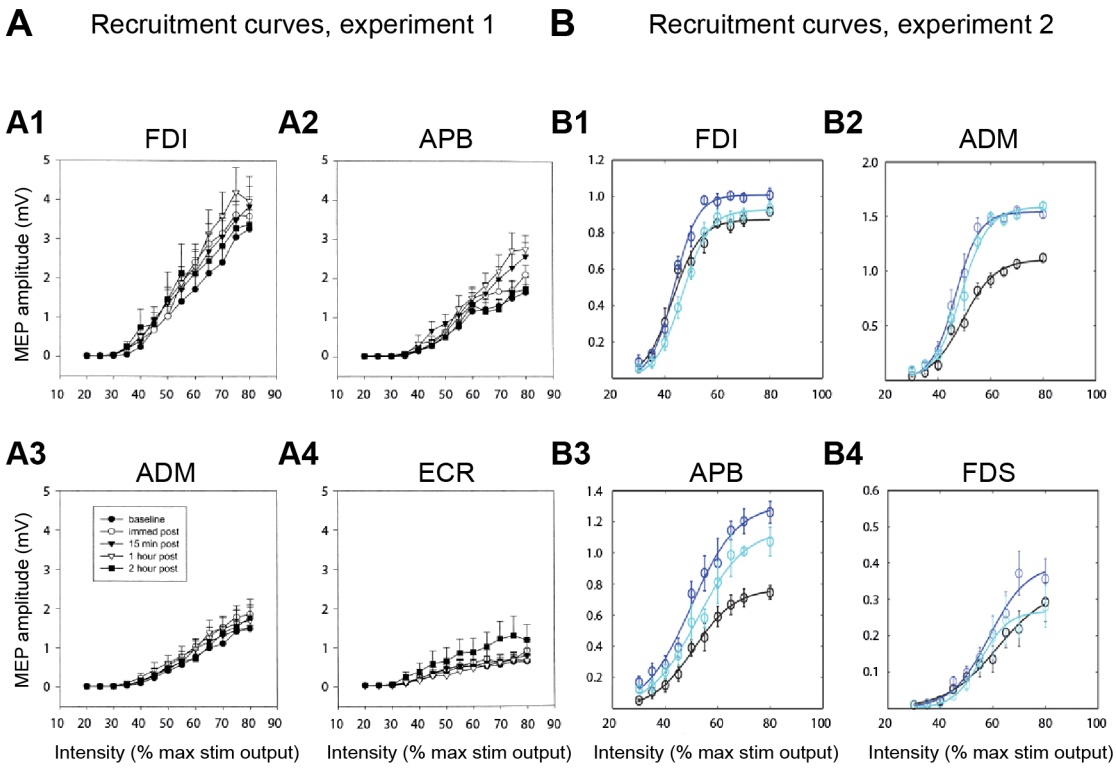

Figure 1.2: *Recruitment curves for 4 intrinsic muscles following* 1 h *of paired associative stimulation to the motor point of two different muscles.* Paired associative stimulation (AS) was applied to the first dorsal interossus (FDI) and abductor pollicis brevis (APB) in experiment 1 (A1-A2). In experiment 2 (B1-B2), AS was applied to FDI and abductor digiti minimi (ADM). Control muscles (no stimulation) were extensor carpi radialis (ECR) and ADM in experiment 1 (A3-A4), flexor digitorum superficialis (FDS) and APB in experiment 2 (B3-B4). Figures of experiment 1 were taken from Ridding and Uy, 2003, whereas experiment 2 represents pilot data from one example subject. Black lines (and data points) correspond to baseline activity with 10 % maximum voluntary contraction (MVC), blue lines 1 h post and the cyan lines 1.5 h post AS. Lines show the sigmoid fit (cf. Equation 1.1) and error bars the 95 % confidence intervals (CIs). In experiment 2, corticospinal excitability changes were topographically less specific in APB due to its proximity to FDI.

A more sensitive and reliable measure in excitability of corticospinal projections is the recruitment curve (Chipchase et al., 2011; Everaert et al., 2010; Ridding and Rothwell, 1997). The recruitment curve (Figure 1.2A1-1.2B4) describes the input-output relationship of MEP modulation induced by changing TMS output intensities (Devanne et al., 1997). The recruitment curve reflects excitability properties of the population of CST neurons, motoneurons and interneuronal

relays like C3/C4 propriospinal neurons (Alstermark et al., 1999) contributing to the MEP. Recruitment curves can mathematically be described with a sigmoid function of the form

$$m(i) = \frac{k}{1 + \exp\left(-r \cdot (i - i_0)\right)}. \tag{1.1}$$

where r is the slope of the function $m(i)$, i are the TMS output intensities and i_0 is the point of reflection (at $50\,\%$ of k). AS applied to different muscles leads to an increase in MEP amplitudes in dependence on the TMS output intensity (Figure 1.2A and 1.2B). Data in part two of Figure 1.2B1-1.2B4 were acquired at the beginning of the experiments described in the present study. The aim of these experiments was to replicate some of the major findings in the literature and to improve the assessments and interventions. In contrast to Ridding and Uy (2003), I recorded recruitment curves at $10\,\%$ MVC to be able to compare corticospinal excitability at a consistent muscle contraction level. More frequently, recruitment curves are determined when the subject is at rest and asked not to perform any muscle contraction. This however, is more difficult to control.

Another effect studied in response to associative and non-associative stimulation relates to the somatotopic organisation of the sensorimotor cortex. The sensorimotor cortex denotes both the somatosensory and motor cortices.

Historically, the sensorimotor cortex is considered to be ordered in a topographic fashion. That means adjacent body parts are represented at adjacent sites within the sensorimotor cortex. This organisation frequently illustrated by the Penfield homunculus seems to hold true in the primary somatosensory cortex (S1) and some gross body part separations in motor cortices. Simple sensorimotor representations do not hold true on a more fine-scale level for the primary motor cortex (M1). Activation of single muscles in M1 may be generated from wide cortical areas (Sato and Tanji, 1989). Furthermore, cortical representations of

muscles overlap (Andersen et al., 1975; Schieber, 2001). The sensorimotor cortex is known to be capable of reorganisation in various conditions (Kleim et al., 1998; Sanes and Donoghue, 2000). It has been found that associative tactile stimulation of digits results in sensorimotor map expansions and greater overlap (Godde et al., 1996). Also AS has been suggested to alter sensorimotor organisation. Schabrun and Ridding (2007) performed cortical mappings of representative muscles before and after the intervention of NAS or AS. Muscle representations were mapped with a cap and the distance between the centres of gravity (CoG) were determined for different muscles. The authors of this study found that after AS cortical representations of the two stimulated muscles overlap more extensively than pre-intervention. NAS or alternating afferent stimulation on the other hand is thought to lead to a stronger separation of the cortical representation of the stimulated muscles, which has been confirmed in patients with focal hand dystonia (Rosenkranz et al., 2009; Schabrun et al., 2009).

The pathophysiology of FHD has been hypothesised to be linked to maladaptive (co-) activation of sensory afferents which lead to aberrant motor-cortical plasticity (Quartarone et al., 2008, 2014; Schabrun et al., 2009; Tinazzi et al., 2000). These characteristics are similar to those artificially induced in healthy human subjects using paired synchronous (associative) motor point or nerve stimulation (McDonnell and Ridding, 2006; McKay et al., 2002; Pyndt and Ridding, 2004; Ridding and Uy, 2003; Schabrun and Ridding, 2007; Schabrun et al., 2009). Although the pathophysiology of FHD has been linked to the above mentioned maladaptive (co-) activation of sensory afferents, there are also alternative explanations regarding the pathophysiology. The pathophysiology of FHD might be located in the brainstem.

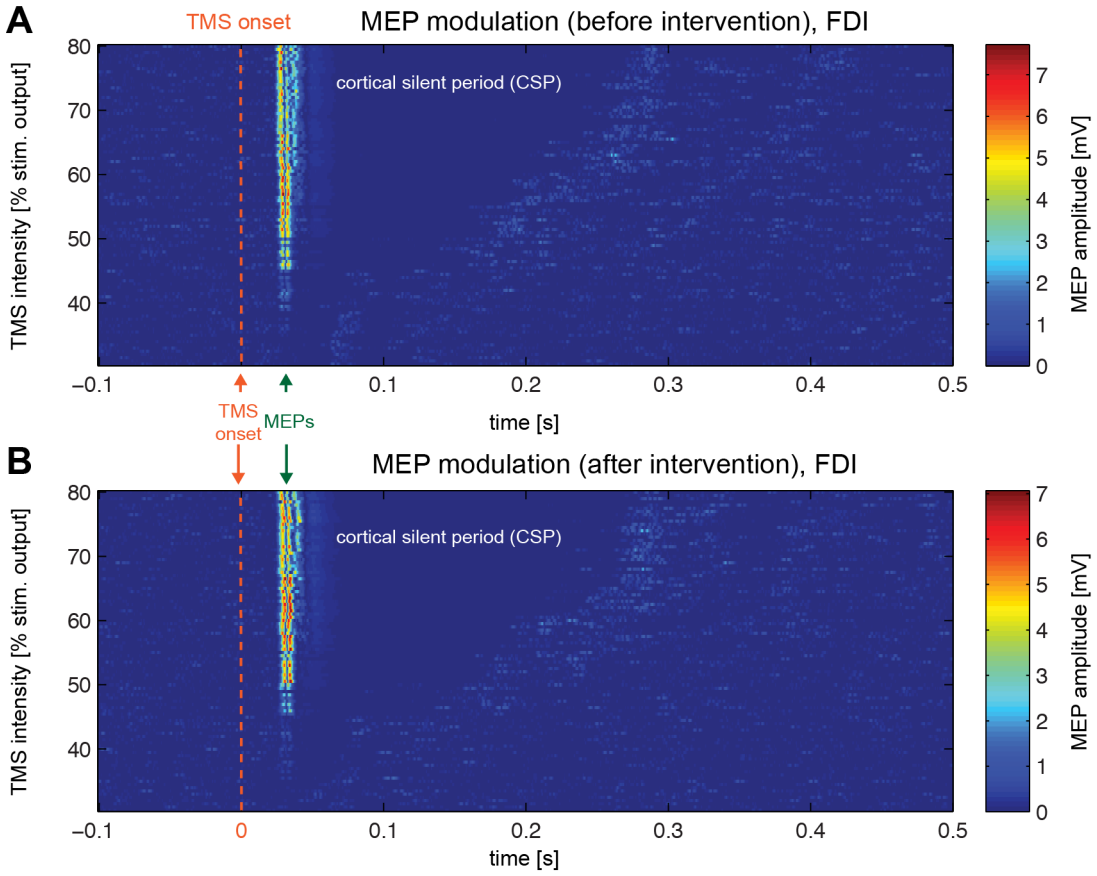

Figure 1.3: *Illustration of MEPs and cortical silent period.* (A) Motor evoked potentials (MEPs) and cortical silent period (CSP) in response to variations in TMS intensity [% of stimulus output] before the intervention. (B) MEPs and CSPs after one hour of synchronous APB and FDI stimulation. TMS was applied at the optimal site for FDI.

Physiological changes in the brainstem cause secondary changes in the cortex, which in turn might lead to aberrant (co-) activation of sensory afferents. Although I am aware of these other possibilities, I will mostly refer to the first hypothesis (Quartarone et al., 2014) throughout the thesis.

Training of a simple motor task can also modify the sensorimotor representation (Butefisch, 2004). Considering that peripheral afferent stimulation leads to cortical reorganisation as well, what is the influence of AS and NAS on motor performance or natural movements?

Peripheral afferent stimulation has been shown to improve motor performance in various tasks like a two-point discrimination task (Godde et al., 2000) or in a grooved pegboard task (McDonnell and Ridding, 2006). Furthermore, peripheral afferent stimulation led to improved motor performance after stroke (Dos Santos-Fontes et al., 2013; Liao et al., 2014; McDonnell et al., 2007), neuropathic pain (McGowan, 2006; Nashold et al., 1982; Schabrun et al., 2013, 2014), spinal cord injury (Lala et al., 2015; Ragnarsson, 2008; Roy et al., 2010) and in patients with dystonias (Hallett, 2011; Rosenkranz et al., 2005, 2009; Schabrun et al., 2009; Sussman, 2015).

Studies which used the associative pairing of a cortical (delivered by TMS) and a peripheral (e.g. median nerve) stimulus, also reported an increase in the motor-cortical excitability as indicated by an increase in the MEP in humans (Stefan et al., 2000) and monkeys (Amaya et al., 2010).

Additionally, Stefan et al. (2000) found a prolonged cortical silent period (CSP) after the associative interventions. The CSP and MEPs of the relevant (during the intervention stimulated) muscle-sites are also increased after synchronous (associative) APB and FDI stimulation (Figure 1.3A and 1.3B).

Several studies reported that the site of the change in excitability or plasticity in response to peripheral ES is likely to be cortical, although plasticity changes could in principle occur along the whole corticofugal pathway including the CST, corticobulbar tract (CBT), any part of the lemniscus pathway (e.g. cuneate nucleus), spinal cord, neuromuscular junction or at the level of the muscle itself.

The assumption of the cortical origin of plasticity is based on observations in imaging studies and findings showing lack of any modulation in responses to peripheral ES on M- (Kaelin-Lang et al., 2002) and F-waves (Ridding et al., 2000) as well as the Hoffmann-reflex (Chipchase et al., 2011; Fernandez-Del-Olmo et al., 2008; Tinazzi et al., 2005).

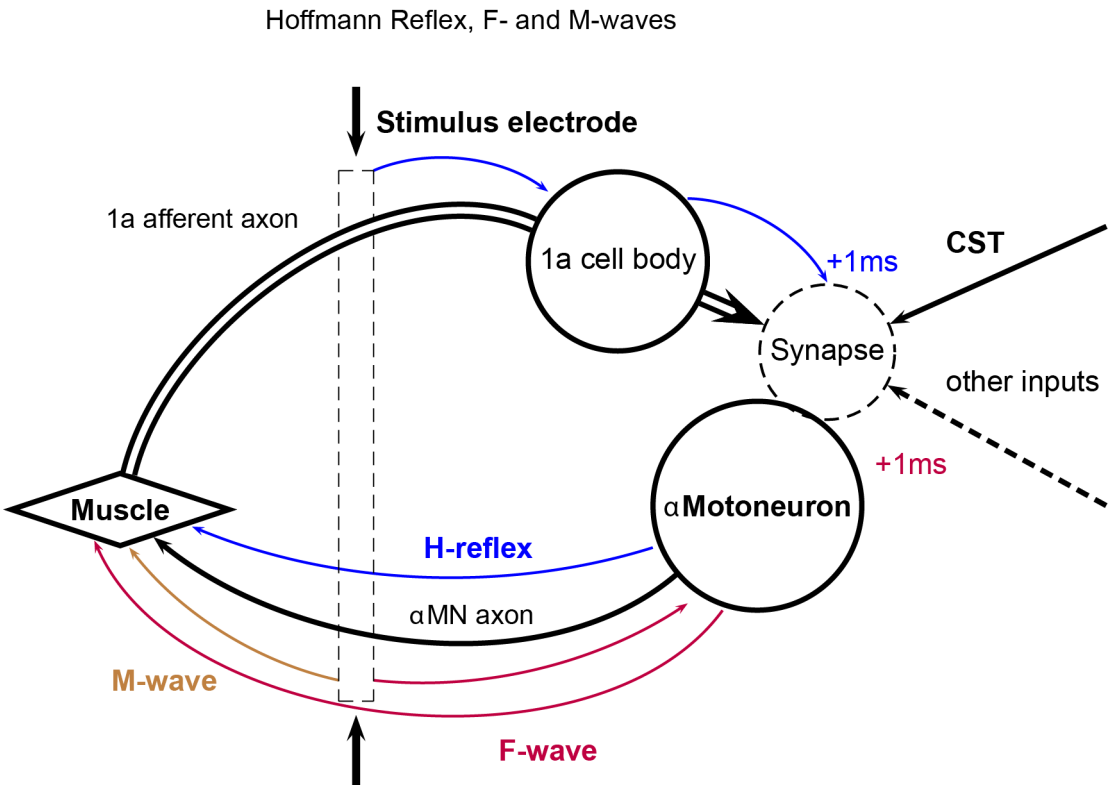

Figure 1.4: *Hoffmann-Reflex, M- and F-waves generated by nerve stimulation.* The M-wave or the compound muscle action potential (brown arrow) is elicited by stimulating the nerve leading to an orthodromic response. At lower stimulus intensities (due to the thicker axons leading to lower thresholds), 1a sensory afferents are activated. APs travel over the dorsal root to the 1a afferent - αMN synapse, where the αMN is innervated, leading to the Hoffmann-reflex (blue arrows). Stimulating the nerve at different intensities will activate αMNs ortho- (brown arrow) and antidromically (red arrow towards the αMN cell body). The response measured in the muscle after antidromic αMN activation causing a back-firing of the neuron is called the F-wave. The synaptic delay is approximately one millisecond and is used for the estimation of the αMN conduction time (cf. Equation 1.2). The αMN synapse stands under various influences: afferent input, direct corticospinal tract (CST) input through cortico-motoneuronal (CM) inputs and other inputs (e.g. corticobulbar projections).

Electrical stimuli delivered over a nerve harbouring efferent axons activate, when enough current is applied, the αMN axon orthodromically. The compound action potential of one corresponding αMN travels along the axon until it elicits an AP in the muscle. The muscle response recorded with an EMG electrode caused by the orthodromic activation of the αMN is called a M-wave (cf. Figure 1.4, brown

arrow). At lower nerve stimulation intensities (due to the thicker axons leading to lower thresholds), the Hoffmann-reflex (H-reflex) is generated (Figure 1.4, blue arrows). The H-reflex relies on a monosynaptic reflex circus involving the 1a sensory afferent and the αMN. The electrical stimulus leads to APs in 1a sensory afferents. These APs enter the spinal cord through the dorsal root (entering the posterior horn), until they cause an activation of the αMN with a synaptic delay of approximately one millisecond. The muscle response measured after 1a afferent and αMN activation is called the H-reflex.

Stimulating the nerve at different intensities will activate αMNs ortho- and antidromically (Figure 1.4, red arrow towards the αMN cell body). The response measured in the muscle after antidromic αMN activation causing a back-firing of the neuron is called the F-wave. This response can only be seen while stimulating with higher intensities. The reason for that seems to be that only the higher threshold (low conduction velocity) fibres are capable of the rebound excitation at the cell body of the αMN.

The latencies of the M- and F-wave (Equation 1.2) can be used to estimate the conduction time $\hat{\Delta}t_{\alpha\mathtt{MN}}$ of an αMN axon (Kimura, 1984).

$$\hat{\Delta}t_{\alpha\mathtt{MN}} = \frac{\Delta t_{\mathtt{CMAP}} + \Delta t_{\mathtt{F-wave}} - 1}{2} \tag{1.2}$$

Considering the physiological mechanisms underlying the M-, F-wave and the H-reflex, no significant modulation in those responses (Chipchase et al., 2011; Fernandez-Del-Olmo et al., 2008; Kaelin-Lang et al., 2002; Tinazzi et al., 2005) does not seem to exclude the spinal cord or motoneurons as possible sites modulated by peripheral afferent stimulation completely.

The neuromuscular junction does not appear to be a likely target for plasticity because the αMN-muscle synapse is a hundred percent efficient synapse. Hundred percent efficient means that in contrast to other synapses in the CNS, every incoming AP leads to a muscle response, whereas in the cortex and spinal cord temporal and spatial summation of EPSPs is necessary to elicit an AP in the postsynaptic neuron.

This efficiency is only diminished in certain neurological conditions like Myasthenia gravis. It would furthermore be possible that changes occur within interneuron populations within the spinal cord, in propriospinal neurons or in other pathways projecting to the αMN synapse (e.g. corticobulbar pathways).

Furthermore, evidence regarding the H-reflex seems to be slightly controversial in the literature as recent studies found H-reflex modulations after an associative intervention (Lamy et al., 2010), which leaves the possible site modulated by peripheral afferent stimulation open for discussion (Ziemann et al., 2008).

Putting the aforementioned evidence in a nutshell, it seems to be likely that the site of an excitability change is cortical, although other locations in the central nervous system (CNS) like the spinal cord, brainstem, thalamus, nucleus cuneatus or nucleus gracilis could be affected by peripheral afferent stimulation as well. To identify sites of plasticity and physiological mechanisms underpinning the assessments frequently used in human and non-human primates, it is essential to investigate ascending and descending pathways (Figure 1.5) of the sensorimotor system. Plasticity and change in excitability are used interchangeably throughout the present text assuming that plasticity-inducing mechanisms lead to excitability changes of relevant neuronal groups.

Neuronal processing in ascending pathways depends on the modality of the sensory stimulus (Table 1.1). Different sensory receptor types (muscle-, thermal-, mechanoreceptors and nociceptors) possess axons, which differ in conduction velocity and diameter.

	Classification		Size		
Receptor	Type	Group	Diameter [µm]	CondVel [$\mathrm{m\,s^{-1}}$]	Modality
MECHANORECEPTORS					
(I). SKELETAL/MUSCLE					PROPRIOCEPTION
Muscle spindle (primary)	Ia	Aα	1-20	70-120	Muscle length & velocity
Muscle spindle (secondary)	II	Aβ	6-12	30-70	Muscle stretch
Golgi tendon organ (secondary)	Ib	Aα	12-20	70-120	Muscle contraction
Stretch-sensitive free endings	III	Aδ	1-6	5-30	Stretch or force
(II). (SUB-) CUTANEOUS					TOUCH
Meissner's corpuscle	RA	Aα, β	6-12	30-70	Stroking
Merkel disk receptor	SAI	Aα, β	6-12	30-70	Pressure and texture
Pacinian corpuscle	PC	Aα, β	6-12	30-70	Vibration
Ruffini ending	SAII	Aα, β	6-12	30-70	Skin stretch
THERMAL RECEPTORS					TEMPERATURE
Cool / warm receptors	III, IV	Aδ, C	1-6	0.5-30	Cold / warm
NOCICEPTORS					PAIN
Polymodal	III, IV	Aδ, C	1-6	0.5-30	sharp pain, burning

Table 1.1: *Somatosensory receptors process information of various modalities in different speeds*. Sensory receptors (mechanoreceptors, thermal receptors, and nociceptors) possess different axon types, diameter and conduction velocities (CondVel) and respond to a variety of sensory modalities. Subcutaneous mechanoreceptors can be further subdivided into rapidly adapting (RA) and slowly adapting (SAI/II) fibres (Andrew and Part, 1972; Kandel et al., 2012; Russell, 1980).

Muscle spindles with axons of the type Ia have a diameter between 1-20 µm and a conduction velocity of 70-120 $\mathrm{m\,s^{-1}}$ (Table 1.1). γ-motoneurons (γMN) on the other hand possess only 3-6 µm thin axons with slower conduction velocities between 15-30 $\mathrm{m\,s^{-1}}$ (Andrew and Part, 1972; Russell, 1980). Other studies report even slower conduction velocities (Eccles et al., 1968).

α-MNs possess slightly thicker axons, which explains the different threshold for eliciting M-waves in comparison to the H-reflex (Figure 1.4).

Additionally, it is important to notice that different ascending pathways are involved in processing modality-specific information.

The spinothalamic tract for example is mainly involved in the processing of pain, temperature and crude touch. These modalities do not play a crucial role in peripheral motor point or nerve stimulation, because intensities used in these studies are often not higher than twice the motor threshold. The motor threshold for stimulation of peripheral nerves or motor points is defined by the intensity which elicits a twitch in the target muscles. These intensities are still below those sufficient to cause a pain perception but still high enough to cause activation of cutaneous receptors at the site of the electrode.

Although such high intensities are not used in studies involving peripheral afferent stimulation, a precise mapping of effects in dependence on variations of these parameters would be beneficial in order to understand underlying mechanisms involving modality-specific pathways (Chipchase et al., 2011).

Other ascending projections to the brainstem or cerebellum (spinocerebellar or cuneocerebellar tract) would in principle be capable of modulating cortical output as well. However, a more relevant ascending pathway is the medial lemniscus or dorsal column system (Figure 1.5).

Information about localised touch, pressure, vibration and joint position is processed by large myelinated sensory afferent nerve fibres (e.g muscle length specific input from 1a spindle afferents). Some of these afferents terminate in the spinal cord and are involved in mono- and transsynaptic reflex circuits (e.g. H-reflex).

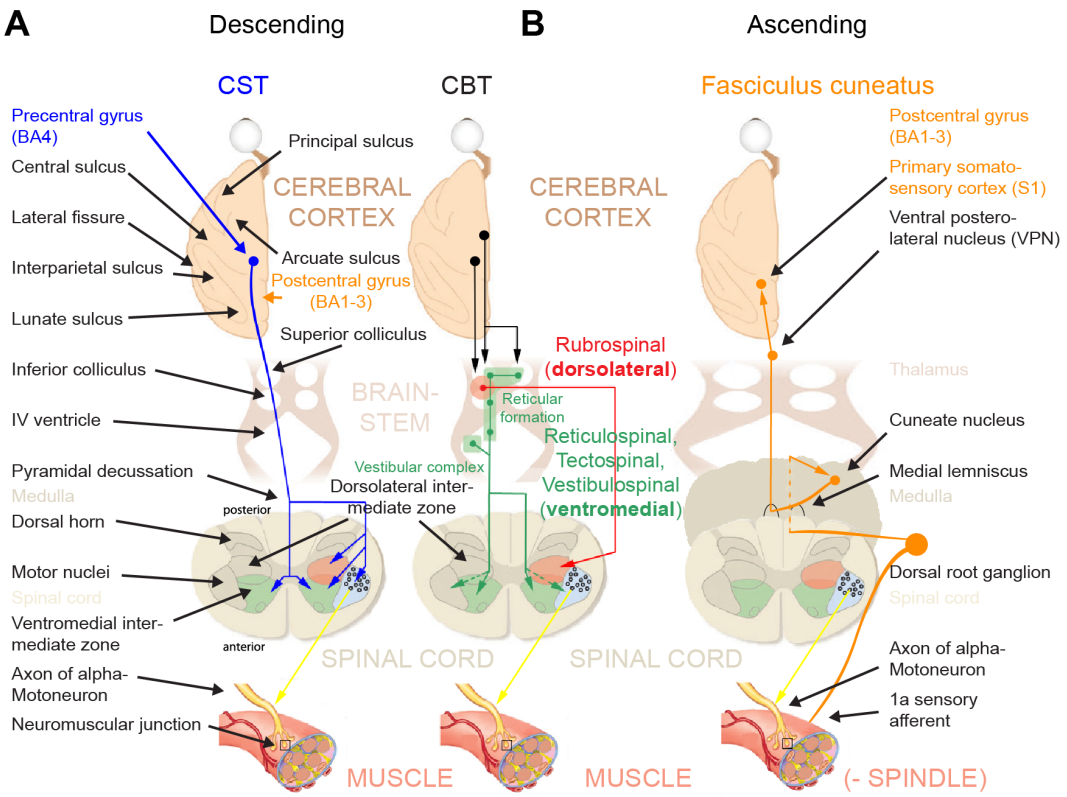

Figure 1.5: *Ascending and descending pathways of the sensorimotor system.* Two important descending pathways (A) are corticospinal (CS) and corticobulbar (CB) tracts. (A) Most fibres of CST project to the contralateral dorsolateral intermediate zone (IZ), ventromedial IZ or directly to motoneurons (cortico-motoneuronal connections) in the ventral horn. CBTs can be subdivided into dorsolateral (rubrospinal; red arrows) and ventromedial (reticulospinal, tectospinal and vestibulospinal; green arrows) pathways (Swanson and Kuypers, 1980). The rubrospinal pathway roughly projects to the contralateral dorsolateral IZ and reticulospinal, tectospinal and vestibulospinal pathways bilaterally to the ventromedial IZ. (B) A sensory 1a afferent enters the spinal cord through dorsal roots, before projecting in dorsomedial fibre tracts to the medulla oblongata. First order fibres possess synaptic contact with neurons in the cuneate nucleus. From there on fibres cross to the contralateral side and project through the medial lemniscus to the VPN in the thalamus before reaching the somatosensory cortex. Figure A modified from Lemon, 2008.

Other afferents are projecting to circuits in Rexed lamina II (Substantia gelatinosa) and are involved in pain inhibition. This might be why peripheral ES has been shown to have an effect in the treatment of pain (Kolen et al., 2012). The majority of the ascending large sensory afferents enter the spinal cord over the dorsal root.

The sensory afferents ascend to the level of the brainstem in the dorsal column (Figure 1.5B). At the level of the medulla, sensory afferents possess synaptic contacts with secondary neurons in the nucleus gracilis (for lower body part-) or nucleus cuneatus (for upper body part information). Because most of the studies investigating peripheral afferent and nerve stimulation focus on intrinsic hand muscles, the focus here is on the fasciculus cuneatus. The cuneate nucleus is capable of modulating somatosensory signals (Witham and Baker, 2011) and the intracuneate circuitry has been mapped in response to afferent stimuli (Soto et al., 2004). Therefore, the cuneate nucleus could be capable of modulating activity in response to peripheral afferent stimulation. The cuneate nucleus becomes even more likely as a site for plasticity considering the given gating mechanisms and the physiological constraints. Activation of various peripheral nerves (e.g. ulnar or median nerve) leads to activation of the same cuneate neuron (Witham and Baker, 2011), which indicates a certain amount of convergence. The median nerve provides motor innervation of the first, second lumbrical muscles and the thenar eminence. Furthermore, it innervates predominantly APB. All other intrinsic hand muscles (e.g. FDI) are supplied by the ulnar nerve.

The cuneate nucleus is subject to cortical control (Aguilar et al., 2003). The corticocuneate connections differ in certain species. In monkeys, the corticocuneate projections are likely to be collaterals of the CST (Cheema et al., 1985), whereas in cats there are separate projections (Bentivoglio and Rustioni, 1986). In both monkeys and cats there is a projection from motor cortex (BA4) to cuneate. In cats the projection from somatosensory cortex includes area 3a

whereas in monkeys it is rather area BA1/2. Altogether this also fulfils some of the requirements being a likely target for modulation due to afferent inputs.

The secondary neuron axons (internal arcuate fibres) project to anterior-medial parts of the medulla. These fibres decussate and build the medial lemniscus, which project to the thalamus where these make synaptic connections in the ventral posterolateral nucleus (VPN). The thalamus might also be modulated by afferent stimulation considering the corticothalamic interconnections. Finally, information will be processed in the primary somatosensory cortex (S1), which receives most of the sensory input. Especially the areas 3a and 3b of S1 possess the capability of changing excitability of corticofugal projections (S1 also possesses CST projections) directly or indirectly via the primary motor cortex (M1) because of its extensive horizontal inter-area connections. These horizontal inter-connections provide a physiological substrate for mechanisms involved in cortical plasticity (Sanes and Donoghue, 2000). Additionally, M1 also receives direct input from the thalamus (Lemon, 1979, 1981). Thereby, AS could modulate cortical activity, which might result in modified output of descending corticofugal outputs. One way to assess a change in S1 excitability in humans is to use scalp electrodes to measure responses to peripheral afferent stimulation by using electroencephalography (EEG). Somatosensory-evoked potentials (SEPs) can be modulated by using different peripheral afferent stimulation protocols like a paired-pulse paradigms. Such a paradigm consists of a conditioning stimulus (CS) followed by another pulse, the test stimulus (TS). Delivering this protocol to the median-nerve depending on specific inter-stimulus intervals (ISIs) leads to changes in SEPs, i.e. changes in N20 or P25 EEG components (Hoffken et al., 2007). The N20 and P25 are the negative deflection of the EEG response at $20\,\mathrm{ms}$ post-stimulus and the positive one at $25\,\mathrm{ms}$, respectively. These can be induced by median nerve stimulation and are used for comparison in various neurological conditions.

Not only ascending pathways should be studied in search of the site (or sites) of plasticity induced by peripheral afferent stimulation. Descending pathways are also important because descending pathways can influence, modulate and filter afferent input (Canedo, 1997). Furthermore, assessment used in humans like the aforementioned changes in MEP size reflect properties and relevant physiological processing sites of descending systems (Figure 1.5A).

The two major projections from the sensorimotor cortex are the corticospinal tract (CST) and the corticobulbar tract (CBT). Although there are other projections to the pons, cerebellum or to the basal ganglia, these do not seem to be of primary importance in the generation of MEPs or modulation in response to afferent stimulation. Furthermore, there are corticothalamic projections which might be of interest (cf. ascending projections to VPN).

There are many cortical areas as possible origins for CST projections in the sensorimotor system (Dum and Strick, 2005). These regions include amongst others the primary somatosensory cortex (S1), primary motor cortex (M1), supplementary motor area (SMA), dorsal and ventral premotor area (dPM and vPM) and the posterior parietal cortex (PPC). Especially S1 (cf. ascending systems) and M1 (crucial in fine finger control) are sensible locations for cortical plasticity induced by afferent stimulation.

In M1, the CST (Figure 1.5, blue arrows) sends descending fibres to the spinal cord from deep layer projection neurons. Most of these fibres cross to the contralateral site at the pyramidal decussation in the medulla. CST neurons build synaptic connections in the spinal cord (SC) with either interneurons in the intermediate zone (IZ) or directly with motoneurons in the ventral horn. The latter are called cortico-motoneuronal (CM) connections. CM connections are uniquely present in human and non-human primate corticospinal systems (Alstermark et al., 2004; Lemon, 2008). CM connections are considered to be the main pathway involved in the generation of MEPs. Therefore, CM connections are crucial for

both the assessment as well as the intervention (peripheral afferent stimulation). CM connections arise from caudal (posterior) parts of M1 (Rathelot and Strick, 2009, new M1) and a small percentage from area 3a (part of S1).

Corticobulbar tracts can roughly be subdivided into dorsolateral (Figure 1.5, red arrows) and ventromedial (Figure 1.5, green arrows) pathways. In addition to this ventromedial / dorsolateral bias, there is also a lot of overlap of projections (Kuypers et al., 1960). Out of these brainstem pathways, the reticulospinal pathway (part of the ventromedial tract) has been found to be able to facilitate hand muscles (Riddle et al., 2009). Riddle and Baker, 2010 found that neurons recorded in the IZ receive common input from both CST and reticulospinal tract. The reticular formation receives sensory input (Baker, 2011) for example over the midventral cuneate nucleus (Leiras et al., 2010), which makes it another possible site for plasticity changes.

In primates, the CST can control fine finger movements also by strengthening connections to propriospinal C3/C4 interneurons (Sasaki et al., 2004). When stimulating the pyramidal tract (PT), C3/C4 propriospinal neurons may be able to control CST output by feedback and feedforward mechanisms (Isa et al., 2006, 2007).

Finally, considering the enormous amount of various types of motoneurons and all intra- and extraspinal interconnections, specific motoneuron subtypes might be altered in response to sensory afferent stimulation (Jessell et al., 2011; Rothwell, 2012).

Before considering the mechanism underlying single-, paired-, associative and non-associative peripheral afferent recruitment, at least two types of stimulation can be differentiated. Electrical stimulation (ES) is often applied through surface EMG electrodes. This leads to transcutaneous activation of both sensory afferents and motoneurons (cf. Figure 1.4). These efferents can send antidromically signals to the synapse of the motoneuron or to close

intra-spinal interneuronal populations. One cell type likely to be innervated would be the Renshaw cell, which can be activated by antidromic activation of motoneurons and by CST projections (Mazzocchio et al., 1994). ES might recruit different neuronal populations depending on its intensity. Stimuli below the motor threshold (stimulus intensity to elicit a twitch in a target muscle) might activate for example propriospinal neurons which possess feedback control over pyramidal tract neurons (Giboin et al., 2012; Lemon, 2008). Another possibility to activate afferents, which has been found to change cortical excitability, is a vibration stimulus (Rosenkranz and Rothwell, 2003). This quality of stimulation mainly recruits afferents via Pacinian corpuscles (cf. Table 1.1). In addition to Pacinian corpuscles, other touch, pressure and stretch sensitive receptors are also activated.

What are the underlying mechanisms responsible for the excitability changes, which can be observed in response to peripheral afferent stimulation? In many animal models, it has been shown that timing (Feldman, 2000), frequency (Dudek and Bear, 1992) and intensity (Barr et al., 1995) of the synaptic inputs can influence the strength of synaptic connections.

The co-activation and coincidence of correlated inputs has been postulated by Hebb (1949) who stated that *[w]hen an axion of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cell firing B, is increased* (page 50).

Physiological mechanisms are functional modifications of already existing synapses and neurons. Structural mechanisms refer to the physical rewiring of cortical (and spinal) circuits by synapse formation, deletion and morphological changes (Feldman, 2009).

A physiological mechanism, which might be present in all excitatory synapses of the mammalian brain (Malenka and Bear, 2004) is the phenomenon of long-

term potentiation (LTP). Hereby, high-frequency (tetanic) stimuli are given to a presynaptic afferent. Furthermore, LTP can be induced by pairing presynaptic low-frequency stimuli with a large post-synaptic depolarisation (Caporale and Dan, 2008).

This phenomenon is the opposite to long-term depression (LTD). LTD is generated by low-frequency stimulation of the sensory afferent either alone or paired with a small depolarisation of the post-synaptic cell (Caporale and Dan, 2008; Dudek and Bear, 1992).

LTP and LTD have most extensively been studied in the hippocampus. The reason for the majority of studies focusing on LTP and LTD in the hippocampus is probably twofold: LTP and LTD have historically been suggested to be the crucial mechanisms for learning and memory (Kandel et al., 2012). Furthermore, the hippocampus possesses very clearly defined intra- and inter-areal connections of well defined cell-types.

One typical experiment would involve the afferent stimulation of Schaffer collaterals influencing one CA1 neurons activity. In these and other experiments several types of LTP and LTD have been identified for different cortical areas. N-methyl-D-aspartate (NMDA) dependent LTP, metabotropic glutamate receptor (mGluR) dependent LTP and other receptor and non-receptor based types of LTP have been described (Malenka and Bear, 2004). There exists variability regarding LTP and LTD mechanisms depending on neuronal cell-types, neuromodulation, network background activity and synaptic location (Sjostrom et al., 2008).

Not only the frequency for the presynaptic stimulation seems to be crucial for the induction of physiological changes, but also the precise timing between pre- and postsynaptic spikes (Bi and Poo, 1998; Markram et al., 1997). Action potentials (APs) and spikes are used synonymously in the present dissertation.

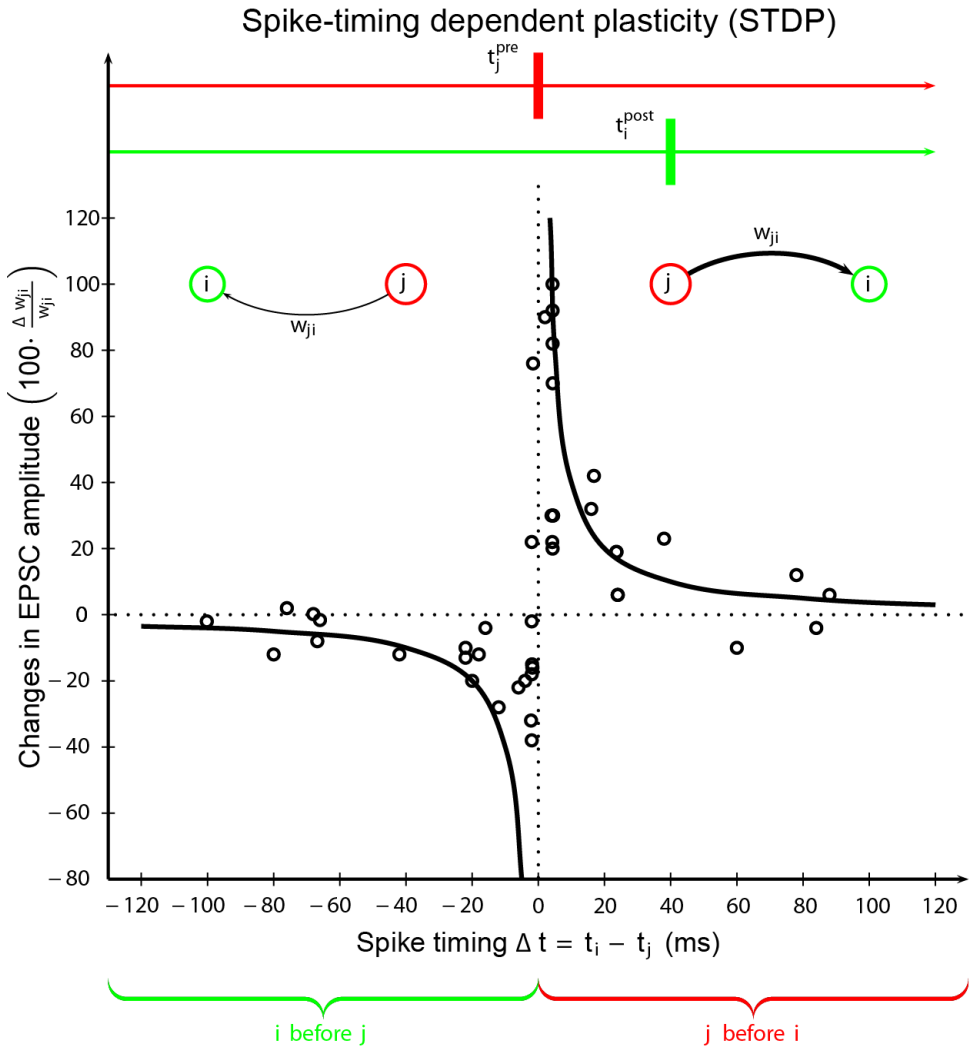

Figure 1.6: *Schematic illustration of spike-timing dependent plasticity (STDP).* The STDP function illustrates the change in synaptic connectivity $\frac{\Delta w_{ji}}{w_{ji}}$ in dependence on the relative timing Δt between pre- (neuron j, red circle) and postsynaptic (neuron i, green circle) spike pairings. Note that the type and parameter of the STDP function depends on the location of the neuron and on whether the pre- and postsynaptic neuron is an excitatory or inhibitory one (Caporale and Dan, 2008; Malenka and Bear, 2004). Experimental data (black circles) taken from Bi and Poo, 1998.

If a presynaptic neuron (Figure 1.6, cell j) fires an action potential before the postsynaptic one (cell i) and if the timing between APs is smaller than approximately 20 ms, the synaptic connectivity $\frac{\Delta w_{ji}}{w_{ji}}$ is facilitated.

Changes in synaptic strength or connectivity can be expressed as the change in excitatory postsynaptic currents (EPSCs).

On the other hand, if the postsynaptic action potential precedes the presynaptic one (Figure 1.6, cell i before j), the EPSC is suppressed within the aforementioned inter-spike interval. The time of presynaptic AP t_j relative to the postsynaptic t_i is determining the strength of synaptic modification.

Assuming the presynaptic neuron j receives $N \in \mathbb{N}$ and the postsynaptic neuron i $M \in \mathbb{N}$ spikes. In this case, the synaptic connectivity Δw_{ji} can be expressed as

$$\Delta w_{ji} = \sum_{n=1}^{N} \sum_{m=1}^{M} W\left(t_i^m - t_j^n\right) \tag{1.3}$$

where $W(t)$ is the STDP function (Gerstner et al., 1996; Zhang et al., 1998).

This function can be expressed by an exponential decay (for j fires before i) of the form

$$W(t) = A_+ = \exp\left(\frac{-t}{\tau_+}\right), \forall t > 0 \tag{1.4}$$

Note that the type and parameter of the STDP functions depends on the location of the neuron and on whether the pre- and postsynaptic neuron is an excitatory or inhibitory one (Caporale and Dan, 2008; Malenka and Bear, 2004).

One cellular mechanism seems to be apparent in various types of spike-timing dependent and independent LTP/LTD: The activation of a receptor (e.g. NMDA receptor) and the increase of postsynaptic calcium levels (Malenka and Bear, 2004). The NMDA receptor (NMDAR) is a likely candidate to be involved

in activating intracellular signal cascades in response to associative or spike-time dependent inputs leading to plasticity changes. The NMDAR serves as a coincidence detector. Presynaptic APs cause the release of the neurotransmitter glutamate and the postsynaptic depolarisation removes the Mg^{2+} block, allowing calcium to enter in the postsynaptic cell (Kandel et al., 2012). Small amounts of intracellular calcium lead to the activation of protein phosphatase I (PPI) leading to LTD, whereas high amounts of calcium activate calcium-calmodolin-dependent protein kinase II (CaMKII) and the induction of LTP. The metabotropic glutamate receptor (mGluR) on the other hand uses voltage dependent calcium channels (VDCC), which leads to an increase in intracellular calcium and the initiation of signal cascades.

In addition to the physiological changes, structural changes at the synapses or dendritic spines can occur and change the excitability of a network (Feldman, 2009).

All of these physiological and structural mechanisms of plasticity might occur in response to peripheral afferent stimulation. LTP/LTD-like mechanisms might change the properties of local neurons in important processing sites of ascending and descending systems. Location and cell-type dependent plasticity inducing mechanisms in isolation or together with modifications at identified sites of the CNS might lead to excitability changes and somatotopic reorganisation observed in human subjects in response to different afferent stimulation protocols.

The present study was designed to shed light on the underlying functional and structural changes as well as the mechanisms in the sensorimotor cortex induced by synchronous (associative) or asynchronous (non-associative) median and ulnar nerve stimulation (intervention) behaviourally and physiologically.

Whereas in studies on human subjects TMS-induced MEPs are used to assess corticospinal excitability before and after synchronous and asynchronous interventions, these measures provide only indirect measurements and limited

evidence regarding the underlying mechanisms induced by prolonged peripheral nerve stimulation (PNS). A similar limitation is true for behavioural and neuropharmacological assessments. These provide important indication about the relevant neurophysiological processess but remain to be verified.

The non-human primate model provides an excellent system to study the underlying cortical mechanisms induced by synchronous as opposed to asynchronous PNS. Extracellular recordings enable the investigation of local effects on identified and unidentified single units in the primary motor cortex (M1). This approach can be used as an assessment of locally-specific neuroplastic changes. Furthermore, the pattern of neuronal firing in conjunction with information about the cell type (PTNs versus unidentified M1 neurons and information regarding the activiation of specific units by peripheral stimuli) can be informative about key processes underpinning the mechanisms accounting for the neuroplastic changes in the sensorimotor system. The macaque monkey provides a particular good animal model because of its neuroanatomical and functional similarities to the human primate, especially with respect to the corticospinal system (cf. Lemon, 2008).

I hypothesis that the prolonged stimulation of the median and ulnar nerve should lead to behavioural, neurophysiological and structural changes in the sensorimotor cortex. The monkey's ability to perform selective thumb and index finger movements should be diminished after the synchronous median and ulnar nerve intervention. After the asynchronous intervention, the subjects should be able to move their digits normally or more selectively. Changes after both types of intervention (asynchronous and synchronous stimulation) should be visible in cell discharges in M1 evoked by peripheral stimulation. Since changes can be observed in TMS-induced MEPs, pyramidal tract neurons (PTNs) should be affected as well. Furthermore, it can be assumed that neuroplatic changes induced by PNS should occur in M1 neurons, which receive activation from all relevant peripheral stimuli. Relevant stimuli in this context are all muscles or

nerves stimulated during the intervention. For neuronal effects induced by the synchronous (associative) intervention, the latencies of the peripheral afferent inputs should be crucial. Finally, structural changes (e.g. changes in Parvalbumin-positive interneurons and perineuronal nets) induced by one week of synchronous (associative) PNS should be quantifiable by laminar-specific changes in cell and net count density.

For the purpose of studying these effects, two macaque monkeys were trained to a skilled and dexterous finger abduction task involving the selective and independent movement of either the thumb or the index finger. Methods for operant conditioning, optimised and refined training procedures, and the use of positive reinforcement training (PRT) techniques are described in Chapter 2.

Subsequently, the behavioural performance in selective thumb and index finger movements was compared before and after one hour of synchronous and asynchronous median and ulnar nerve stimulation. The synchronous intervention led to an increased number of errors and decreased performance measures. The number of errors decreased and the performance increased after the asynchronous intervention for both monkeys. The effects of the interventions on the behavioural performance are described in Chapter 3.

Next, cell responses of identified and unidentified neurons in primary motor cortex (M1) were assessed in response to singe-site EDC, median and ulnar nerve stimulation. The neuronal discharges of stable M1 units were compared before and after both interventions (synchronous versus asynchronous median and ulnar nerve stimulation). The population response of M1 units possessed a characteristic intervention-dependent response difference profile. Classification analysis of M1 neurons based on its activation by afferent input revealed important candidates for mediating neuroplastic effects induced by the interventions. LTP-like mechanisms might be responsible for mediating

these changes. The neuronal responses in M1 after the associative and non-associative interventions are described in Chapter 4.

In addition to the functional effects caused by these interventions, the structural changes in response to one week of synchronous (associative) median and ulnar nerve stimulation were assessed using immunohistochemical techniques. Using a novel automated detection algorithm, the laminar distribution of parvalbumin-positive interneurons, perineuronal nets (PNNs), and the co-localisation of these two entities were analysed on the stimulated (contralateral) in comparison to the control (ipsilateral) sensorimotor cortex. The results revealed significant differences on the stimulated sensorimotor cortex with respect to the laminar-specific distribution and co-localisation of these entities. The immunohistochemical techniques, cell and net detection algorithms, and laminar-specific results are described in Chapter 5.

Finally, the results of the present study are compared and discussed in the context of potential underlying mechanisms leading to functional and structural plastic changes in the sensorimotor cortex induced by peripheral afferent nerve stimulation. The discussion of these mechanisms and implications are described in Chapter 6.

Chapter 2. Refinement of training procedures: training two macaque monkeys to perform skilled and selective abduction movements of instructed digits

2.1. Summary and key findings

- Non-human primates are an ideal model to study independent dexterous finger movements (e.g. Schieber, 1991) and the importance of these has been stressed by a number of neurological conditions (e.g. focal hand dystonias, Hallett, 2011).
- To study these, behavioural training of two female macaque monkeys was refined utilising concepts of transfer, association and motor learning.
- Generalisation and transfer learning was enhanced by an increased number of familiar objects and procedures, and mostly positive reinforcement training (PRT) techniques were applied to condition monkeys to perform a finger abduction task.
- The qualitative results imply that transfer learning building on familiar objects and procedures was effective for most of the training procedures.
- Quantitative analysis of the monkey's weight, correct trial number, and performance indicated training procedure dependent effects. The monkey's weight and correct trial number were correlated between monkeys and the subjectively assessed stress level correlated with the total number of correct trials.
- The present study suggests several approaches to refine training procedures and discusses the use of a centralised database incorporating qualitative and quantitative measures to study cross-subject effects to improve training standards, the animal's welfare and performance.

2.2. Introduction

Human and non-human primates are distinct among mammals in terms of their ability to reach (Castiello et al., 1993; Gail and Andersen, 2006; Mason et al., 2004), grasp (Brochier et al., 2004; Castiello, 2005; Iwamura and Tanaka, 1996), and manipulate objects (Novak et al., 1993; Parker and Gibson, 1977; Westergaard, 1992). Even more, primates are uniquely able to move fingers (Sasaki et al., 2004; Schieber, 1991; Soteropoulos et al., 2012; Witham and Baker, 2015) independently and to use digits to handle, grip and manipulate small objects dexterously (Baker et al., 2003a; Jackson et al., 2003; Schieber, 1991).

The ability to perform independent finger movements has functionally been linked to the existence of direct monosynaptic corticospinal connections from the primary motor cortex (M1) to motor neurons in the spinal cord (SC) in human and non-human primates (Bennett and Lemon, 1996; Lemon, 2008; Olivier et al., 1997). However, in rehabilitation and recovery after corticospinal lesions or neurological conditions like stroke, other corticofugal (Sasaki et al., 2004) or brainstem pathways including the reticulospinal tract (Baker, 2011; Riddle et al., 2009) might take over some of these functions. Structurally, corticomotoneuronal (CM) cells are located caudally in the primary motor cortex (new M1, Rathelot and Strick, 2009).

Considering these functional and structural similarities between human and non-human primates with respect to dexterous finger movements, monkeys are an excellent model to study skilled finger movements and its corresponding kinematics and (neuro-) physiology.

This is reflected by a high number of studies using a non-human primate model to study highly skilled dexterous finger movements while performing a precision grip task (Baker et al., 2003a; Jackson et al., 2003; Lane and Dunnett, 2011), interacting with a modified Brinkman board (Freund et al., 2006),

or performing flexion or extension movements with each finger independently (Schieber, 1991).

The importance of investigating dexterous finger movements is even further underlined by studies investigating neurological conditions impairing the independent motion of the digits (Hallett, 2011). Examples of these focal hand dystonias (FHDs) are conditions commonly referred to as musician's dystonia (Chang and Frucht, 2013; Furuya and Altenmuller, 2013; Rosenkranz et al., 2005; Sussman, 2015) or writer's cramp (Braun et al., 2003; Hallett, 2006; Marsden and Sheehy, 1990).

Teaching a monkey a complex motor task, i.e. a finger abduction motion, involves several requirements, which enable successful motor learning. These include the practice, increase and maintenance of the monkey's motivation, cooperation, health, attention, feedback, control and focus.

Learning should therefore be optimised and automated by practice. Motor learning consists of a cognitive phase requiring focus and attention, an associative phase where novel skills are practised and unwanted behaviour is suppressed, and an automated autonomous phase in which movement patterns can be performed in a less attentive manner (Fitts and Posner, 1967). In a more recent review, motor learning has been distinguished into the following three parts: First, sensory information is extracted from all task relevant features and processed. This has to be done in an efficient way to be able to react to those sensory cues immediately. In the second cognitive part, decision making strategies are formed, which select an appropriate movement repertoire. In the final part, control mechanisms (e.g. feed-forward control) are in place during performing an action (Wolpert et al., 2011). Trying to enhance and optimise all of these parts, might increase the effectiveness of the training and lead to long-term plastic manifestations in the monkey's brain (Dayan and Cohen, 2011).

Furthermore, observing movements can be effective in acquiring complex motor patterns (Gatti et al., 2013).

Monkeys are usually trained using operant and classic conditioning techniques (Bloomsmith et al., 1998; Bloomsmith et al., 2007; Laule et al., 1996). More recently, a number of studies using positive reinforcement training (PRT) techniques reported improvements in the monkey's cooperation, husbandry and management routines (Coleman and Maier, 2010; Fernstrom et al., 2009; Graham et al., 2012; Laule et al., 2003; Perlman et al., 2012). Additionally, positive reinforcement training (PRT) can reduce undesirable, stereotypic behaviour (Bourgeois and Brent, 2005) and aggression (Minier et al., 2011).

The success of PRT training might closely depend on how consistently it is applied to a particular project or facility wide (Perlman et al., 2012).

Monkeys which were trained according to this approach showed improved health, welfare, motivation, cooperation and consequently performance (Coleman and Pierre, 2014; Minier et al., 2011; Perlman et al., 2012).

Increasing the monkey's health and welfare, reducing any potential stress, and refining training techniques to optimise the efficiency of monkey training applies the principle of the 3Rs (replacement, reduction and refinement) into practice (Russell and Burch, 1959). Reassessing and improving training procedures and methodology is therefore an important objective throughout the behavioural training of the present study. In fact, training itself can be an enrichment (Westlund, 2014), positively engaging the monkeys into diversified stages in training.

Monkeys are capable of building trans-situational and generalised correct response strategies during behavioural training which can enhance learning in novel situations (Warren, 1974; Washburn and Rumbaugh, 1991). This phenomenon is often called transfer or association learning. The degree and

success of transfer learning depends on the number of identical elements in the new context (Woodworth and Thorndike, 1901). More recent studies utilising a Pavlonian instrumental transfer learning hypothesis, state that cues rather than identical objects in the novel context are crucial and that association learning is reinforced using PRT (Cartoni et al., 2013). Furthermore, motivation has an influence on transfer (Pugh and Bergin, 2006) and reinforcement learning (Dayan and Balleine, 2002). Therefore, increasing the monkey's motivation by positively reinforcing crucial steps in learning might increase the monkey's overall performance. The neuronal mechanism of transfer learning has been studied (Obayashi, 2004). Transfer learning was applied to acquiring new motor skills (Choi et al., 2001) and to discrimination tasks (Schrier, 1966). It is important when switching from irrelevant to relevant features (Schrier, 1971).

Referring to the first part of motor learning (Wolpert et al., 2011), namely the sensory information extraction, it can be useful to use multi-modal sensory stimuli to indicate correct behavior. The learning effect can be even more enhanced by using a conditioned reinforcer (for example clicker) in conjunction with a positive reinforcer, namely the reward (Gillis et al., 2012). This feedback mechanism can help shaping the behaviour by enhancing processing in the cognitive phase of motor learning.

Another crucial aspect for the optimisation of the monkey's welfare and motivation, is reducing the stress level. This becomes especially apparent when considering that increased stress can cause neurological diseases like depression (Caspi et al., 2003; Pittenger and Duman, 2008) and mood disorders (Russo and Nestler, 2013). Before being able to evaluate the monkey's stress level, it is critical to assess the monkey's personality, anxiety and temperament in advance to the behavioural training since these aspects contribute to training success (Coleman, 2012; Coleman and Pierre, 2014; Coleman et al., 2005). A very recent study showed that stress can be reduced by optogenetically activating hippocampal cells associated with a positive memory (Ramirez et al., 2015). Behaviourally,

stress can be reduced by PRT (Lambeth et al., 2006) and by giving the subject choice and control over parts of the behavioural training (Roma et al., 2006). The latter has been shown to increase cognition and performance. This can be achieved by increasing the number of familiar objects, procedures and routines.

Managing primates and interacting with them sometimes requires the use of restraints (Bliss-Moreau et al., 2013; McMillan et al., 2014). Several improvements to training routines can be applied in this context. PRT can be applied to restraint training (Bliss-Moreau et al., 2013) and effectiveness can be increased by utilising sensory desensitisation techniques (Clay et al., 2009; Laule et al., 2003).

Although a PRT approach is in general favourable for most of the training routines, sometimes combining PRT with negative reinforcement training (NPRT) can be an effective alternative (Wergard et al., 2015).

In the present study, I trained two female macaque monkeys to perform a skilled motor task involving abduction movements of the index finger and the thumb. The motion of those was instructed by multi-sensory cues (tactile, visual and auditory).

The training was designed to improve aspects of the behavioural training by using mostly a PRT approach. Training procedures were refined under consideration of principles involving motor, transfer and association learning. Desensitisation and restraint techniques were applied and whenever possible replaced with more beneficial procedures and thereby increasing the monkey's health, welfare, cooperation, and performance.

All training procedures were qualitatively and quantitatively evaluated and analysed. Effects of subjective stress levels, cross-monkey correlations within particular stages of the behavioural training along with other physiological measures were analysed.

The results revealed that using a PRT approach together with establishing a constructive set of familiar objects, routines and training procedures enhanced training progression and motor learning. Alternative approaches for neckbar restraint training enabled stress-reduced and voluntary cooperation. The monkey's correct trial number and weight progression were training-procedure dependent and cross-monkey correlations existed. The subjectively assessed stress level correlated with the total number of correct trials.

These findings might lead to the development of several methods to improve the behavioural training of primates on complex motor tasks. The correlation effects and the influence of stress on training performance across the two monkeys underline the importance of a generalised (ideally faculty wide) database monitoring physiological parameters, cross-monkey effects, and indicators of performance. Such a system could along with a standardised scoring scheme incorporating (partly subjective) measures like the monkey's stress levels, anxiety status, and personality assessments reveal important correlations within the training process. This would increase the monkey's welfare and performance, and might help designing new effective ways of training primates based on predicted performance.

2.3. Materials and methods

2.3.1 *Animals*

Subjects for the current study were two female macaque monkeys (monkey S was six years and monkey U four years old). The average weight for monkey S was $6.30\,\mathrm{kg} \pm 0.04\,\mathrm{kg}$ and $4.76\,\mathrm{kg} \pm 0.04\,\mathrm{kg}$ for monkey U (mean $\pm$ SEM).

These monkeys were purpose-bred (Prescott et al., 2012a) at the medical research council (MRC) funded centre for macaques (CFM). Monkey S was born

in July 2008 and monkey U in January 2010 (Table 2.3).

Both monkeys were housed with each other during the time course of the behavioural training and the subsequent electrophysiological recordings. Monkeys at the comparative biology centre (CBC) are either housed in pairs or in small groups (e.g. four monkeys). This is important for the monkey's welfare and has some implications in terms of enrichment leading to desired and effective behaviour (Baker et al., 2012; Gilbert and Baker, 2011)

The monkeys had free access to water (and sometimes fruit juice), were usually fed with biscuits and a forage mix of seeds. Fruits and vegetables were usually given during the training sessions as rewards (Figure 2.2C). On non-training days, for example on the weekends, monkeys received specific fruits and vegetables as part of their diet.

After their arrival at CBC, monkeys got used to their cage environment for a few weeks before initial interactions and training occurred.

Monkeys participated in environmental enrichment routines performed at CBC involving the usage of toys and mirrors (Figure 2.1B). Foraging has beneficial effects in terms of the animals' general enrichment, increased locomotion (Griffis et al., 2013), training memory and associations (Glavis-Bloom et al., 2013). Therefore, monkeys received on a regular basis seeds and cereals mixed into the saw dust of their home cages. Prior to the implantation of a headpiece for head fixation (Baker et al., 1999; Lemon and Prochazka, 1984), monkeys had access to buckets filled with water as another form of environment enrichment (Robins and Waitt, 2011). These water buckets were utilised to give the monkeys the opportunity to swim and interact with the water.

During the time course of the training, monkeys moved to a new housing unit once in April 2013. Further home cage environmental changes took place in May 2012, September 2013 and July 2014.

Monkeys received a radio frequency identification (RFID) tag (Datamars, Bedano, Switzerland) in June 2012 for registering the monkeys' presence within a cage enabling access to an automated feeder.

Regular health checks and veterinary care were provided by the CBC. The monkeys got acclimatised to husbandry and research staff since their arrival at CBC in April 2012.

All experimental procedures were carried out under authority of licenses issued by the UK Home Office under the Animals (Scientific Procedures) Act 1986 and were approved by the Animal Welfare and Ethical Review Board of Newcastle University.

2.3.2 *Training procedures*

In the following, I am going to explain the different training procedures and intermediate steps in the monkeys' training until the final (finger abduction) task. Training procedures started in May 2012 after the monkeys got acclimatised to their environment (e.g. home cages) and to the weekly (cleaning and feeding) routines performed at the CBC.

Initial home cage interaction and carabiner training

The first training goal was adapting the monkeys to (human) interactions and thereby to decrease stress levels (Morgan and Tromborg, 2007), increase their confidence (Coleman, 2011; Waitt et al., 2002) and social behaviour (Baker, 2004). In fact, interactions with human caretakers increase the monkey's welfare and avoid undesirable behaviour like self-injury or vocalisation (Manciocco et al., 2009). In this context, different modes of vocalisation should be differentiated.

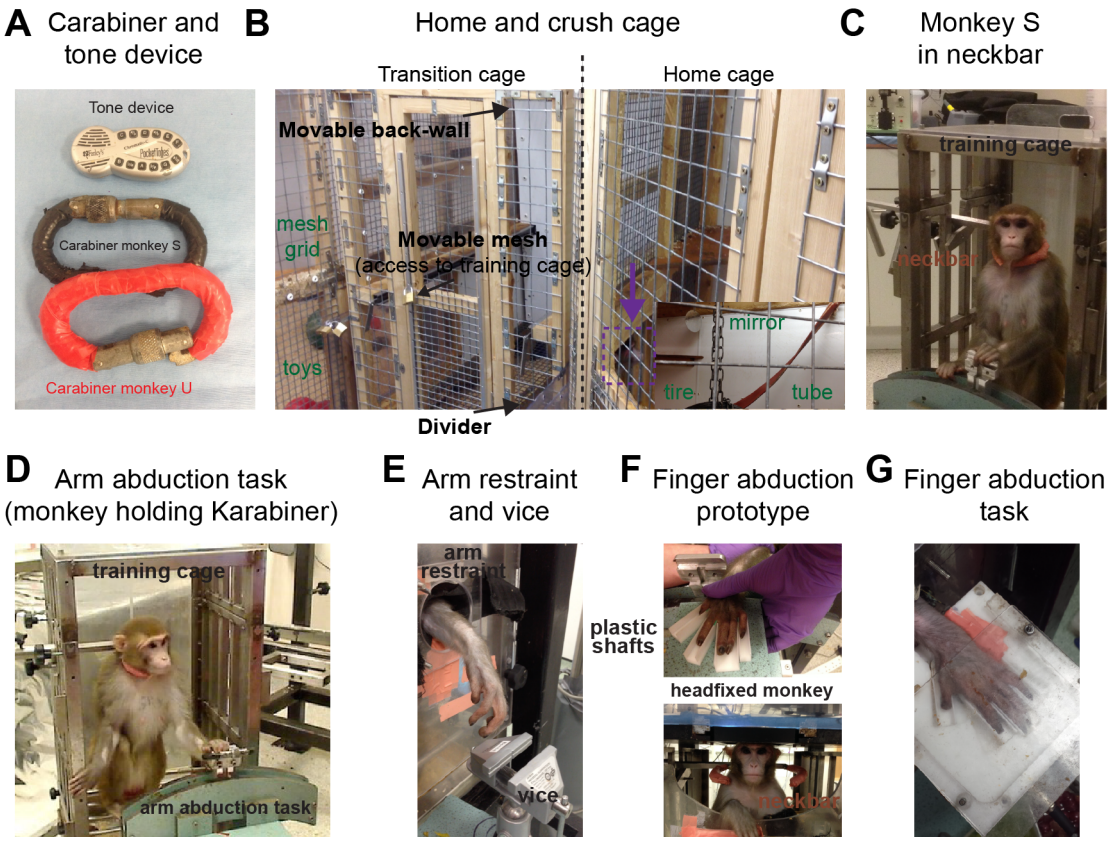

Figure 2.1: *Targets, objects, training equipment and setup.* (A) Tone devices and coloured carabiner (black: monkey S, red: monkey U). (B) Transition and home cage environment. Toys and features of environmental enrichment are shown in green. The purple arrow is showing a monkey moving from the transition to the home cage. The transition between these can be closed by using a divider (black arrow). (C) Monkey S in the training cage with the neckbar and the arm abduction task (carabiner not attached). (D) Monkey performing arm abduction task (carabiner attached). (E) Monkey in arm restraint. The vice for attaching the finger abduction task is positioned in front of the training cage on a movable table. (F) Monkey positioned its fingers in plastic shafts of the finger abduction prototype (top). Head-fixed monkey with opened neckbar waiting for the task. (G) Finger abduction task. Monkey positioned its hand voluntarily under a transparent plastic lid.

Whereas *warning* calls and vocalisation in response to injury or danger could be indicators of stress and discomfort, other vocalisations might possess a beneficial quality like calls for food (Hauser and Marler, 1993).

I used a coloured carabiner (Figure 2.1A) as a target allocated to each individual monkey during the first training stage. Reaching for or touching target objects

can be trained very quickly (Laule et al., 2003). I performed training sessions in a small group of four monkeys. Carabiners were attached to their home cages. The home cage denotes the cage in which the monkeys were *housed*. Monkeys received a fruit reward of a particular size (Figure 2.2C) each time the monkeys touched or held on to their assigned carabiner. Monkeys were not rewarded (neither punished when holding on to another carabiner (carabiner with a different colour). In this context *negative punishment* might refer to removing the individual's carabiner from the home cage and thereby stopping the possibility for receiving any reward.

Operant conditioning techniques were used to train the monkeys to the desired behaviour (Laule et al., 2003).

During all stages of the training, I tried to favour PRT to negative reinforcement or positive and negative punishment. PRT strategies are beneficial reducing the animal's stress level, increase the monkey's collaboration, increase training efficiency (Fernstrom et al., 2009), and increase the animal's welfare (Coleman and Maier, 2010). Positive reinforcement training (PRT) can be defined as a strategy in operant conditioning, which by adding a desired stimulus, item or object *enhances* the subject's behaviour. Positive punishment leads to a *reduction* in the behaviour caused by adding an undesirable (e.g. noxious) stimulus, item or object. Negative reinforcement training (NRT) on the other hand side aims for enhancing the subject's behaviour by removing a undesirable stimulus, item or object. Negative punishment causes a reduction in the behaviour caused by removing a desired stimulus, item or object. It is important to stress that positive and negative in this context of behavioral psychology describe adding and removing an entity rather than a qualitative description.

Although a PRT strategy was favourable throughout the training process, sometimes it was necessary to show the individual monkey the consequences of particular and unwanted (not compatible with the training goal) behaviours. This was for example necessary during the arm restraint training. In these situations consequences in form of adding additional objects were used (e.g. positive punishment: adding constraint or limiting space). In some situation a mixed positive and negative reinforcement training program can be favourable (Wergard et al., 2015). This was for example the removal of the back wall of the transition or training cage (negative reinforcement) in combination with a target object and a reward (positive reinforcement).

In addition to the coloured carabiner, I used a tone device (Figure 2.1A) to bridge the monkeys' correct behaviour (touching and holding the carabiner) with the reward (Laule et al., 2003). In a number of studies, a clicker was used for this purpose, but here I used a tone device (Farley's Chromatic C Pocket Tones) as a conditioned reinforcer to the positive reinforcer (fruit reward). This had several advantages: A different tone could be allocated to each individual monkey and transfer of skills to similar or different contexts was possible.

I used both the individually allocated coloured carabiners and the tone device as familiar objects to enhance transfer and association learning (Warren, 1974; Washburn and Rumbaugh, 1991). For this purpose every step in the training was designed to build on these familiar objects (Figure 2.3A and 2.3B).

Training sessions were performed from Monday to Friday and whenever possible at consistent times of the day. Frequent training with macaque monkeys has been shown to be most effective when performed regularly and at consistent times (Fernstrom et al., 2009).

Transition cage and divider training

Being able to move the monkey to another cage to separate an individual from the group is sometimes necessary in the daily research routine or for planned and unexpected health checks and examinations. The next stage in training was the transition cage training. The transition cage enables the possibility to minimize the space moving the back wall forwards and thereby moving the monkey to a different position or cage. Additionally, a closer examination of the monkey for health assessments is possible in this position.

The aim for the transition cage training was to be able to move the animals voluntarily to a different cage, where the monkeys could be shut in (and thereby separated from other monkeys) with a divider. Some facilities have procedures in place forcing the monkey actively (mostly by positive punishment) to a desired location. This procedure increases the stress levels and brings the monkey to the training session already in an upset state. Therefore, it can be beneficial to make the monkey move to another cage in a calmer, more playful and effective way. For this purpose I used familiar targets and cues in this new situation (Figure 2.3A and 2.3B). Furthermore, I used operant conditioning to desensitise the monkey reacting to touching and moving the divider. Likewise, including familiar objects (targets) and an already familiar training routine (carabiner training) to a novel context increased the monkey's confidence in this new situation. But not only the targets were familiar to the monkeys, also the tone that was used for bridging the desired behaviour to the reward served as a familiar cue. Objects and cues, which were transferred to the next stage were colour, tone (auditory cue), target (carabiner), divider, and additional interpersonal cues (Figure 2.3A and 2.3B).

Training cage training

The next desired training outcome within the overall training process was to be able to move the monkey to the laboratory within a training cage. A training cage is a smaller movable cage, which can be modified for experimental purposes. As in the context of the divider training, being able to move the monkey to another room is also beneficial from a medical perspective. Furthermore, the custom made training cages can be dissembled which allows for task-specific adaptations (see Figure 2.1C and 2.1D). Thus, monkeys had to learn to enter the training cages and to get used to the limitations in space. Since monkeys are not used to such limitations in space, this could be a potential stress factor within the overall training. Similar to the transition cage training, which served as the transition step between the home cage and training cage training (this is the location where monkeys entered the training cage), this step could be performed by forcing the monkey into the training cage. My goal was to minimize all potential stress factors by applying the following principles: Use of familiar objects, targets and cues to condition the monkey to the new situation by positively reinforcing the wanted behaviour (Coleman and Maier, 2010). Therefore, I started attaching the training cage to the transition cage, which basically built a tunnel between the transition and the training cage. I alternatingly positioned the carabiner on the transition cage in close proximity to the training cage and at various places within the training cage. Thereby I was gradually rewarding the monkey to enter the training cage by using familiar training routines. At a later stage in training, monkeys had access to an automated feeder. Since this automated feeder was incorporated into a training cage which itself was attached to the transition cage, the feeder training served a similar advantageous effect: Getting the monkey comfortably and voluntarily entering a smaller space. Both the carabiner and the feeder training served in this situation the purpose of forming a positive association with the training cage. After the monkeys were more comfortable with the training

cage and actually fully entering it, monkeys gradually got used to be shut into the cage with another divider similar to the one used within the last training step. Comparable desensitisation routines (touching, moving and shaking the divider) were used to achieve this intermediate goal.

Lab training

At this stage it was possible to relocate the monkeys to the laboratory to perform a training session in a different environment. It was essential to rely on familiar objects and routines (Figure 2.3A-2.3C) in this context. Since the new context can cause stress, it was important to get the monkeys used to the different environment deliberately. Initially these training sessions lasted only for 10 to 20 minutes before sequentially increasing the session length. Thereby the monkeys received constantly more rewards and accordingly an increased amount of food over time. Furthermore, the monkeys got used to the laboratory environment. It is absolutely crucial, especially in novel situations, to be able to rely on as many familiar objects and routines as possible. These include in addition to the aforementioned objects also people, clothes, toys and other monkeys. Therefore, I decided to perform the training in the laboratory initially with a smaller group of two monkeys. This had not only the advantage of increased confidence among the subjects, but also made use of the previously established group training dynamics.

After a few weeks of training in the laboratory, the monkeys were slowly introduced to individual training sessions. Although this resulted in the loss of one known aspect of the training, the monkeys by now were much more confident in their new environment and the laboratory itself in addition to the normal (carabiner) training routines.

Towards the end of the training in the laboratory, monkeys got introduced to a

relevant item used in the next stage of the training, the neckbar (Figure 2.3C, 2.3D and 2.3F). At the beginning, monkeys were rewarded whenever they were touching the neckbar.

Neckbar training

After the monkeys got used to touching the neckbar, the neckbar was moved into close proximity to the monkey. The monkeys still got rewarded for touching the neckbar. After the monkeys got fairly confident with the neckbar, the training routine was slightly modified. The monkeys were instructed to hold on to their carabiner while the opened neckbar was positioned behind the monkey's head. Next, the monkeys were rewarded whenever their neck was touching the neckbar behind them.

Using a reward tone and thereby bridging the correct behaviour to the reward additionally enforced this correct behaviour. By that, the neckbar itself and the act of positioning their head within it became effectively reward associated.

This procedure required two trainers to be involved in one training session. One person would sit in front of the training cage and reward the monkey for the previously described correct behaviour. The other person would sit at the side of the training cage and hold the neckbar into the desired position.

Involving two trainers in the training procedure was especially important for the next aspect of training. Here the monkeys got used to slowly closing the neckbar. This was accomplished by deliberately indicating to the monkey that the correct behaviour in this situation involved waiting for the trainer to move the neckbar before the tone and finally the reward was given.

The final and most crucial step in the neckbar training involved being able to completely close the neckbar. Before this stage in training, the monkeys were

already very confident with the neckbar being almost completely shut.

When shutting the monkey completely into the neckbar, it was essential to counteract any potential stress evolving from this situation. Therefore, the monkeys were heavily rewarded when the neckbar was completely shut. Initially the time one monkey spent in the neckbar was deliberately kept short. This time was gradually increased. The next few training sessions after closing the neckbar completely were the most crucial ones. Monkeys were a bit wary about the neckbar at first before the positive value (reward) of positioning the head into the neckbar prevailed.

After a few weeks of neckbar training, the monkeys got more comfortable in this situation. Next, the neckbar was fixed at the side of the training cage. The training cage could now be safely disassembled (removing the front door and the top of the cage).

Arm abduction task

The benefits of transfer learning as explained in the context of familiar objects and procedures can also be applied to procedures involving teaching a desired motion (motor behaviour). Considering that the final goal within the monkey training is teaching the monkeys to perform (selective) finger abduction movements, intermediate training steps were designed to teach the monkey general training concepts as well as motion specific ones.

Therefore, the goal for the next stage in training was to teach the monkeys the concept of an abduction movement. Rather than starting immediately with skilled abduction movements of the fingers, monkeys were taught to perform this kind of motion with their preferred arm. This was for both monkeys the left arm.

For the purpose of teaching the monkeys the arm abduction movement, a movable metal bar was positioned in front of the training cage. At the top of this custom-made bar it was possible to attach the carabiner used in previous stages of the training. Therefore, it became immediately apparent to the monkey to hold on to the carabiner. Since the resistance of how easily the metal bar moves could be adjusted by using various kinds of springs, a very low resistance was chosen initially. By that, whenever the monkey touched the carabiner, the metal bar moved consequently resulting in a correct trial and a subsequent reward.

The resistance was gradually increased throughout the training sessions teaching the monkey to perform an actual (abduction) movement rather than just holding on to their carabiner. Soon only trials were rewarded in which the monkey performed a complete arm abduction movement all the way to the side of the task (Figure 2.3C and 2.3D).

Because the monkeys tried to initiate a motion as quickly as possible to minimise the time to the next trial (and thereby the reward), it was important to introduce an instructed delay period. At first this time period was of arbitrary length simply rewarding any delay before the motion onset. To teach the monkeys the instructed delay before the motion onset more systematically, a vibration cue was introduced.

Vibration cue and instructed delay training

To be able to teach the monkeys the instructed delay period more reliably a rod-shaped vibration disk was positioned within the metal bar where the carabiner was attached. This vibration disk could be manually activated by the trainer causing the whole metal bar including the carabiner to vibrate.

To enforce the delay before the motion onset, monkeys were rewarded for holding on to the carabiner, but not moving, by using low value rewards. These were

usually pieces of apple (cf. Figure 2.2A-2.2C). After a very short delay period, the vibration was activated and monkeys were immediately rewarded for any kind of movement (even before completing the full abduction movement) with a high value reward (cf. Figure 2.2A and 2.2B).

Trials in which the monkey moved before the cue were not rewarded and the metal bar including the carabiner was returned to its start position (the middle position). After the monkeys started to form the mental association between the vibration cue and the motion onset, just holding on to the carabiner was not rewarded anymore.

Next, the overall movement in response to the vibration cue was refined with respect to performing the complete abduction movement. The trial number and the session length were gradually increased.

Until this stage of the training, the monkeys got familiar with the behavioural relevant target (carabiner), the auditory (delivered by the tone device) and tactile (vibration) cues, the training routine including the neckbar, the laboratory environment (Figure 2.1A, 2.1C and 2.1D) and the rewarding scheme (Figure 2.2A-2.2C).

Before actually making the transfer from the arm to the finger abduction movement one more essential preparatory step needed to be taught to the monkey. This involved fixing (restraining) the monkey's preferred arm to a position where it can be safely placed into the movable plastic shafts.

Arm restraint training

Before actually fixing the monkey's arm into the desired position, it was essential to prepare the monkey to accept direct physical interaction in the form of holding the monkey's hand and arm.

This was achieved by operant conditioning and positively rewarding the monkey whenever the instructor touched the monkey's hand or arm.

The arm restraint consists of a sleeve and a bar, which can be fixed to the side of the training cage. At the stage where the monkey got used to an experimenter holding its arm, the sleeve was positioned around the monkey's arm while rewarding the monkey's cooperation. Once the monkey got confident with this procedure, the sleeve was actually positioned around the monkey's elbow and the arm restraint was fixed to the side of the training cage. Once the arm was completely fixed, the monkey was rewarded with high value rewards (Figure 2.2A and 2.2B). The length of this fixation was stepwise increased. Towards the end of the arm restraint training the monkeys got presented with the plastic shafts before moving on to the next step in training.

Finger abduction prototype

To support the transfer from the arm abduction task to the finger abduction movement, another intermediate step in training was added.

For this purpose a simpler version of the final finger abduction task was utilised to improve the monkeys hand and finger position. Hereby, monkeys were only rewarded when each digit was positioned in its corresponding plastic shaft.

Initially it was necessary to help the monkey to position its fingers in the corresponding plastic shafts by manually guiding fingers to the correct targets.

During the time period of the finger abduction prototype training the monkeys learnt to use the plastic shafts and to position their fingers comfortably in these. Thus, they were able to form a positive association with the task itself.

The final stage in the finger abduction prototype training was selectively rewarding whenever the monkey positioned its hand correctly and performed a motion with any of the digits. This formed a solid basis for the final training stage.

Finger abduction task

The final stage in training involved a custom made task capable of measuring the finger position, applying a resistance (force) to the finger abduction motion, and delivering a focal finger-specific vibration cue. The electronic circuits controlling the vibration, the motors and the position encoders were software controlled and could therefore be modified to fit the needs of the current stage in training (see Section 2.3.5).

In addition to the above-mentioned sensors and encoders, coloured LEDs that were positioned on top of the thumb, index and the little finger were used as additional cues to the vibration ones. These LEDs were not positioned directly on top of each digit but rather on top of a transparent plastic piece (lid) positioned over the monkey's hand.

The initial stage of the finger abduction task training was exactly the same as described before in the context of the finger abduction prototype training. Monkeys were instructed to position their fingers in the corresponding plastics shafts. Whenever the monkey performed any movement with any finger, the monkey received a reward.

After the monkey got used to this new training device, the training focused on the motion of the index finger only. The vibration cue for the index finger was activated and the motor forces for this finger were kept at low values. Thus, the slightest movement of the index finger in response to the vibration cue resulted in a correct trial.

Although initially the monkey moved all fingers equally to achieve the correct trial, this behaviour changed over time once the monkey learnt the meaning of the cue (association to movement initiation).

To promote a full finger abduction movement, the motor forces were increased so that the monkey had to overcome both an initial as well as a constant second force (spring constant).

After the monkey achieved a reasonable performance level, another preparation had to be practised before switching the finger abduction task to another digit.

Since the monkey's strategies for accomplishing this task varied substantially between individuals and due to the fact that the fingers tend to slip out of their corresponding plastic shafts, it seemed beneficial to provide support for those fingers. This was achieved by using a plastic lid fixed on top of the fingers. But instead of introducing another restraint, the monkeys were taught to slide their hand and thereby their fingers voluntarily into the plastic shafts under the coverlid. At this time, the finger abduction task was already highly associated with the rewards, which increased the monkey's motivation.

Next, the selectivity of the index finger movement was improved. Gradually decreasing the threshold values of the non-instructed fingers and increasing the threshold value for the instructed finger accomplished this. The thresholds were defined as the minimum force values registered by the force encoders in response to a finger abduction movement.

After a reasonable force, motion, selectivity and performance level was established, the focus of the training moved towards the thumb. The training strategy in this context was exactly the same as described in the paragraph about the selective index finger movements. At the beginning it was necessary to minimise the force values of the thumb and thereby reinforcing any motion of the new finger. The threshold values of the non-instructed digits were temporarily

deactivated. Instead of presenting the monkey with an error tone for each non-instructed motion, only correct trials were reinforced by using a tone (same frequency as the already familiar one from earlier stages in training) and the subsequent reward. Once the monkey grasped the correct behaviour, the force values were again increased. After that the threshold for the non-instructed digits was introduced.

After only a few weeks of training the thumb, the final stage in training the finger abduction task was reached. The last part in training focused on alternating between the index finger and the thumb. For this purpose it was essential to stress the importance of the tactile cue. This was accomplished by introducing the LEDs. This was occasionally stressed even more by manually pointing to the instructed finger. The values regarding the selectivity of the non-instructed fingers were reduced during this stage of the training. After a couple of training sessions the monkeys were able to associate between the multimodal cues and the desired movement. At this point in time the training could be optimised with respect to the selectivity of the movements.

After reaching the desired selectivity the force values of the motors were increased to increment the effort required to move the instructed fingers. Finally, the instructed delay and the holding time (the time during which the abduction against the motor force was maintained) were increased.

Surgical preparation

Monkeys were implanted with head pieces enabling electrophysiological recordings and atraumatic head fixation. Atraumatic head fixation refers to the monkey's learnt behaviour of acclimatising to the immobilisation of the head during experimental procedures. In addition to the implant of the head piece, monkeys were implanted with electromyography (EMG) electrodes enabling

recordings of task relevant and control muscles. For details regarding the surgical procedures see Section 4.3.2.

Head fixing the monkey

After surgical wounds were fully healed, the behavioural training continued.

For electrophysiological recordings it is essential to be able to fix the monkey's head into a static position enabling stable recordings from single neurons within the cerebral cortex. The fixation of the monkey's head is usually accomplished by fixing screws on top of the monkey's head piece to a solid frame. Since this head restraint technique can be quite unusual for the subject and cause potentially stress, the goal during this stage of the training was to build a positive association with the head fixation process by effectively rewarding the monkey while being head-fixed. The time, while the monkey was head-fixed, was gradually increased.

Other procedures

In addition to the aforementioned training procedures, a few minor additional training procedures were taught. These included laboratory interactions especially post surgery, which usually involved target (carabiner) training. For example, the monkeys were taught to offer any hand (or foot) for examination within the training cage. Another training procedure was related to the automated feeder training. As assessment for the effect of the automated feeder training, monkeys performed for two weeks a simple button press task. Training sessions regarding the button press task were performed by two different trainer (which were already familiar to the monkeys). The length of these sessions was kept short to approximately $30\,\mathrm{min}$. Procedures regarding the automated feeder and

the related assessments did not interfere with any of the main training procedures and were not further analysed within this thesis.

Rewarding scheme, fruit hierarchy and restricted feeding

At the start of the overall training an assessment of the fruit reward hierarchy for each individual monkey was performed (Figure 2.2A-2.2C). Quite substantial differences regarding the preferred food type for each individual monkey were identified. Knowing the exact ”value” of each particular reward type enabled an improved and more consistent rewarding regime. By sorting the different kinds of fruits and nuts into a weighted order, the subject's performance was enhanced and the session length increased. Furthermore, the size of each piece of food was kept reasonably consistent.

Before committing to a strictly hierarchical rewarding scheme different rewarding strategies were tested. These included a mixed and partially mixed rewarding scheme. The strictly hierarchical rewarding scheme however seemed to be the most effective for monkey S and U.

Within the hierarchical rewarding scheme the number of pieces per food type was stepwise increased. Therefore, monkeys were conditioned to a specific number of rewards per type, which made an approximation of the total number of trials per session possible.

This strategy did also help to provide consistency for the monkey's food expectation for an individual training session.

Knowing the exact value of each kind of food helped shaping the difficulty level with respect to the task parameter throughout the training session. More difficult parts of the task or new aspects could hereby be introduced towards the end of a training session when the monkey already performed high numbers of trials.

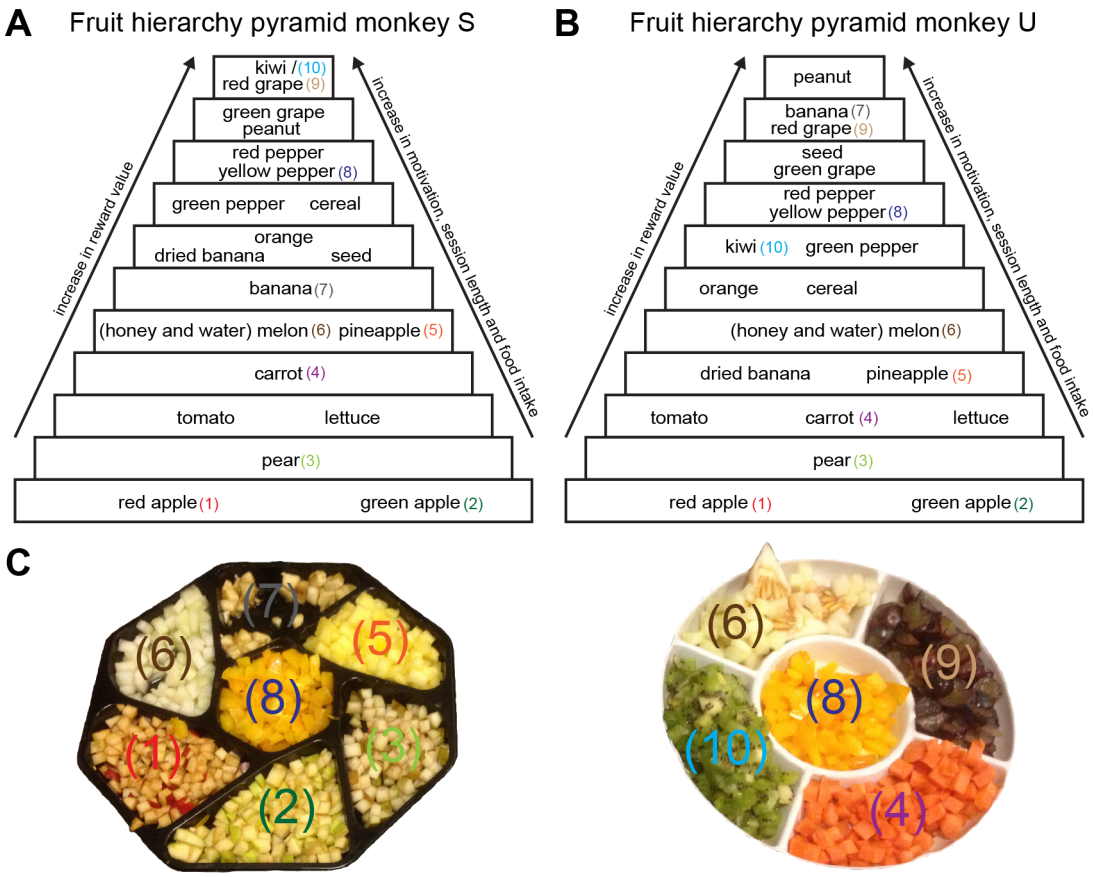

Figure 2.2: *Fruit hierarchy assessment.* (A) Fruit hierarchy pyramid for monkey S. (B) Fruit hierarchy pyramid for monkey U. In a typical training session 10 different reward types were given (cf. coloured numbers). (C) Selection of fruits for an example training session. The piece size was kept constant for each type of fruit. Note that red grapes which are among the highest value rewards were intentionally kept in bigger pieces to increase the motivation within the last few trials of each training session even more.

Using the highest rewards for introducing new aspects of the task turned out to be a reliable approach.

The monkey specific fruit hierarchy was determined by the following method: Monkeys were offered two alternatives of food presented on the trainer's left and right hand. The food chosen first by the monkey was registered. Whether the food was presented on the trainer's left or right hand was randomised. This procedure was repeated 5 times for each pair of fruit or nuts. Using this method (binary choice test), it was possible to create the ranked order of fruit shown in

the monkey specific fruit hierarchies (Figure 2.2A and 2.2B).

During the time course of training, monkeys were fed in their home cages with biscuits and seeds. They had free access to water and occasionally fruit juice.

Building a set of familiar objects and procedures

Monkeys are capable of learning concepts, semantics and behavioural routines which they can apply to different contexts (Warren, 1974). Since the success of applying these in a new context depends on the number of familiar objects (Woodworth and Thorndike, 1901) in these novel situations, it is favourable to introduce many familiar objects and utilise them in subsequent training procedures (Figure 2.3A and 2.3B).

Therefore, the various training stages were designed to incorporate this principle. During the first stage of the training, namely the home cage carabiner training, monkeys got used to the coloured carabiner and the presentation of a tone used to enforce the desired behaviour (touching the carabiner). Nevertheless, the above mentioned principle did not only apply to objects. Familiarity and trust to people (including animal trainer, research, technical and husbandry staff) was absolutely essential and determined the success of the behavioural training (Baker, 2004; Coleman, 2011; Manciocco et al., 2009).

The set of familiar objects (Figure 2.3A and 2.3B) was further increased with training procedure specific objects, tools and toys ranging from instrumental to behaviourally relevant and task-related objects. Early introduced ones became more familiar, reinforced and semantically associated. Thus, these objects could also be used to signal or emphasise specific parts of a training session. Additionally, relations and association chains could be established.

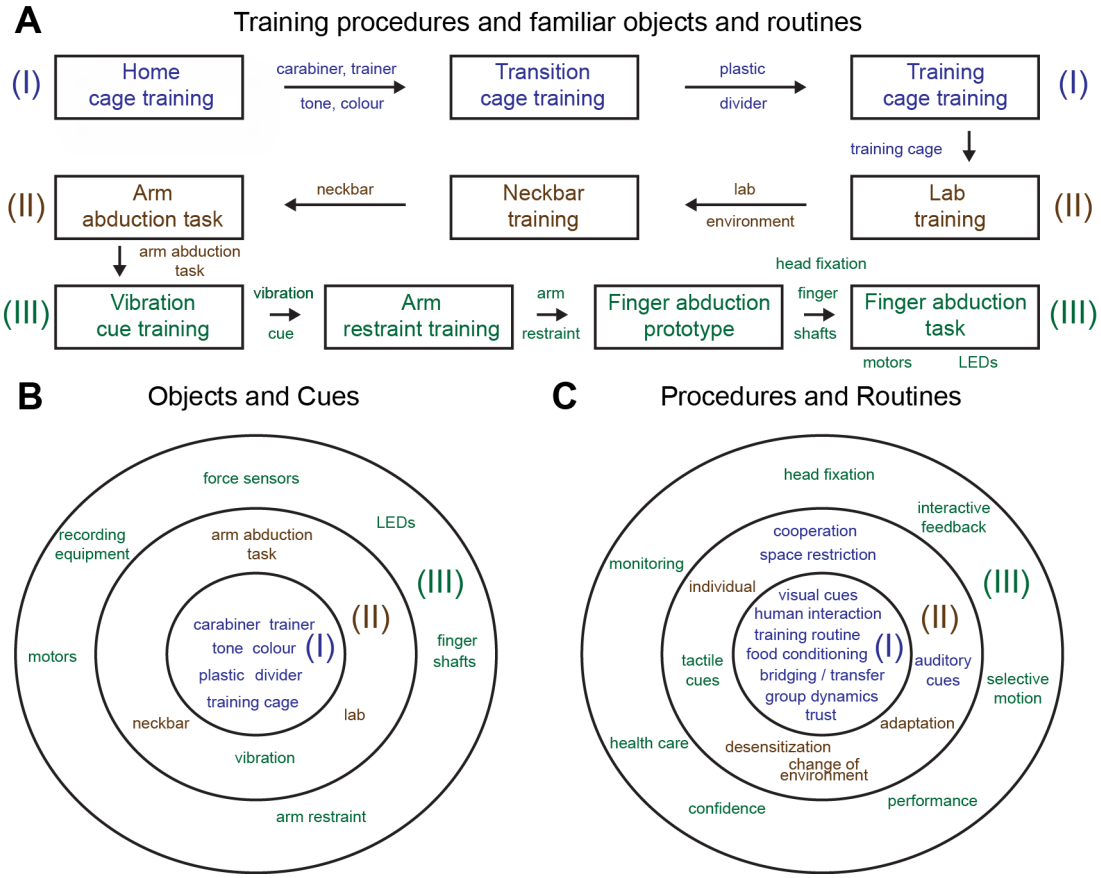

Figure 2.3: *Set of familiar objects and routines*. (A) Overview of all training procedures and transitions divided into stages (I-III). Each arrow indicates the transition from one procedure to the next. In each stage of training (procedure) new objects were introduced and thereby increasing the set of familiar objects (B). The amount of objects and targets increased from early (I) to later (III) stages in training. (C) During the time course of the training the set of familiar objects, procedures and concepts was equally increased. Both objects and procedures served as building blocks for the next subsequent training procedures.

Showing the monkey a target (carabiner) prepared the monkey to perform (or not perform if it is the wrong target or colour) an action (holding, touching or pulling on the carabiner). The correct behaviour was enforced by a signal tone before a reward was given. Within this association chain the (familiar and semantically associated) objects were assigned to relations. These relations, routines, procedures and associations (Figure 2.3C) could be taught to the monkey. Furthermore, these routines and procedures became familiar themselves, could be transferred to novel contexts, and increased the monkey's

cognition, performance and welfare.

Another aspect important in the context of familiar objects and routines is teaching the monkey to have control over these items and concepts. Choosing to interact with a target or to utilise it, will have an expected (effective) outcome. This expectation itself could positively reinforce the monkey's motivation and learning process. Furthermore, giving monkey the choice and control over situations has been shown to reduce the stress level (Roma et al., 2006).

2.3.3 *Subjective personality assessment, stress and social hierarchy*

Monkeys live together within a social dominance hierarchy (Varley and Symmes, 1966). This hierarchy is associated with the monkey's serotonin, dopamine and cortisol levels (Riddick et al., 2009; Weiger, 2007; Yodyingyuad et al., 1985). Therefore, it is crucial to assess the monkey's individual social rank within its group as early as possible before starting to focus on specific training methods. Knowing the social rank helps to design experiments and training procedures. Furthermore, the social rank does affect the animal's stress level and thereby influences the training effects (Devilbiss et al., 2012; Faraji et al., 2014). From an animal welfare point of view, reducing the stress level (by interactions and prioritisation based on the assessed hierarchy) increases the animal's wellbeing and reduces the risk of disease in non-human (Reimers et al., 2007; Riddick et al., 2009; Sapolsky, 2005) and human primates (Lundberg, 2006). At the monkey's arrival at CBC in April 2012, monkey U and S were housed in a group of four monkeys together with monkey T and Y. Due to instabilities within their social dominance hierarchy (monkey S and Y were competing for dominance), the monkeys were split into groups of two monkeys early 2013.

Score	Category key word	Description
0	calm	calm, relaxed and/or very cooperative
1	normal	no signs of distress, discomfort and/or nervousness
2	fidgety	fidgety, hectic, impatient and/or skittish
3	upset	upset, challenging and/or aggressive towards cage mates or trainer
4	nervous	nervous, very excited, very fidgety and/or very skittish
5	pain	clear distress and discomfort, signs of pain, (self-) injury and/or pronounced stereotypic behaviour

Table 2.1: *Stress assessment and scoring system*. The monkey's stress level was subjectively assessed based on a scoring system. The stress level was assessed at the end of each training session.

Within the group of two, monkey S was the clearly dominant one whereas monkey U was submissive. Additionally, monkey U as being the youngest one always indicated very submissive behaviour. At the same time, monkey U was not very confident and acted fidgety alone and among its cage mates. Monkey S on the other side was confident, relaxed and calm. Training procedures and interaction training, increased the monkeys' confidence and reduced the subjective stress level.

The fidgety behaviour of monkey U was reduced with training but persisted throughout the time course of training.

Since the monkey's individual temperament affects training performance and success (Coleman et al., 2005), using a PRT approach with increased interaction and enrichment (Figure 2.1B), familiar objects (Figure 2.3A and 2.3B), and training routines (Figure 2.3C) improved the monkey's temperament beneficially, enhancing the training efficiency.

The monkey's stress level was assessed within each training session based on a scoring system (Table 2.1) ranging from calm to very stressed behaviour.

Score	Evaluation	Description
0	did not work	procedure did not work at all
1	did not work with restrictions	procedure might have worked with more time
2	neutral	procedure did work, but not sure whether improvement
3	slight improvement	procedure worked, slight improvement
4	improvement	procedure worked well
5	strong improvement	procedure worked perfectly

Table 2.2: *Criteria for subjective training procedure assessments*. Scoring system for assessing the training procedures.

2.3.4 *Qualitative assessments and evaluation criteria*

While quantitative data was analysed based on statistics, qualitative data was assessed based on the following criteria. The beneficial and unfavorable aspects of a particular procedure, method or strategy during the training process were summarised and whenever possible subjectively categorised based on a scoring system (e.g. Table 2.1). Potential confounding factors were discussed and interpreted within the appropriate context.

Furthermore, each training procedure was evaluated based on the overall outcome of these particular stages in training. The evaluation was performed using a scoring system (Table 2.2). The scoring system included evaluations ranging from no improvements until various degrees of progress.

Additionally to this subjective scoring system, the advantages and disadvantages of each stage in training were summarised (Table 2.4).

2.3.5 *Data acquisition and analysis*

Data acquisition consisted of acquiring two kinds of data: qualitative aspects of each training s(ssion were sampled within laboratory diaries. Quantitative training parameter were sampled using a custom written Python (Rossum, 1995) library (Python, Version 2.7.9, `https://www.python.org`) with the graphical user interface (GUI) library `Tkinter` (Tkinter, Version 2.4, `https://wiki.python.org/moin/TkInter`). For more details about qualitative assessments see Section 2.3.4.

Parameters like the monkey's weight, the length of the training session, the correct trial number, the number of errors, (if applicable) the amount of drugs given for a particular medical condition, the amount of food intake, and the training procedure category (Figure 2.3A) were sampled.

In addition, some of the qualitative parameters like the monkey's stress level were assessed based on a subjective scoring system (Table 2.1).

All of these parameters were saved and transferred to a normalised relational database (Codd, 1970). For this purpose a `SQL` database was used (SQLite, Version 3.8.10, `http://www.sqlite.org`). Database creation and query-based access (for sample queries see appendix A) were realised using the Python toolkit and object relational mapper `SQLAlchemy` (SQLAlchemy, Version 1.0.5, `http://www.sqlalchemy.org`).

Several parameters including the total number of training sessions, weights, correct trial numbers were visualised (cf. different coloured marker for specific training categories, Figure 2.4A, 2.4B, 2.5A, 2.5B, 2.6A and 2.6B).

The monkey's training performance $\rho_{\mathtt{training}}$ was expressed as the ratio between the correct trial number $n_{\mathtt{trials}}$ per session length $l_{\mathtt{session}}$ in $\mathtt{min}$:

$$\rho_{\texttt{training}} = \left(\frac{n_{\texttt{trials}}}{l_{\texttt{session}}} \right) \tag{2.1}$$

The correlation of parameters between monkeys was calculated using the Pearson product-moment correlation coefficient r_{xy} (Pearson, 1895)

$$r_{xy} = \frac{(\sum_{i=1}^{n} x_i \cdot y_i) - (n \cdot \overline{x} \cdot \overline{y})}{(n-1) \cdot s_x \cdot s_y} \tag{2.2}$$

for n data points, with the means $\overline{x}$ and $\overline{y}$, and the standard deviations s_x, s_y.

Statistical significance testing of datasets with different length (cf. Figure 2.10A and 2.10B) was performed using a two-sample t-test ($p < 0.01$).

2.4. Results

Training procedures were performed from May 2012 until December 2014 (Table 2.3). Training specific objectives and methods were defined (Table 2.3), familiar objects and procedures extended (Figure 2.3A-2.3C), and qualitatively (Table 2.4) and quantitatively (Figure 2.4-2.10) analysed and assessed.

2.4.1 *Transfer learning effective for most of the training procedures*

Home cage carabiner training and early interactions built the foundation for subsequent training procedures

The home cage carabiner training was effective for both monkeys. After only a few days of target training, monkey S and U learned the idea of holding the carabiner. The initial wariness decreased quickly within a few weeks. Monkeys learned the association with a coloured target. Therefore it was possible to move the monkeys with the carabiner to desired locations, e.g. another cage. Performing this stage of training in a small group helped enhancing the learning process.

When the monkeys were confident with holding on to their carabiner, a few trials in which the trainer touched the monkey's hand or just held the hand before delivering a reward were performed. Being able to touch or hold the monkey's hand has a number of advantages. This can be used for medical assessments and examinations. Behaviourally, holding the monkey's hand can distract its attention during various training procedures and routines. Furthermore, being able to hold the monkey's hand and arm enables assessments for sensory receptive fields essential for testing the response properties of identified neurons recorded from primary somatosensory cortex (S1). In total, 30 home cage carabiner training sessions were performed for monkey S and 34 for monkey U.

	Procedure / event dates		Number of sessions			Objectives and methods	
Key events & training procedures (→)	Started	Finished	Monkey S	Monkey U	Goal of session / event	Methods / procedures used	Objects introduced
Birth (MS)	2008, Jul	n.a	n.a	n.a	n.a	n.a	n.a
Birth (MU)	2010, Jan	n.a	n.a	n.a	n.a	n.a	n.a
Arrived at CBC	2012, Apr	n.a	n.a	n.a	n.a	n.a	n.a
→ Home cage carabiner training	2012, May	2012, Jun	30	34	Initial interaction, mobility, tone association	PRT, targets, operant conditioning, bridging	Coloured carabiner, tone device
RFID tag	2012, Jun	n.a	n.a	n.a	Monkey detection (automated feeder)	Anaesthesia	n.a
→ Transition cage training	2012, Jul	2012, Aug	35	32	Mobility, tone association, separation	PRT, target, operant conditioning, bridging	Carabiner, tone device, divider, transition cage
→ Training cage training	2012, Aug	2012, Sep	21	21	Mobility, shifting	PRT, target, bridging	Carabiner, tone device, divider,training cage
→ Lab training	2012, Sep	2012, Sep	28	15	Mobility, shifting, lab environment	PRT, target, bridging, desensitisation	Carabiner, tone device, divider, training cage
→ Neckbar training (MS)	2012, Sep	2012, Nov	23	n.a	Voluntarily entering neckbar	PRT, target, desensitisation, restraint	Carabiner, tone device, training cage, neckbar
→ Neckbar training (MU)	2012, Sep	2012, Dec	n.a	36	Voluntarily entering neckbar	PRT, target, desensitisation, restraint	Carabiner, tone device, training cage, neckbar
→ Arm abduction task (MS)	2012, Nov	2013, Jan	30	n.a	Learn arm abduction movement	PRT, arm abduction training, bridging	Carabiner, tone device, manipulandum
→ Arm abduction task (MU)	2013, Jan	2013, Jan	n.a	13	Learn arm abduction movement	PRT, arm abduction training, bridging	Carabiner, tone device, manipulandum
→ Vibration cue training (MS)	2013, Jan	2013, Apr	52	n.a	Learn arm abduction movement, vibration cue	PRT, arm abduction training, cue conditioning	Carabiner, tone, arm abduction, tactile cue
→ Vibration cue training (MU)	2013, Jan	2013, Apr	n.a	50	Learn arm abduction movement, vibration cue	PRT, arm abduction training, cue conditioning	Carabiner, tone, arm abduction, tactile cue
MRI scan (MS)	2013, March	n.a	n.a	n.a	M1 and S1 localisation, medical examination	Anaesthesia, MRI	n.a
→ Arm restraint training (MU)	2013, May	2013, May	n.a	1	Arm restraint	PRT, restraint, operant conditioning	Carabiner, tone device, arm restraint
→ Finger abduction prototype (MS)	2013, Apr	2013, Jul	40	n.a	Finger abduction, plastic shafts, finger positioning	PRT, desensitisation, selectivity	Tone device, plastic finger shafts, arm restraint
→ Finger abduction prototype (MU)	2013, Apr	2013, Jul	n.a	41	Finger abduction, plastic shafts, finger positioning	PRT, desensitisation, selectivity	Tone device, plastic finger shafts, arm restraint
→ Arm restraint training (MS)	2013, Jun	2013, Jun	5	n.a	Arm restraint	PRT, restraint, operant conditioning	Carabiner, tone device, arm restraint
MRI scan (MU)	2013, Jul	n.a	n.a	n.a	M1 and S1 localisation, medical examination	Anaesthesia, MRI	n.a
→ Finger abduction task (MS)	2013, Jul	2014, May	132	n.a	Finger abduction, cue, alternate index & thumb	PRT, visual & tactile cue, auditory feedback	Finger abduction manipulandum, arm restraint
→ Finger abduction task (MU)	2013, Jul	2014, Dec	n.a	197	Finger abduction, cue, alternate index & thumb	PRT, visual & tactile cue, auditory feedback	Finger abduction manipulandum, arm restraint
Headpiece & EMG implant (MS)	2013, Oct	n.a	n.a	n.a	Electrophysiological recordings, EMGs	Anaesthesia, surgery	n.a
Headpiece & EMG implant (MU)	2014, Apr	n.a	n.a	n.a	Electrophysiological recordings, EMGs	Anaesthesia, surgery	n.a

Table 2.3: *Overview of key events and main training procedures*. Key events and main procedures are ordered chronologically. Events and procedures for monkey S (MS) are highlighted with a light blue and for monkey U (MU) with light brown background. Events and procedures which equally apply to both monkeys are shown on a white background. Training procedures are emphasised in green colour and with an arrow (→).

The transition cage training helped separating monkeys

Moving one particular monkey by using its coloured carabiner to the smaller transition cage worked as expected. Touching the divider at first resulted in a fleeing behaviour (towards the home cage), but later monkeys got desensitised to this.

Later in training this behaviour reversed. After a while the monkeys got more reactive to touching the divider.

This behaviour became more apparent when the monkeys changed the unit. The change in behaviour coincided with several incidences (e.g. non consistent methodology regarding the transition cage between researcher, monkey trainer and husbandry staff). 35 training sessions focused on the transition cage training for monkey S and 32 for monkey U.

The training cage training increased the monkey's mobility

The training cage training enabled moving the monkeys to a different environment (for example the laboratory).

The familiar objects and procedures served as foundations (i.e. a basis of entities and concepts) on which subsequent training sessions could be built upon. Indeed the use of familiar targets increased the monkeys' confidence, cooperation and motivation.

In contrast to moving to the transition cage, the monkeys entered the training cage voluntarily and with less hesitation the majority of time during the whole training process despite of some small incidences. 21 training cage sessions were carried out for both monkeys.

The training in the laboratory introduced a new environment

Moving the training to the laboratory enabled practising skills in a new environment with novel settings.

After an initial uncertainty, the monkey's subjective stress level decreased whereas the confidence and active exploration increased. The use of familiar objects and procedures helped promoting this effect. However, monkeys did not start to pay attention to the task immediately. At first they spent time exploring their new environment and got easily distracted by noises or unknown voices. In these situations, monkeys held commonly on to their carabiner while listening, observing or exploring the environment. 18 target training sessions in the novel laboratory environment were accomplished with monkey S and 15 training sessions with monkey U.

Conditioning the monkeys to enter the neckbar voluntarily

A substantial number of training sessions (23 for monkey S and 36 for monkey U) focused on teaching the monkeys to enter the neckbar voluntarily.

Monkeys got used to the presence of the neckbar. Afterwards, monkeys were conditioned to accept this object in close proximity. This led to an entirely positive association with the object (neckbar). This was accomplished within a few training sessions and worked equally well for both monkeys.

Next, the neckbar became behaviourally relevant by making itself a target. Early in training it was necessary to actively touch the monkeys neck with this object, delivering the bridging (enforcing) tone, before giving the reward. This way the link between touching the neck and the reward was formed.

Subsequently, the monkey learnt to position its head voluntarily within the neckbar to receive a reward. Desensitising the monkey not to react while the neckbar was moved or gradually closed remained behaviourally relevant until it was possible to completely close the neckbar.

At the stage where the monkey was comfortable resting in the neckbar, the time in the neckbar and the overall session length was gradually increased. The neckbar training resulted in the desired goal of reduced stress levels and increased cooperation by applying the described procedure using a PRT approach. However, while monkey S entered the neckbar according to the trained approach, monkey U started to hesitate entering the neckbar the described way. Due to this reason, I spent a couple of additional training sessions on teaching monkey U to offer its left hand through the bars of the training cage. After this behaviour became an usual training routine towards the end and the beginning of the training sessions, monkey U was conditioned to let the trainer hold its hand while positioning the neckbar. These additional steps in training resulted in the above mentioned difference in training sessions between monkey S and U.

Holding the arm of monkey U for the process of positioning the neckbar seemed to calm its behaviour (see Section 2.3.3). Therefore, it was beneficial to apply this approach to releasing the neckbar at the end of each training session as well.

Arm abduction task as preliminary step in association learning

The arm abduction task was designed to teach the monkeys important concepts, paramount for the subsequent sessions until the final stage, namely the finger abduction task. These included getting the monkeys used to the usual training routine (entering the lab, positioning the neckbar, disassembling the training cage, positioning a table in front of the training cage), before the monkey was presented with the actual task (in this case the arm abduction task). In total 30 arm abduction

task training sessions for monkey S and 15 for monkey U were performed before moving on to the next stage (Figure 2.3A) in training.

Since the monkeys were already strongly conditioned to touching and holding the carabiner, attaching it to the metal bar of the arm abduction task (see Figure 2.1C) immediately led to the expected behaviour (moving the bar with the carabiner) and focused the monkey's attention to the new task.

Using a low resistance of the springs holding the metal bar made these easily movable. In fact, whenever the monkey was touching the carabiner, the bar moved. Thereby, the monkey got used to the motion and the resistance could be increased within the next few training sessions (by changing the springs holding back the metal bar). At the end of this training stage, the monkeys were able to perform clear and skilled arm abduction motions and the tactile cue could be introduced.

Introducing the tactile cue

When the monkeys were confident with the arm abduction task which increased their set of familiar objects (Figure 2.3B) by adding the task itself, the metal bar, the movable device, in addition to procedures and routines (Figure 2.3C) related to the training sessions, the vibration cue was introduced.

The strong vibration disk motor caused a distinct vibration of the metal bar and consequently the carabiner. Whenever the vibration was activated and the monkey moved subsequently, the (bridging) tone (using the already familiar tone device, cf. Figure 2.1A) was given, and the monkey was rewarded. The delay before the presentation of the tactile cue was then increased. Monkeys were not rewarded when the bar was moved without prior cue presentation and the bar was returned to its starting position by the trainer.

2.4 Results

After a few training sessions monkeys started forming an association between the tactile cue and the movement initiation (or at least learnt to delay the motion until the presentation of the tactile cue).

In total 52 training sessions focused on the vibration cue arm abduction task training for monkey S and 50 for monkey U.

Positioning the monkey's arm in an arm restraint

Before being able to concentrate on the finger abduction task, the monkeys had to be brought into a position which enabled a flexible adjustment of the monkey's hand and thereby enabling the monkey to work in a comfortable position.

This stage of the training was prepared by getting the monkey used to someone touching and holding their arm during previous training procedures (see home cage interaction and neckbar training). Therefore, it was almost instantly (5 training sessions for monkey S and 1 for monkey U) possible to hold the monkey's arm while positioning a fabric sleeve around the monkey's arm.

In the following, the monkey's arm was fixed to the side of the training cage and subsequent training sessions focused on the finger abduction prototype. The length of the first few sessions focussing on the finger abduction prototype training and at the same time the arm restraint was moderately increased.

Finger abduction task prototype learning

The finger abduction prototype served as a preliminary step to teach the monkeys to perform finger abduction movements.

As a first step, monkeys were rewarded for positioning their fingers in movable plastic shafts. Fingers were actively guided by the experimenter. Once the fingers were in place, monkeys got the correct behaviour signal tone and a reward.

After initial familiarisation to the new object, the plastic shafts were moved while the monkey's fingers were correctly positioned within the plastic shafts (passive movement). The auditory (bridging) tone was given whenever a movement was performed.

Finally, monkeys were only rewarded whenever the monkey moved any finger correctly (active movement). 40 training sessions for monkey S and 41 for monkey U focused on teaching the monkey to perform an abduction movement with any digit. This led to a reward. Both monkeys had a tendency to preferentially use their index finger.

Finger abduction task and selectivity

The transfer from using the finger abduction prototype (with the movable plastic shafts) to the final finger abduction manipulandum worked without any noticeable problems. Both monkeys immediately positioned each of their digits in the corresponding plastic shafts.

The motor forces were disabled at early training stages, so that every movement of any finger resulted in a correct trial. The control of the task, counting the correct trials and errors, and registering the position signal were captured electronically.

2.4 Results

Once the monkeys were moving any digit (both monkeys moved preferentially the index finger), the motor forces were turned on introducing a low resistance. Therefore, the movement became more apparent and pronounced.

At first, the threshold for the thumb and index finger was deactivated before a very high threshold was set. After a couple of training sessions the threshold was approximated to be close to the maximum force observed in the monkey's uninstructed fingers. Therefore, the monkey failed (an error tone was presented and the monkey was not rewarded) only a few times. At the same time the threshold for the minimum force required for the instructed finger was regularly increased. In the following the threshold for the uninstructed digits (thumb and index) were decreased until the force threshold values were below the threshold of the instructed finger. This method led to more selective movements and worked equally well for both monkeys (except for the fact that more training sessions were required to teach monkey U the selectivity).

The same approach was taken when teaching the monkey to move the thumb selectively. Starting with no force values and actively enforcing the thumb movement (passive movements were necessary at first), the monkey moved the thumb in a couple of trials which was strongly enforced with positive and high value rewards (cf. Figure 2.2A and 2.2B). A refinement of the thresholds and regular increases of the motor forces were performed until the monkey reached a reasonable performance.

Next, the training was directed to teach the monkey alternating between instructed digits (thumb and index). This required the monkey to take the vibration (or the visual: LEDs on top of the transparent plastic) into account. For this purpose, the thresholds for the non-instructed digits were increased again to reduce the number of errors at the start and to promote an effective training session. These thresholds were increased again in the following training sessions

to increase the selectivity. Monkeys learnt to incorporate the cue to perform differential movements whenever the thumb or the index finger was instructed.

Although the monkeys were able to perform selective thumb movements, the movements of the index finger were not that selective when alternating randomly between these fingers. Due to the limited time allocated to this part of the project, the stage of the training before starting the electrophysiological recordings was as follows: Both monkeys were able to perform very selective movements with the thumb. Whenever the index finger was instructed, monkeys did move the index finger more strongly (higher force values) but also the thumb (although less pronounced in comparison to trials where the thumb was instructed). These distinct patterns for index and thumb movements served as assessments for subsequent error rate analysis (see Chapter 3).

In total 132 sessions for monkey S and 197 sessions for monkey U focused on the finger abduction task. 36 % of these sessions for monkey S and 52 % for monkey U focused on training the finger abduction task until the final performance level was reached and electrophysiological recordings began.

Overall, the training for performing distinct finger abduction movements worked well for both monkeys. More training sessions were required for monkey U. This was partly due to the fact that the size of the plastic shafts was not optimal for the hand shape of monkey U. Furthermore, a modified arm restraint had to be used for monkey U to enable a better and more comfortable arm and hand position. The angle and ergonomics of monkey U's hand were optimised by shaping the manipulandum using sleek tape.

2.4.2 *Summary of qualitative results regarding training procedures*

Overall teaching the monkeys the desired behaviour, namely to move the index finger or the thumb selectively whenever instructed did work within the assigned time frame.

Using a positive reinforcement training (PRT) approach worked well for most of the training procedures. However, occasionally (for example in the context of the transition cage and arm restraint training) it was necessary to combine this approach with negative reinforcement training (NPRT).

Of all training procedures, 5 training procedures were evaluated as being an improvement (or strong improvement) to conventional training routines or using objects and methods in which the transfer of (skill) learning worked particularity well (Table 2.4).

Within these procedures the introduced concept (e.g. the conditioned target, behaviour or association chain) was learnt by the monkey within the predicted time frame and a transfer of these to a novel context was possible. This was for example the case for the carabiner training for which the monkeys learnt the associated colour and tone within a few training sessions. These concepts could be applied to subsequent training procedures and enabled immediate task specific skill development in the context of the arm abduction task. Similarly the finger abduction task prototype set important foundations regarding finger, hand positioning, and the critical behaviourally relevant concepts (e.g. finger abduction motion). The remaining training procedures led to minor or no improvements in comparison to conventional routines.

Using the fruit reward hierarchy schedule (Figure 2.2A and 2.2B) improved both monkeys' motivation and built an important tool when introducing the training concepts towards the end of particular training sessions.

Training procedure	Score	Advantage	Disadvantage
Home cage carabiner training	4	A high number of training concepts trained in a short amount of time (e.g. target training, conditioning, association learning, bridging, mobility).	No real disadvantage considering the efficient way this procedure can be taught.
Transition cage training	1	Moving the monkey with their coloured carabiner individually to a different cages or in this case the transition zone between home cage and training cage environment is very useful.	This procedure requires a very strict application of the target paradigm. Any person working with the monkeys (even with neighbouring ones) has to pursue similar training procedures. Desensitisation to the divider seems to be very monkey (temperament) dependent.
Training cage training	3	The training cage practise enabled us to move the monkey to a different environment, cage or into the laboratory. Using targets to move the monkey into the smaller training cage increased the monkey's confidence and positive association with the cage.	Although monkeys were very cooperative moving into the training cage, desensitising the monkey to react to moving the divider of the training cage would have required more time.
Lab training	3	Using familiar targets and objects in a new environment increased the monkey's confidence. After a short delay, both monkeys independent of their personalities started interacting.	Required a few sessions to interact with the monkey in the laboratory environment. This delay is however affordable.
Neckbar training	5	Training the monkey to enter the neckbar voluntarily worked very well for both monkeys. It decreased the monkey's stress level and increased its confidence. No need to force the monkey into the neckbar.	This procedure required some time to teach the monkeys. Although time consuming, it can be worth delaying the overall training procedures in favour of the monkey's increased cooperation long-term.
Arm abduction task	4	The arm abduction task was an appropriate task to teach the monkeys the arm abduction motion and interaction with a task. Using the already familiar target (carabiner) made it possible to transfer already known concepts which increased the learning speed.	Although important general skills could be taught applying this training procedure, I am not sure how much transfer learning to the actual finger abduction movement was inferred.
Vibration cue training	3	Building on the already familiar concepts from previous training sessions, it was sensible to integrate a vibration cue into the previously used setup. Movement initiation in response to the cue could be taught successfully.	Similar critics than for the arm abduction task. Several useful general skills were taught, but I am not sure how much the monkeys could transfer the idea of the vibration cue to the novel context (finger abduction task)
Finger abduction prototype	4	The finger abduction prototype training enabled us to teach important concepts regarding the monkey's hand and finger position as well as the idea of the concept of motion.	Maybe a bit time consuming, but worth the effort. Problems arose primarily due to non-optimal plastic shaft dimensions.
Finger abduction task	4	The custom made manipulandum allowed for precise modifications of the task relevant parameters. Improving the abduction movement, movement initiation in response to the cue, selectivity and motor force could be trained in a controlled manner.	A long learning process. It took a while until both monkey's grasped the idea of alternating finger movements in response to the cue.

Table 2.4: *Evaluation and scoring of training procedures*. The main training procedures were evaluated according to a scoring system (see Table 2.2).

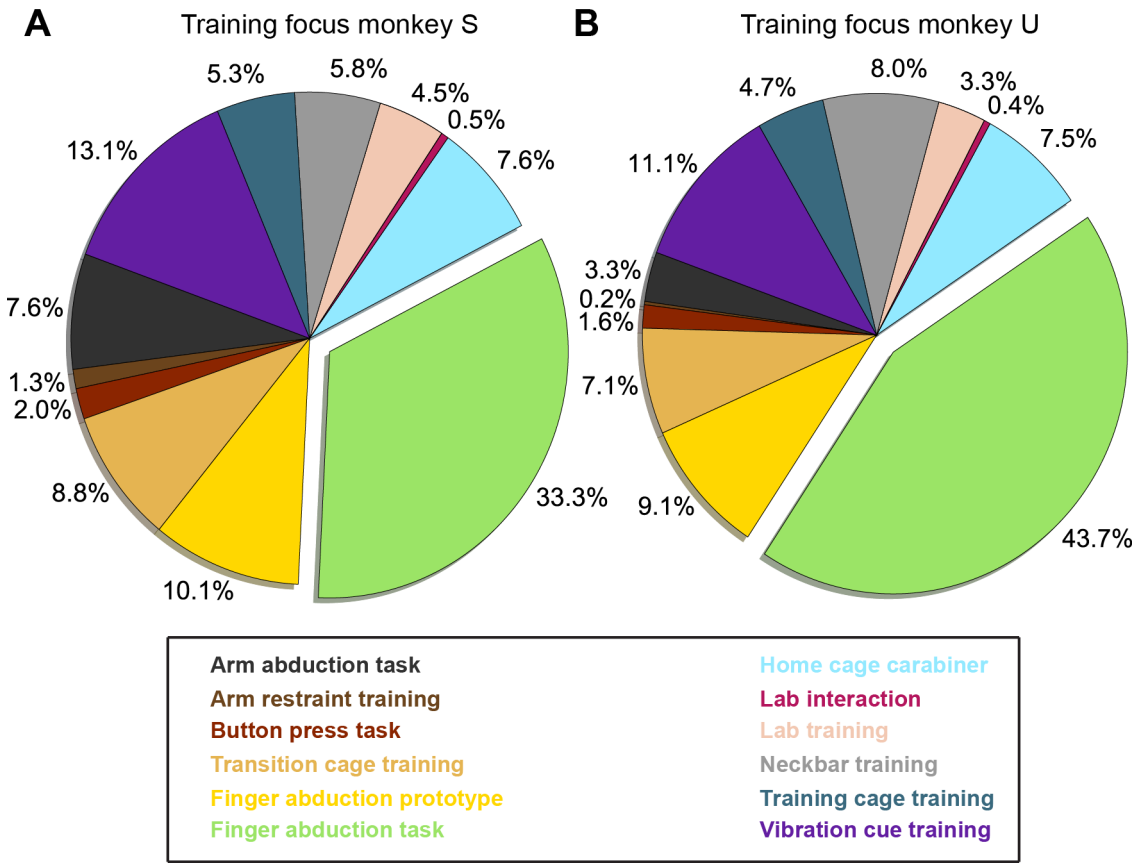

Figure 2.4: *Focus of training*. (A) Percentage of training sessions focused on a particular training procedure (coloured wedges) for monkey S. (B) Percentage of training sessions focused on a particular training procedure (coloured wedges) for training monkey U. Different training categories are colour coded. In total there were 396 training sessions for monkey S and 451 for monkey U. The majority of sessions focused on the final (finger abduction) task.

2.4.3 *Similar focus on particular training procedures*

The majority of training sessions (33 % for monkey S and 43 % for monkey U) focused on the finger abduction task (Figure 2.4A and 2.4B). Less than (monkey S 36 %) and about half (monkey U 52 %) of these finger abduction training sessions were required to to reach an acceptable performance level.

All other training procedures were taught in similar proportions (cf. Figure 2.4A and 2.4B) between monkeys. These similar amount of sessions permitted more specific comparisons between training procedures for both monkeys.

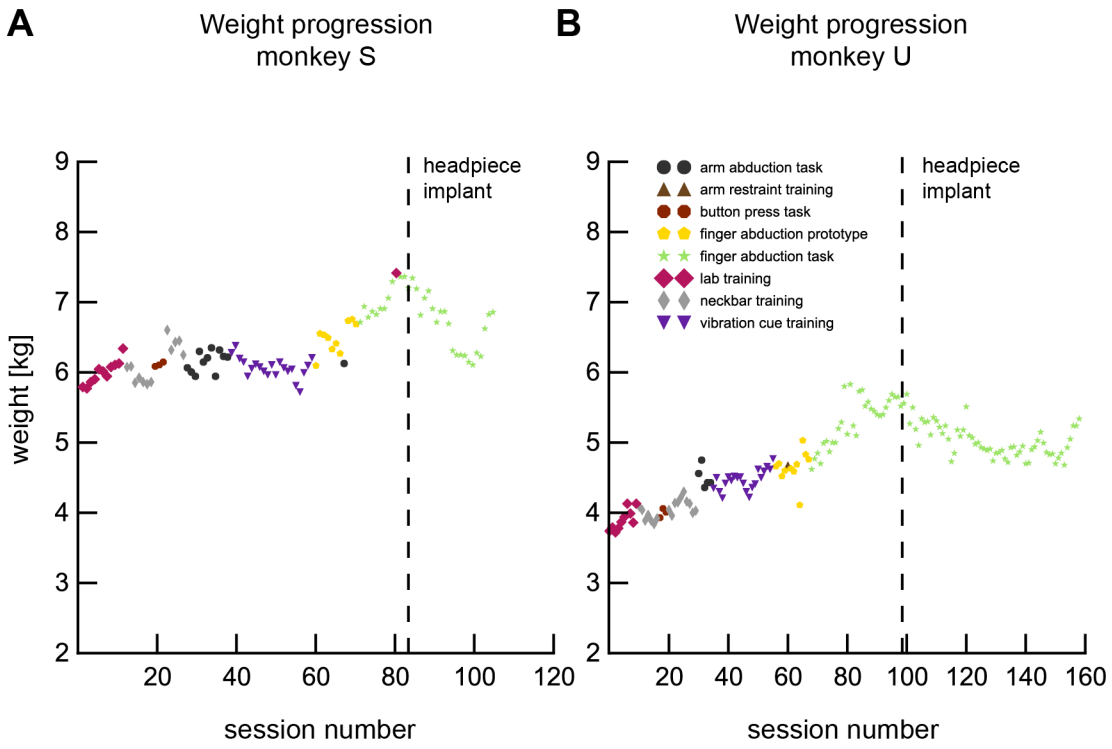

Figure 2.5: *Weight progression over time for monkey S and U.* (A) Weight progression for monkey S. (B) Weight progression for monkey U. Each training procedure is shown in a different colour (cf. Figure 2.4A and 2.4B) and marker. The time of the headpiece and EMG implant are marked by the dashed black line.

2.4.4 *Continuous weight increase throughout the training procedures*

The weight of both monkey S and U increased throughout the time course of all training procedures (Figure 2.5A and 2.5B). The weights of both monkeys decreased after EMG and headpiece implants before a rebound of the weights towards an increase were observed (Figure 2.5A and 2.5B). Local weight progression extrema were training procedure dependent (cf. coloured markers indicating specific training procedures in Figure 2.5A and 2.5B) and similar between monkey S and U. Highest weight values were observed before EMG and headpiece implant surgeries (cf. dashed black lines Figure 2.5A and 2.5B). Average weights were $6.31\,\mathrm{kg}$ for monkey S and $4.77\,\mathrm{kg}$ for monkey U. Standard weight deviations were $0.41\,\mathrm{kg}$ for monkey S and $0.51\,\mathrm{kg}$ for monkey U.

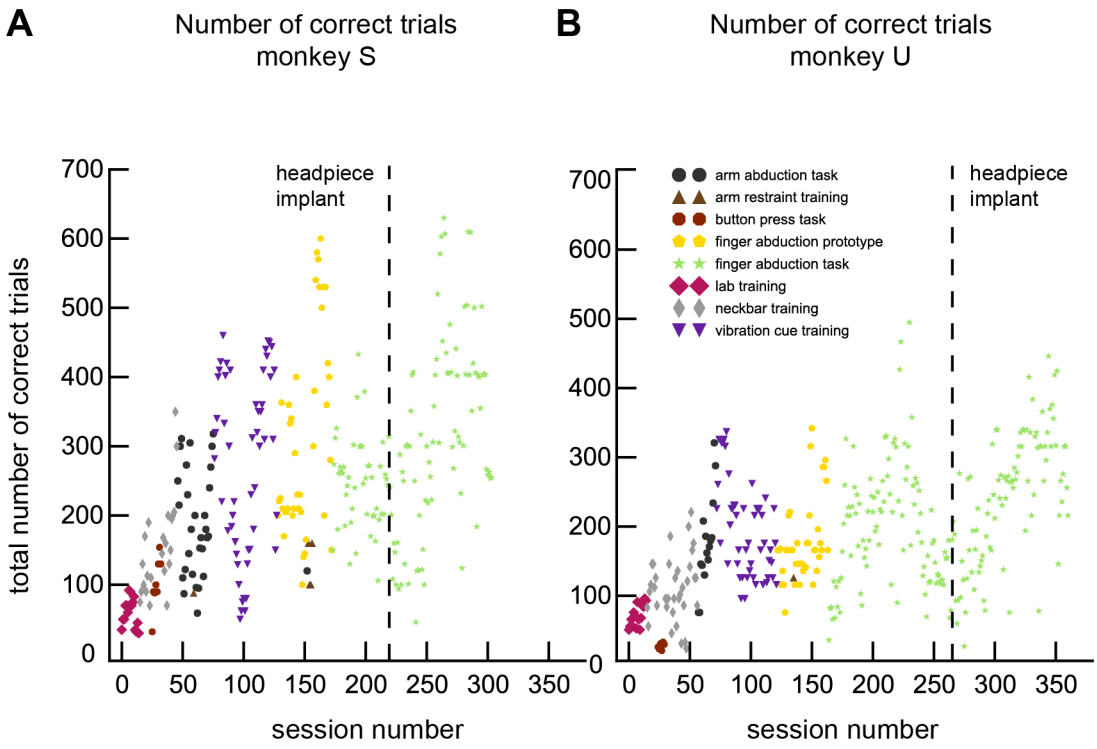

Figure 2.6: *Number of correct trials over time.* (A) Number of correct trials for monkey S. (B) Number of correct trials for monkey U. Each training procedure is shown in a different colour (cf. Figure 2.4A and 2.4B) and marker. The time of the headpiece and EMG implant are marked by the dashed black line.

2.4.5 *Continuous but variable increase in the total number of correct trials throughout training procedures*

The total number of correct trials increased throughout the time course of training procedures for monkey S and U (Figure 2.6A and 2.6B). The number of correct trials was variable and training procedure dependent (coloured marker Figure 2.6A and 2.6B). After EMG and headpiece implants (black dashed line in Figure 2.6A and 2.6B) correct trial numbers increased but remained variable.

The average number of correct trials was 251 for monkey S and 173 for monkey U. The standard deviation of the number of correct trials was 134 for monkey S and 91 for monkey U.

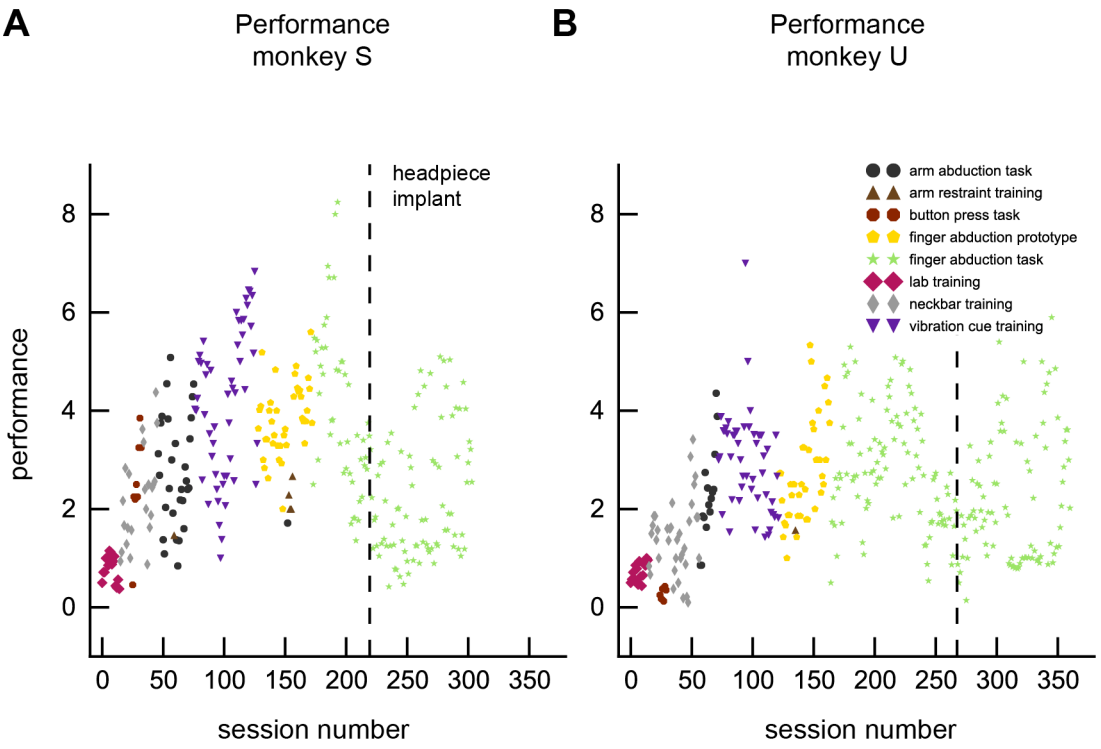

Figure 2.7: *Training performance over time.* (A) Training performance for monkey S. (B) Training performance for monkey U. Each training procedure is shown in a different colour and marker. The time of the headpiece and EMG implant are marked by the dashed black line. The training performance is the total number of correct trials divided by the total session length in min.

2.4.6 *Continuous but variable increase in training performance throughout training procedures*

The overall training performance (the total number of correct trials divided by the total session length in min) increased throughout the training procedures for monkey S and U (Figure 2.7A and 2.7B). Performances decreased temporarily after EMG and headpiece implant surgeries (black dashed lines, Figure 2.7).

The average training performance for monkey S was $3.04\,\mathrm{trials/min}$ and $2.38\,\mathrm{trials/min}$ for monkey U. The standard deviation of the training performance varied substantially from $1.58\,\mathrm{trials/min}$ for monkey S and $1.25\,\mathrm{trials/min}$ for monkey U throughout the training procedures.

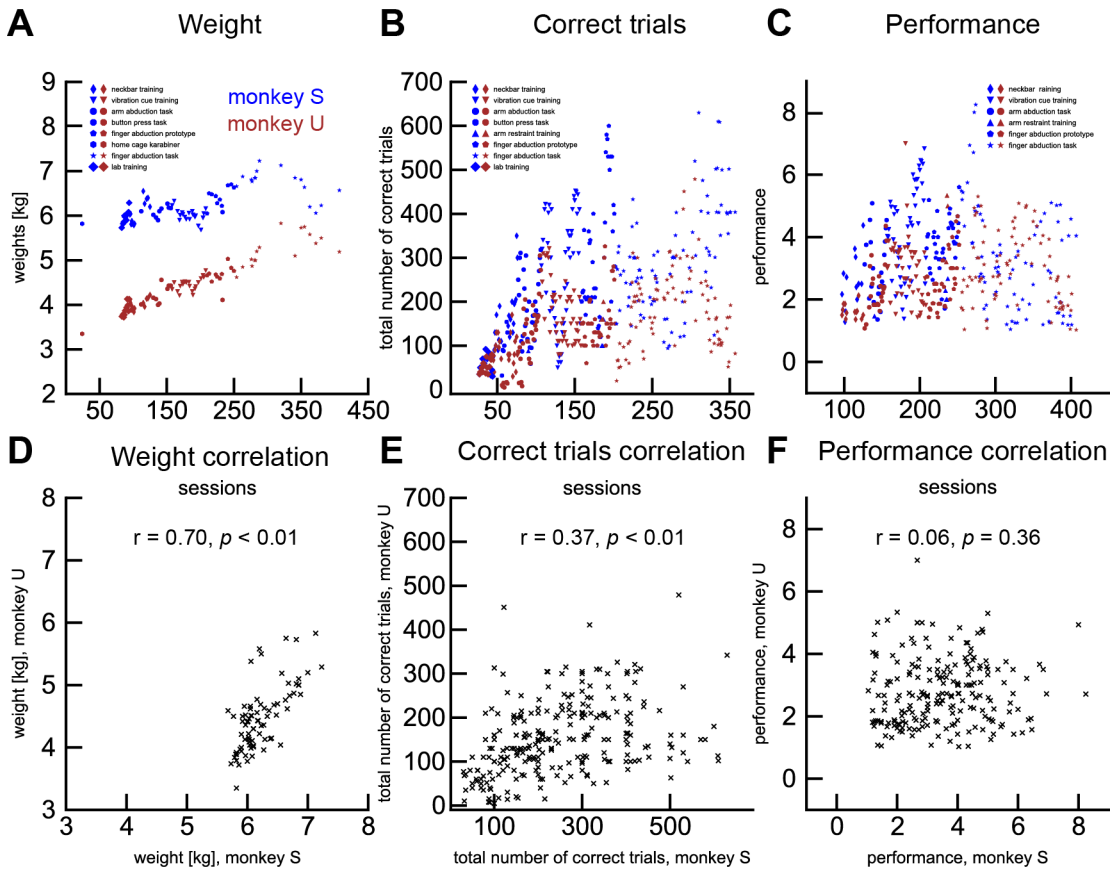

Figure 2.8: *Correlation of weight, trials and performance between monkeys.* (A) Weight progression over training sessions for monkey S (blue marker) and U (brown marker). (B) Correct trial number progression over training sessions for monkey S (blue marker) and U (brown marker). (C) Training performance for monkey S (blue marker) and U (brown marker). Note that the marker shape is the same as in previous figures and indicating the different training procedures (cf. Figure 2.5A and 2.5B for example). (D) Correlation between weight values between monkey S and U. (E) Correct trial number correlation between monkey S and U. (F) Correlation of training performance between monkey S and U. The training performance is the total number of correct trials divided by the total session length in min. The Pearson correlation coefficient r and the significance p are given for each correlation. Only data with entries for both monkey S and U on the same training day (session) are shown.

2.4.7 *The monkeys' weights and the number of correct trials were correlated between monkeys*

The monkeys' weights and the total number of correct trials were correlated between monkey S and U (Figure 2.8A-2.8F). All of the training sessions in which

values for the weight, number of correct trials and training performance were captured for both monkeys on the same day, were compared between monkey S (Figure 2.8A-2.8C, blue marker) and monkey U (Figure 2.8A-2.8C, brown marker). Data captured on the same day for both monkeys were chronologically ordered before calculating correlations. Different training procedures are indicated by different marker shapes similar to those used in previous illustrations (e.g. Figure 2.5).

For all of these parameters (weights, correct trials and training performances) the linear correlation coefficient r (Pearson correlation coefficient, $p < 0.01$) between monkeys was determined and visualised (Figure 2.8D-2.8F).

The correlation coefficient revealed a clear correlation between the weight progression of the two monkeys (Figure 2.8D).

The total number of correct trials was weakly but significantly correlated between monkey S and U (Figure 2.8E).

The training performance was not significantly correlated between monkey S and U (Figure 2.8F).

The training procedure specific modulation of weights, correct trials and training performance will be compared in the next section.

2.4.8 *The total number of correct trials and the overall training performance depended on the training procedure*

The total numbers of correct trials and the training performances were different depending on training procedures and similar for both monkey S and U (Figure 2.9B and 2.9C).

The weight did increase continuously between sequential training procedures for monkey S and U (Figure 2.9A). Not much weight variations within and between

training procedures were observed for monkey S and U (see gray error bars showing the average weight values ± standard error of the mean (SEM) in Figure 2.9A).

The total number of correct trials depended on the training procedure (Figure 2.9B). The total number of correct trials increased from the home cage carabiner to the vibration cue training for monkey S (blue bars, Figure 2.9B) and monkey U (brown bars, Figure 2.9B). The only exception was the button press task.

This type of training was performed by a different trainer and with a different session length (see Section 2.3.2) which explains the difference in total trial number.

The total correct trial number for the arm restraint training decreased before gradually increasing until the final stage of the training (finger abduction task). This trend in correct trial number progression per training procedure was reflected equally for both monkey S and U. The average correct trial number per training procedure was mostly higher for monkey S than U. The only exception formed the arm restraint training in which monkey U performed better.

A similar training procedure specific trend could be observed when analysing the training performance for monkey S and U (Figure 2.9C).

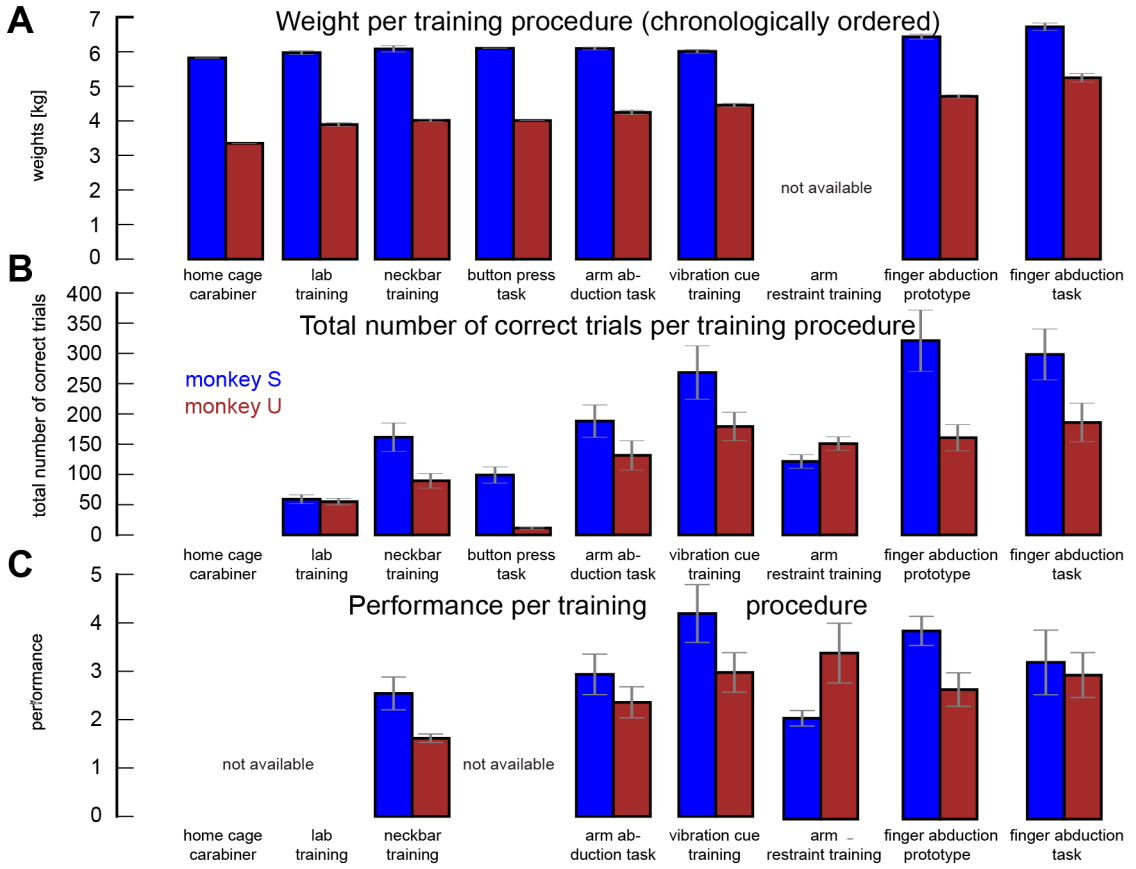

Figure 2.9: *Comparison of weights, trials and performance between monkeys and training procedures*. (A) Weight per training procedure for monkey S (blue) and U (brown). (B) Total number of correct trials per training procedure for monkey S (blue) and U (brown). (C) Training performance per training procedure for monkey S (blue) and U (brown). Training procedures are ordered chronologically. The gray lines are showing the mean value $\pm$ SEM. Some training procedures were not available due to lack of data values within these categories. The training performance is the total number of correct trials divided by the total session length in min.

The training procedure regarding the button press task was not available due to lack of session length data. The only difference regarding the trend (change of magnitude of either the total number of correct trials or training performance) could be observed regarding the arm restraint training. Up to this stage in training performances increased gradually for monkey U but not S (Figure 2.9C).

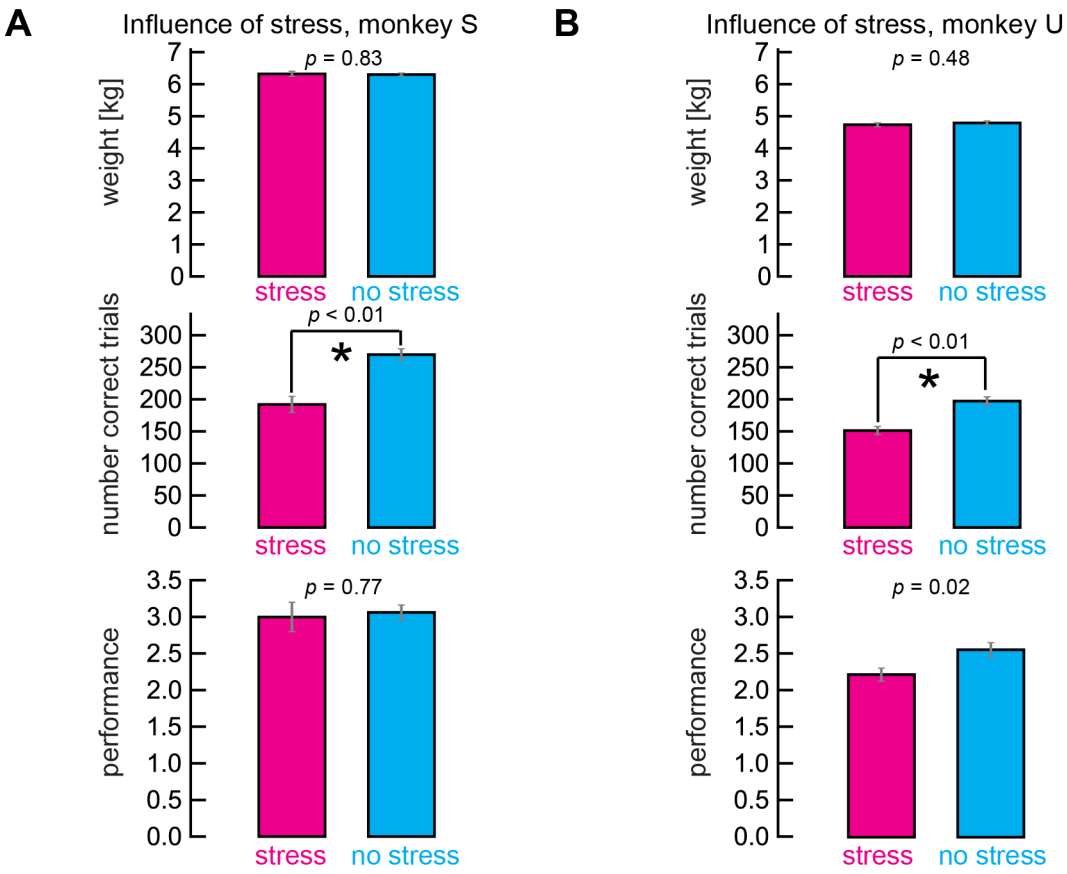

Figure 2.10: *Effects of subjective stress on weight, correct trials and performance for monkey S and U.* Effects of subjective stress for monkey S (A) and monkey U (B). Each parameter (weights, correct trials and training performance) was compared between sessions in which the monkey's behaviour was rated as stressful (magenta) as opposed to not stressful (cyan). Significant differences are marked with an asterisk ($p < 0.01$, two sample t-test).

2.4.9 *Subjective stress level influenced correct trial numbers*

The monkeys' subjective stress level influenced the total number of correct trials for monkey S (Figure 2.10A, second row) and U (Figure 2.10B, second row).

Whereas subjective stress of the monkey did not lead to any significant changes in weight (Figure 2.10A) or training performance (Figure 2.10C), a significant effect of stress on the total number of correct trials was present for monkey U (two sample t-test, $p < 0.01$) and monkey S (two sample t-test, $p < 0.01$).

Key development / achievement	Effects and conclusion
Designing training sessions based on familiar objects and procedures	Context-dependent introduction of objects, items and procedures produced facilitated learning and provided additional tools to shape the subject's behaviour.
Food hierarchy based on subject's individual preference	Individually assessed reward hierarchy enabled a better control of the incentive value and thereby the subject's motivation.
Training focus analysis and concentration on particular key training procedures	Designing training procedures and transitions by allocating a comparable number of session with a specific objective for all subjects involved.
Analysing measurable quantitative parameter like trial numbers and weight in relation to training procedures	Quantitative parameter other than trial number and error rate can be indicative of training success.
Cross-subject correlation analysis of behavioural and training procedure specific parameters	Correlation analysis of training procedure specific parameters of subjects receiving the same behavioural training revealed cross-subject interactions, which might be used to assess and predict trainings performance.
Home cage interaction and the neckbar training using PRT	Redesigning training procedures like the initial home cage interaction (introduction of individually-assigned and coloured carabiner and an allocated bridging tone) and the neckbar training (using positive reinforcement rather than positive punishment) reduced the subject's stress level and increased its welfare, confidence, motivation and cooperation.

Table 2.5: *Summary of key developments during the process of the behavioural training.* Key training procedures and techniques, which were applied during the behavioural training of two macaque monkeys and classified as effective for the overall training progress.

2.5. Discussion

The present study refined a variety of training procedures which were designed to teach two macaque monkeys to perform a skilled motor task involving the selective motion of the index finger and the thumb. Training procedures built on principles of desensitisation, classic and operant conditioning, PRT, motor, transfer and association learning (Bloomsmith et al., 2007; Coleman and Pierre, 2014; Perlman et al., 2012; Warren, 1974; Washburn and Rumbaugh, 1991; Wolpert et al., 2011). Due to the primates' capability of generalisation (Warren, 1974), particular objects and items were familiarised, utilised, and incorporated into subsequent training procedures (Figure 2.1A-2.1G, 2.3A-2.3C, and Table 2.3). An optimised rewarding scheme was used to enhance motivation and performance (Figure 2.2A-2.2C). Quantitative analysis revealed increased weight, correct trial number, and training performance

progression (Figure 2.5A, 2.5B, 2.6A, 2.6B, 2.7A and 2.7B) over time. These quantitative parameter were training category dependent (Figure 2.9A-2.9C). Between-monkey correlation analysis showed a clear correlation between the monkey's weight, a weak but significant correlation between the monkeys' correct trial numbers, and no significant correlation between the monkeys' training performances (Figure 2.8A-2.8F). Finally, the monkeys' subjective stress levels were assessed by an evaluation system (Table 2.1) and effects on the above mentioned quantitative parameters were analysed (Figure 2.10A and 2.10B). A summary of key developments in behavioural training is shown in Table 2.5.

2.5.1 *Training procedures were designed to build on common principles*

The training procedures were planed with the final goal to teach two monkeys to perform a finger abduction task (Figure 2.1G). All of these procedures were based on principles in motor, transfer and association learning (Warren, 1974; Washburn and Rumbaugh, 1991). Target training has been shown to be an effective method in the context of positive reinforcement training (Coleman et al., 2005; Fernstrom et al., 2009; Gillis et al., 2012; Laule et al., 2003; Perlman et al., 2012). Therefore, the first stage in training was designed to condition the monkey to recognise and interact with a target that can be transferred to a novel context. A coloured carabiner which was individually assigned to each monkey within the training group was chosen. This had several advantages: The monkeys learnt to identify and engage with their individual targets, and interference due to the monkey's social dominance hierarchy (Riddick et al., 2009; Varley and Symmes, 1966) was avoided. Correct behaviour including touching and holding the carabiner, and in later training sessions holding the monkey's arm was positively reinforced using a primary reinforcer (fruits, nuts or other rewards, see Figure 2.2A-2.2C). In addition to the primary reinforcer, a secondary reinforcer (Phillips et al., 2003; Prescott et al., 2012b; Westlund, 2012a) was used. Instead of using a clicker

(Gillis et al., 2012), a tone device (see Figure 2.1A) was used to *bridge* (or link) the correct behaviour to the positive primary reinforcer. A different tone was used for each monkey and therefore another specificity of the secondary reinforcer was introduced. The colour of the carabiner as well as the consistent gesture (and body posture of the trainer) served basically as tertiary reinforcer (Westlund, 2012a). After a short time monkeys were able to be moved to desired locations (e.g. another cage). The home cage carabiner target training accustomed the monkeys to interact with the trainer. These procedures represent an important step towards applying refinement strategies to training methods (3Rs, Russell and Burch, 1959), husbandry, interaction and (initial) training when applied faculty wide (Perlman et al., 2012).

The transition cage training was performed with the goal to be able to move the monkey to the transition cage (Figure 2.1B) voluntarily by positioning their initially assigned carabiner within the transition cage. Monkeys were meant to hold on to their carabiner while closing the divider (Figure 2.1B). For this purpose a desensitisation (Clay et al., 2009; Laule et al., 2003; Wergard et al., 2015) strategy was used. Whenever, the instructor (trainer) touched (or progressively moved) the divider and the monkey held on to their carabiner (without moving back to the home cage), the monkey received a reward. This strategy worked well initially. However, over time the monkey's behaviour with respect to voluntarily moving into the transition cage deteriorated. Researcher and technical staff used different approaches and training methodology in the context of some training procedures, animal handling and with respect to the targets (e.g. carabiners). This might have resulted in a few inconsistencies, which affected the overall trainings process. Positive punishment parameter (person entering the home cage, the use of the back-wall itself, etc.) accumulated over time, which could have overweight the positively reinforced aspects. Additional training sessions would have been required to re-establish the positive link (reinforcement) to particular objects and procedures.

Teaching the monkey to enter the training cage voluntarily worked in terms of moving the monkey to the desired location (training cage, Figure 2.1C and 2.1D) by using the primary (coloured carabiner) and secondary (tone) reinforcer. However, when touching and slightly moving the divider of the training cage monkeys returned to the transition (or home cage). Due to this reason, this training procedure was slightly modified in the following way: The target was positioned within the training cage, monkeys entered voluntarily the training cage, the divider was closed without hesitation, and the target training was continued as usual. This procedure became a routine and positioning the coloured carabiner as a cue was not necessary anymore in subsequent sessions. The tertiary (coloured carabiner in this case) reinforcer was replaced with the positioning of the training cage itself and by opening (cue) the training cage. Monkeys entered the training cage voluntarily and an increased confidence and motivation to interact while the monkey was in the cage could be observed. Although the cues (carabiner, positioning the cage, and opening the divider) were linked to the primary reinforcer (reward) positively, it is possible that apart from these positive and reinforcing aspects, negative ones (e.g. avoidance of the transition cage) contributed (Wergard et al., 2015). Nevertheless, the training cage became linked to a positive association, which might have led to the beneficial effects regarding the animal's behaviour.

Being able to build on familiar and positively conjugated objects (carabiner, tone, training cage; see Figure 2.3A-2.3B) beneficially contributed to a successful transfer to a novel environment (laboratory). After initial exploratory behaviour, the monkey's confidence rapidly increased.

Especially successful was the novel approach taken regarding the neckbar (restraint) training. Since many experimental and behavioural setups in research environments require monkeys to be restraint (Bliss-Moreau et al., 2013), several approaches to restraining could be taken. One way of applying the neckbar could imply the use of positive punishment aspects (e.g. minimising the monkey's

space), negative reinforcer (e.g. removing the plastic back-wall) or to position the neckbar as quickly as possible. These approaches have several disadvantages. The monkey does not have the choice and control of entering the neckbar voluntarily which might diminish the monkey's performance (Roma et al., 2006). Furthermore, performing any kind of fast movements (quickly positioning the neckbar) might behaviourally be perceived as potential danger (fast movement as a threat, Stankowich and Blumstein, 2005). This could affect the monkeys stress level, welfare and accordingly the performance. Therefore, monkeys were training to enter the neckbar voluntarily using a PRT approach. Monkeys were involved in a simple target training while the neckbar was positioned in close proximity to the animal. By touching the monkeys gently and in a slow manner with the neckbar and by delivering a subsequent reward, desensitisation was applied and a positive link to the new object was established. Using a secondary enforcer (tone) whenever the monkey was actively positioning its neck into the neckbar linked to the primary enforcer (reward) conditioned the monkey to the desired behaviour (entering the neckbar). With the second monkey however, an additional familiar procedure (Figure 2.3C) had to be introduced. Holding the animal's arm before monkey U positioned its head into the neckbar served both as a tertiary reinforcer as well as an expansion of the training routine. The neckbar training is a good example that restraint training routines can be reinforced positively (Bliss-Moreau et al., 2013) without impacting on the overall time schedule of a particular research project (Table 2.3 and 2.4).

Transfer and association learning by increasing and utilising the set of familiar items and procedures (Figure 2.3A-2.3C) were applied into practice when introducing a novel behavioural task (arm abduction task). Attaching the target (carabiner) to a novel device, semantically linked a task relevant aspect to the new device (task). This increased the monkey's confidence and learning progress. However, the effects of teaching a monkey a general concept (abduction motion) with the goal to reintroduce this concept in a different context (finger abduction

task), has to be studied with a higher number of animals. Equally introducing a novel sensory cue, i.e. a vibration cue (Table 2.2) has to be further evaluated.

A mixture of positive and negative reinforcement training (NPRT) together with desensitisation was effective for introducing the arm restraint routine. This procedure together with earlier arm holding conditioning enabled positioning the monkey's hand in the plastic shafts of the finger abduction task (Table 2.1F and 2.1G) to teach the animal to perform finger abductions. Considering that both monkeys had a preference for the use of the index finger, its motion was preferentially reinforced. Reinforcing aspects of the behavioural training (which are experimentally acceptable and in no contradiction to the training objectives) which are chosen by the subjects, permit the monkeys to *control* parts of their progression. This aspect of choice and control might lead to increased performance (Roma et al., 2006). For the same reason, the training and the tasks (including the manipulandum) have been designed to accomplish the monkey's preference for instance with respect to the monkey's preferred hand use. After the monkeys were comfortable with the plastic shaft and moving *any finger* they have *chosen*, training focused on the (final) finger abduction task. Concepts including a tertiary reinforcer (tactile and visual cue) were introduced. A secondary reinforcer (tone controlled and delivered by a computer program) was used to *bridge* the correct behaviour to the primary positive reinforcer (rewards, see Figure 2.2A-2.2C). Movement of instructed selective abduction movements with the index finger and the thumb were successfully taught. Selectivity and performance could have been improved even further with additional training time. Many concepts, training routines, familiar objects and procedures (Figure 2.3A-2.3C) enhance the learning process by applying principles of transfer and association learning (Warren, 1974; Washburn and Rumbaugh, 1991). A mostly PRT approach (Perlman et al., 2012) combined with occasional elements of NPRT (Wergard et al., 2015) proved effective for refining the monkey's learning process and increasing the monkey's welfare and performance.

2.5.2 *A refined hierarchical rewarding scheme to prolong the subject's performance and motivation*

The use of primary reinforcer in classic and operant conditioning of an animal to a desired behaviour is a common technique applied in behavioural animal sciences (Prescott et al., 2010). Usually either food or fluid rewards are used as a primary reinforcer (Prescott et al., 2010; Westlund, 2012a). The choice between these often depends on the experimental design (Prescott et al., 2010; Prescott et al., 2012b). The value of food as a primary reinforcer becomes immediately apparent when considering its behavioural and ecological status in the evolution of human and non-human primates. For example chimpanzees have shown remarkable skills in remembering the location of food for a long time (Mendes and Call, 2014).

Using food restriction to increase the animal's motivation should be carefully assessed and amended if possible (Prescott et al., 2010). This is however not applicable to all studies (Prescott et al., 2010) and discussion about the need of food restriction in teaching animals behavioural tasks emerged (Prescott et al., 2010; Prescott et al., 2012b; Westlund, 2012a; Westlund, 2012b). The main discussion focussed basically on two questions: Is it possible to use secondary reinforcer without the use of a primary reinforcer? And can a variable rewarding scheme (only rewarding an animal after a random number of correct trials) work to condition an animal to a desired behaviour?

In this context, it has been argued that the mere presentation of the secondary reinforcer can cause the desired behaviour (Ikemoto and Panksepp, 1999; Westlund, 2012a). Whereas this might be true for simple behaviour, when acquiring more complex tasks the motivational content of a tertiary or secondary reinforcer might not be enough. It has furthermore been speculated that a secondary (conditioned) reinforcer might activate cortical networks involved in reward expectation and emotional processing (Schultz et al., 2000; Seymour and

Dolan, 2008), and that a secondary reinforcer might activate the *seeking* system (Alcaro and Panksepp, 2011; Phillips et al., 2003), which leads to increased motivation (Panksepp, 2005; Westlund, 2012a). Whereas this might be true, the incentive value can fade over time when not positively enhanced by strengthening the link to the primary reinforcer (at least occasionally).

To address these questions and to refine the monkey's welfare and training performance, I performed several pre-assessments regarding the rewarding scheme. But before being able to utilise a primary reinforcer effectively, it is important to assess the value of a particular reward. Since the effect of a reward type (Prescott et al., 2010) can be subject dependent, the same can be true for the incentive value (desirability) of a particular reward and thereby influencing the monkey's performance (Wu et al., 1986). Furthermore, it has been shown that a higher value reward does have behavioural consequences, i.e. decreased reaction times (Watanabe et al., 2001). Prescott et al., 2010 suggested to study species specific literature and seek advice with consulting veterinarians, but the incentive value of a reward can be assessed empirically. Therefore, a monkey-specific fruit hierarchy (Figure 2.2A-2.2C) was determined by using a simple binary choice test (for details see Section 2.5.2). In the following, I tried to use a variable rate rewarding scheme (Schoenfeld et al., 1956; Westlund, 2012a) for several training procedures with monkey S and U. I found that the higher the attentional demand or task complexity, the more effective the fixed rate rewarding scheme (Prescott et al., 2010) for both monkeys. This is in line with findings in behavioural (neuro-scientific) research reporting advantageous effects of fixed rate rewarding schemes for more complex tasks (Prescott et al., 2012b). Additionally, I tried to use a mixed rewarding scheme (randomly giving any type of reward in a random order). This led to reduced overall trial numbers for both monkeys. I did not try to skip the primary reinforcer completely, since the monkey's performance was already reduced when using a variable rate scheme. Applying a strict food restriction might not be necessary for all training procedures

(Prescott et al., 2010; Westlund, 2012a), but whenever a complex behaviour is taught, additional motivational incentive of the primary reinforcer can be beneficial and sometimes even necessary.

Considering that the rate of reward should be carefully considered (Prescott et al., 2010), task-specific constraints and subject-dependent incentive values and the order of reward types (Figure 2.2A-2.2C) should be carefully assessed. The rate of reward delivery also depends on the size of the food reward (Figure 2.2C), and should be kept as consistent as possible. Using an incremental food size would be feasible for instance and can be utilised whenever additional motivation is required to learn e.g. a novel behaviour.

Referring back to the debate regarding the two questions above, I would argue that the level of food restriction depends on the particular training setup, the complexity of the task, the objectives regarding a desired behaviour, the time frame and desired performance level. Nevertheless, refinements within training procedures of intermediate complexity would be possible and should be carefully assessed. From a welfare point of view, once the animal is fully trained, food (and fluid) restrictions should be decreased, enabling the gain of weight (Toth and Gardiner, 2000) if the animal is mature. This however can be accomplished with increased training (or increased food size of higher order rewards, Figure 2.2C) as well. Considering that the monkey would constantly increase its weight and reach obesity when an ad-libitum food access would be applied, a restricted feeding regime was implemented. Furthermore, ad-libitum rewarding schemes can lead to decreased performance (Taffe, 2004).

Although many studies use the animal's preferred food for classic and operant conditioning (Crofts et al., 1999), training routines optimizing the precise animal-specific incentive value of rewards are often lacking. Utilising this hierarchy of rewards (Figure 2.2A-2.2C) together with an incremental (or partly incremental)

rewarding scheme, enables the refinement of training procedures to optimise the animal's motivation, welfare and performance.

2.5.3 *Building on familiar objects and procedures to enhance the monkey's confidence and performance*

Increasing the monkey's training performance and welfare at the same time is not necessarily a contradiction. This becomes apparent when regarding behavioural training itself as a form of enrichment (Westlund, 2014). Identifying factors which both lead to increased performance and to the animal's wellbeing are those which effectively engage the monkey into a behaviour that leads to success and rewards (physically and psychologically). Performance is increased when supporting the subject's choice and control (Roma et al., 2006). Therefore many scientific studies involving the behavioural training of non-human primates could utilise these features in designing studies and tasks. One way of achieving these joint objectives is applying principles of transfer and association learning (Warren, 1974; Washburn and Rumbaugh, 1991) to experimental design. PRT does not only help improving the behavioural training of specific aspects in training, it also leads to increased cooperation, motivation and thereby enrichment through the training process. Therefore, it can be sensible to build training procedures on familiar objects and behavioural cues (Cartoni et al., 2013). These can be utilised to establish association chains enabling a smooth transfer of skills to novel situations. Not only familiar objects (Figure 2.3A and 2.3B) but also more abstract concepts including routines and procedures (Figure 2.3C) can become a motivational enforcer itself. Using this approach, new associations can be formed to a new context or skill. Over the time course of training, the set of familiar objects and procedures increased (Figure 2.3B and 2.3C) which also increases the amount of the subject's control and familiarity over the training. This itself increases the monkey's confidence, motivation, welfare and performance.

2.5.4 *Homogeneous focus of behavioural training for both monkeys*

To be able to compare training effects between monkeys, comparable numbers of training sessions addressing a specific procedure have to be performed. This was the case for both monkeys (Figure 2.4A, 2.4B, and Table 2.3). The most noticeable difference with respect to the allocation of training procedure focus between monkeys was within the neckbar training and finger abduction task. Since an additional training step has been added to the neckbar training (see Section 2.4.1) and the training of the finger abduction task required setup modifications for monkey U, the amount of additional training sessions for monkey U were within an expected range. In some cases the transition from one procedure to the next did not depend solely on the monkey's training performance but also on task-setup depending constraints (building a modified arm restraint, building a task specific manipulandum, etc.). Therefore, the number of training sessions cannot be consulted when defining training success in all instances. The finger abduction task could have been taught earlier. The delay was depending on equipment-specific constraints. Overall, the focus on particular training procedures was fairly balanced between monkeys.

2.5.5 *The increase of the monkey's weight depended on the particular training procedure*

Monkey's weight increased for both monkeys through the training process (Figure 2.5A and 2.5B) despite the fact that a moderate but consistent food restriction was applied. The monkey's weight changes with age (Tigno et al., 2004), restricting food is essential to prevent obesity, increase the monkey's performance (Taffe, 2004), and to enhance the effects of primary reinforcer (the food reward) in the context of operant conditioning. Especially when using a PRT approach the high incentive value of the primary reinforcer (food) itself becomes more apparent under restricted feeding regimes. Under such a

feeding regime, the weight progression can be indicative of training performance (indirectly reflecting the number of rewards received for correct behaviours within a training session). Furthermore, the weight progression is an important factor when monitoring the monkey's welfare (Prescott et al., 2010). Due to this reason, the weight progression was monitored and the training procedure dependently analysed (Figure 2.5A and 2.5B).

The increase in weight (slope) for both monkeys was most pronounced when moving to the finger abduction task (Figure 2.5A and 2.5B). This is partly correlated with the total number of correct trials for this training procedure (Figure 2.6A and 2.6B) but not entirely.

Both monkey's weight decreased after the headpiece implant surgery (black dashed lines, Figure 2.5A and 2.5B) due to a number of reasons. Prior and immediately after the headpiece implant surgery monkeys were fasted for the surgical intervention itself and for subsequent medical assessments performed under anaesthesia. Some of the surgical implants itself (e.g. the EMG electrodes) required a recovery period in which wounds associated with the surgical intervention could heal. Therefore, no training sessions (just home cage and lab interaction sessions) were performed. This is another reason why despite ad-libitum food in this time period a sudden decrease in weight occurred. In the following training sessions, session length was gradually increased. Thus, monkeys had gradually the opportunity to reach a higher number of correct trials and therefore rewards.

2.5.6 *Total number of correct trials as welfare and performance indicator*

The total number of correct trials increased for both monkeys throughout the training (Figure 2.6A and 2.6B). The number of correct trials was highly training-procedure dependent (Figure 2.6A, 2.6B, and 2.9B). Progression of subsequent

training procedure dependent correct trial numbers was equally affected for both monkeys (Figure 2.9B). These can be explained by different degrees of complexity for various training procedures. Furthermore, different tasks consisted of different inter-trial lengths. For example a correct trial in the context of the neckbar training (holding the target, positioning the head in the neckbar, waiting for neckbar movement before receiving the reward) required more time than a trial for the arm or finger abduction task.

Furthermore, fluctuations in training performance caused by increasing difficulty (e.g. by adjusting task specific parameter) are reflected within the correct trial number progression. Within most training procedures the difficulty increased gradually. This led to a temporary decrease in correct trial number, before the final performance level within a training stage was reached. From there on, the correct trial number increased before the transition to the next training procedure occurred (Figure 2.6A and 2.6B).

Additionally, there are many aspects which might influence the overall correct trial number including incidences, environmental factors, changes in group dynamics, colony events, the animal's mood, the animal's stress level, health and physiology related parameters. The total number of correct trials is also affected by the session length. And although the monkey influenced the session length usually by motivational factors and inter-trial rate, sometimes it was sensible (e.g. whenever a novel and difficult part of the task was introduced) to keep the over all session length short and thereby the subjective experience of the session positively associated.

2.5.7 *Training performance depended on the training procedures but remained similar between monkeys*

Due to the dependence of the total number of correct trials on the overall session length, the ratio between the total number of correct trials over the total

session length was used as a measure of the monkey's *performance* (Figure 2.1A and 2.1B). The advantage of this measure becomes evident when comparing the progression of the correct trials (Figure 2.6A and 2.6B) to the progression of the training performance (Figure 2.1A and 2.1B). When relying on the first, one might infer that one of the highest trial numbers (and performance) was reached during the finger abduction prototype task for monkey S (Figure 2.6A, yellow marker), that the performance (based on the number of correct trials) was consistently lower for monkey U than for monkey S (Figure 2.6A and 2.6B), and that in all training procedures extreme variations in performance appeared. All of these observations are biases introduced by various session lengths. When consulting the training performance progression (Figure 2.1A and 2.1B), these phenomena diminish: The higher performance for monkey S during the finger abduction prototype training had to do with an increased session length, performance between monkeys was more similar than expected when relying on the correct trial number alone, and extreme outliers in trial numbers were introduced due to very short and long training sessions, respectively. However, apart from outliers and a baseline shift the overall progression remains similar and training category (procedure) specific (Figure 2.7A and 2.7B). Further accuracy in the measure of performance could be achieved when including a more precise measure of session length. The performance could be expressed as the number of correct trials per task length and errors (and attempts) could be included as well. These data however, are more difficult to acquire especially in early training stages in which a computer-assisted performance acquisition is more difficult. The use of automated feeder training in the home cage environment could include a more quantitative measure of training performance at earlier stages in training.

2.5.8 *Weight progression and correct trial number was correlated between monkeys*

When inspecting the weight curves for the two monkeys (Figure 2.5A and 2.5B), a similar progression profile is noticeable. This raises the question of how correlated these two curves are within sessions where both monkeys were trained on the same day (Figure 2.8A). Correlation analysis revealed a moderately strong linear correlation between the weights of the two monkeys (Figure 2.8D). On the one hand side, this might be expected considering that both monkeys had the same access to basic food (e.g. seeds and biscuits) in the home cage environment. However, since weight progression is highly training dependent (Figure 2.5A and 2.5B), this could imply that performance (and thereby trial numbers) were correlated between monkeys. In other words, the change in performance (e.g. as a result of motivation, mood, or events in the animal unit) might affect the performance and thereby the weight of the other monkey as well.

Therefore, the same correlation analysis was performed for the number of correct trials (Figure 2.8B and 2.8E). A weak but significant linear correlation, indicates an overall relation between the total number of correct trials between monkeys. The strength and the variance of this correlation is training procedure dependent (for example a stronger correlation between monkeys for the arm abduction task and the vibration cue training in comparison to e.g. later training stages, Figure 2.8A).

Analysing the linear correlation between the monkeys' performances however, did not reveal any significant linear correlations between monkeys (Figure 2.8C and 2.8F).

This could mean that the weak correlation in the total number of correct trials between monkeys was caused by correlated total session lengths, or that the

total session length was not a precise enough parameter to calculate the overall performance. In either case, there are some correlations between physiological and training-specific parameter (e.g. constraints defined the particular training procedure, cf. Figure 2.9A-2.9C) which should be carefully considered when monitoring the monkey's training performance and defining the short-term and mid-term training objectives.

2.5.9 *Subjective assessment of the monkey's stress level and its influence on trial number and training performance*

Subjectively monitoring the monkey's stress level according to a scoring system (Table 2.1) is important in assessing the monkey's welfare and predicting its performance capability. Considering that the monkey's personality (Coleman, 2012) and anxiety level (Coleman and Pierre, 2014) influences the monkey's performance, it is very likely that the monkey's stress level is equally affecting it. And indeed, stress levels have been shown to affect performance in human (Drummond et al., 2000; Robert and Hockey, 1997) and non-human (D'Aquila et al., 1994; Hackman et al., 2010; Luine et al., 1994; Lupien et al., 2009; Willner, 1997) subjects. The decrease in performance could be related to the aforementioned anxiety level leading to the subject being more easily distracted leading to decreased attention and focus (Willner, 1997). Since pre- and postnatal parental stress does affect human and non-human offspring (DiPietro, 2004), stress presumably influences other members living and interacting in a close social environment as well. Therefore, monitoring the monkey's stress level quantitatively (e.g. measurement of cortisol levels or electrocardiography) and qualitatively (behavioural and psychological assessments) can be informative and valuable in predicting performance level and learning rate. Whereas the monkey's subjective stress levels had no influence on the monkey's weight (Figure 2.1A and 2.1B, first row), the average number of correct trials was generally increased in sessions in which the monkey's behaviour was assessed as being calm and

non-stressed in comparison to more stressful sessions (Figure 2.10A and 2.10B, second row). This difference was significant for both monkeys ($p = 0.01$, two sample t-test). Although the average performance in non-stressful training sessions was slightly higher for both monkeys, this difference was not significant. The difference between the effect of subjective stress on correct trial number and training performance could be related to the session length (cf. discussion in Section 2.5.7). The monkey's stress level could have motivated the trainer to keep the training session shorter and thereby reduced the overall correct trial number. On the other hand side, the overall session length might not be accurate enough to account for stress-induced differences as expressed by the training performance ratio. Nevertheless, the subjective stress level does have an effect on the behavioural training, the animal's welfare and motivation, and therefore on performance and training success.

2.5.10 *A centralised database for qualitative and quantitative measures to refine training procedures and the animal's welfare in the context of applying the 3Rs to improved behavioural training*

The framework of the 3Rs (Russell and Burch, 1959) has become an ethical, legislative and code-of-practice framework defining good standards in animal experimentation. It is a robust framework for improving the animal's welfare, validity of studies, and assessments of translational research (Graham and Prescott, 2015). Harm- and cost-benefit (Animal Procedures Committee, 2003. Review of cost-benefit assessment in the use of animals in research `https://www.gov.uk/research-and-testing-using-animals`) analysis should be performed prior to any scientific study involving the use of animals. Although the replacement and reduction (in the original sense of replacing animals with other species or alternative approaches, and reducing the number of animals used per study) is difficult in (neuro-scientific) research since many basic and translational research questions rely on fundamental understanding of the

(neuro-) physiology, refinement of procedures can be applied to many aspects of animal experimentation (Blakemore et al., 2012). Nevertheless, it is possible to redefine or interpret the first two concepts slightly differently. *Replacement* of training involving negative reinforcement strategies with positive classic and operant conditioning like PRT, *reduction* of the number of unfavorable (e.g. stressful) aspects of training, and *refinement* of training procedures could lead to achieve a maximum benefit (and reduction of any potential harm and distress). To achieve these objectives it is essential to seek for conceptual refinements as well as concrete implementations of these into practice. Soundly planned, behavioural training can incorporate crucial concepts of transfer, association, and motor learning (Warren, 1974; Washburn and Rumbaugh, 1991; Wolpert et al., 2011). The use and study of these can improve standards in animal-based research involving many veterinary, behavioural, and experimental routines. Procedures reevaluating the incentive value of primary reinforcers (cf. food hierarchy assessment, e.g. Figure 2.2A-2.2C) commonly used to condition animals to a desired behaviour could help to enhance the beneficial effect of those. Steps of improved transfer and association learning could involve the use of more familiar objects and routines (Figure 2.3A-2.3C) giving the animal increased control (Roma et al., 2006) over the experiment which itself increases the monkeys confidence and motivation. The effectiveness of these is even more enhanced when applied consistently throughout the facility involving everybody being in contact with the subjects. This requires a consistent training and facility-wide implementation of routines. On the downside, this can imply increased costs and time effort for staff and researchers (Prescott et al., 2005). However, more careful assessments of the training benefits (including increased learning process, performance and time efficiency) should be performed to investigate whether these outweighs the potential costs and effort.

Key development / achievement	Other / conventional approaches	Advantages over other / conventional approaches
Designing training sessions based on familiar objects and procedures	A) Starting with final task immediately (no intermediate steps).	To A) Stepwise learning builds confidence and skills gradually. Starting with the final task immediately requires teaching the subject many general and task specific skills (overload of training objectives).
	B) Transition between quite different (sub-) tasks.	To B) Increase in training time and effort because loosing the advantage of building on already familiar concepts. Potentially increased stress because of many "novel" situations.
	C) Separation between the training of general and task-specific skills.	To C) Planning and training of both general and task-specific skills enables consistent training methodology and building on solidly established foundations. Consistency in training and methodology might lead to a reduction in the subject's stress level.
Food hierarchy based on subject's preference	A) Hierarchical rewarding scheme: not based on the subject's preference.	To A) The subject might lose motivation at particular stages in the hierarchy and perform trials more slowly or stop completely. The overall trial number might be reduced due to skipping of stages with a lower incentive value.
	B) Hierarchical rewarding scheme with variable number of rewards per food type.	To B) Variable number of type of food per reward type might have distinct effects. Increased numbers of trials of types with low incentive value might lead to lower overall trials numbers. Increased numbers of trials of types with a high incentive value might lead to increased overall trial numbers but reduced consistency. A constant and consistent trial number per reward type supports conditioning the subject to take a consistent number of one particular food (e.g. fruits) independent on alterations in the subject's motivation or slight differences in daily preferences.
	C) Mixed / random rewarding scheme.	To C) Mixed or random rewarding schemes are not appropriate for controlling the incentive value of particular reward types and exclude the possibility of gradual training steps (e.g. gradually increasing the incentive value with task difficulty). Subject's will try to deny particular reward types. Overall higher trial numbers with constant hierarchical rewarding scheme.
Training focus analysis and concentration on particular key training procedures	Training on specific objective until the subject grasps the principle and reaches a certain performance level (variable length per subject).	Allocating an equal time frame for each individual requires very consistent training and assessment of methodologies to ensure the completion of objectives in time.
Cross-subject correlation analysis of behaviour and training procedure specific parameters	Only the individual's progress counts. Training effects are independent to other subjects.	Performance of one subject might allow for prediction of another subject's performance. Therefore, variations of performance can be included into short and mid-term training plans. This might lead to increased efficiency and less redundancy of training sessions.
Home cage carabiner and tone device and neckbar training using PRT	A) No teaching of general skills and concepts.	To A) This might lead to an overload of new skills necessary for more complex tasks. A gradual acquisition of skills and concepts strengthens the subject's confidence and motivation.
	B) Splitting training to general and task-specific ones.	To B) Splitting sessions into general and task-specific sessions might result into less consistent behaviour. Some general skills and concepts (e.g. tones, targets) can be incorporated in task-specific sessions.
	C) Home cage interaction without carabiner and tone device.	To C) This would have the advantage of getting the monkeys used to the trainer and researcher. This procedure can be performed in the same amount of time using the tone device and an individually assigned target. This way the task is more clearly defined for the subject and it is possible to convey crucial and transferable concepts.
	D) Neckbar training by positive punishment (either neckbar itself or additional object to limit space).	To D) Might need less training sessions for the subject to get used to this procedure. However, it certainly increases the monkeys stress level. Depending on the subject's personality and on other potentially stressful aspects of the training this approach might promote learnt helplessness. Therefore, a PRT approach in neckbar training is favourable.

Table 2.6: *Key developments of the present study in comparison to conventional approaches.* Key training procedures and techniques, which were applied during the behavioural training of two macaque monkeys in comparison to other conventional approaches.

In the context of refinements, it could be beneficial to build a concise and detailed documentation of physiological, husbandry, and training related quantitative and qualitative data within a centralised database. This data would enable an improvement of conventional approaches as well as training methodologies. A comparison of the effective key developments of the present study to alternative approaches is shown in Table 2.6.

Quantitative measurable data could include training specific details (e.g. trial numbers, errors, performance scores, number or rewards) acquired by the trainer and physiological (and medically) relevant parameters (e.g. results from blood tests, weight, amount of food, drug doses, heart rate, blood pressure) documented by the veterinarians or husbandry staff.

Combining all of these information would enable to improve the standard of welfare and experimentation. For example, considering that training itself is enrichment (Westlund, 2014), and that a monkey refusing to work (or showing decreased performance) can be an early indication of health issues (Smith et al., 2006), these could in conjunction with medical assessments (blood samples etc.) lead to early diagnosis crucial for the animal's health, training and performance. Furthermore, subjective measures of the monkey's (emotional) state (e.g. anxiety, personality, stress levels) could be expanded and combined with objective physiological assessments leading to more informed and valid conclusions. Training could be designed or adapted (e.g. difficulty level or focus of training) based on subjective assessments (cf. effects of stress on behavioural training, Figure 2.10A and 2.10B) and cross-subject effects (Figure 2.8A-2.8F) could be included. The latter could be expanded to investigate group effects of these parameters and how group dynamics affect health and performance. Conclusions of these analysis could be incorporated into standardised training concepts, refined schedules, and even be used to predict the monkey's performance to enhance standards in animal experimentation.

Chapter 3. Influence of peripheral nerve stimulation on task performance

3.1. Summary and key findings

- Non-invasive methods relying on stimulating pairs of afferent nerves have been used to induce plastic changes in the sensorimotor cortex.
- By synchronous (associative) stimulation (AS) of two afferent nerves, excitability changes in the motor cortex occur as indicated by studies reporting changes in motor evoked potentials (MEPs) elicited by transcranial magnetic stimulation (TMS). Furthermore, motor-cortical representations of the synchronously stimulated muscles extend and overlap after AS. By using asynchronous (non-associative) stimulation (NAS) overlapping motor-cortical representations can be temporarily separated in focal hand dystonia (FHD) patients (Schabrun et al., 2009).
- Since AS and NAS are considered to affect predominantly the corticospinal projection to target sites stimulated during the intervention, it would be of interest to assess the task performance by studying selective movements differentially involving stimulated target sites.
- To compare the effects of peripheral nerve stimulation on task performance, I trained two macaque monkeys to execute selective finger movements with the thumb and index finger. The task performance was compared before and after one hour of AS or NAS of the median and ulnar nerve.
- Both monkeys showed an increased number of errors after AS and decreased number of errors after NAS. Similarly, both monkeys' performance decreased after AS and increased after NAS. The difference of performance change between AS and NAS was significant for both monkeys. Monkeys' performance speed decreased after AS and NAS.

3.2. Introduction

Non-invasive methods relying on stimulating pairs of afferent nerves have been used to induce plastic changes in the sensorimotor cortex (Godde et al., 1996; McDonnell and Ridding, 2006; Pyndt and Ridding, 2004; Ridding and Uy, 2003; Rosenkranz et al., 2009; Schabrun and Ridding, 2007; Schabrun et al., 2009).

By synchronous (associative) stimulation (AS) of two afferent nerves (or motor points, for details about the different stimulation sites, see introduction of Chapter 4) excitability changes in the motor cortex occur as indicated by studies reporting changes in motor evoked potentials (MEPs) elicited by transcranial magnetic stimulation (Godde et al., 1996; McDonnell and Ridding, 2006; Pyndt and Ridding, 2004; Ridding and Uy, 2003; Schabrun and Ridding, 2007).

Repetitive stimulation of those nerves has a potential in rehabilitation and treatment of neurological disorders like stroke or spinal cord injury. Especially a neurological condition commonly referred to as focal hand dystonia (FHD) has been hypothesised to be linked to maladaptive (co-) activation of sensory afferents which lead to aberrant motor-cortical plasticity (Quartarone et al., 2008, 2014; Schabrun et al., 2009; Tinazzi et al., 2000). These characteristics are similar to those artificially induced in healthy human subjects using paired synchronous (associative) motor point or nerve stimulation (McKay et al., 2002; Ridding and Uy, 2003; Schabrun and Ridding, 2007; Schabrun et al., 2009).

The importance of the co-activation of peripheral afferents to induce plastic changes in the sensorimotor cortex is further stressed by studies investigating the effects of motor practice (Butefisch et al., 2000; Classen et al., 1998; Karni and Bertini, 1997; Karni et al., 1995; Nitsche et al., 2003; Nudo et al., 1996; Oza and Giszter, 2015; Rioult-Pedotti et al., 1998; Schwenkreis et al., 2005). These use-dependent plasticity (UDP) effects after motor practice on motor-cortical excitability are frequently reflected by studies reporting changes in MEPs

(Ackerley et al., 2007; Butefisch et al., 2000; Liepert et al., 1999) after motor training.

Liepert et al. (1999) studied the effects of synchronised (and desynchronised) thumb and foot movements. The authors found that the motor-cortical representations as indicated by TMS-assessed CoG maps of the cortical APB and foot representation moved closer together after 120 synchronous thumb and foot movements. Schwenkreis et al. (2001) found a shift of the N20 SEP dipole after synchronised thumb and shoulder movements. They concluded that plastic changes in the contralateral S1 occurred due to synchronised proprioceptive input.

The use-dependent and by synchronous (associative) peripheral nerve stimulation induced motor-cortical plasticity are both considered to be initiated by a co-activation of peripheral afferents. Considering this conceptual similarity, what effect on the subject's ability to perform a (skilled) motor task could be expected after repetitive synchronous (associative) peripheral (or motor point) nerve stimulation?

Godde et al. (1996) reported improvements in a spatial discrimination task after tactile co-activation of two digits. McDonnell and Ridding (2006) noted that a group of subjects which received synchronous (associative) stimulation of APB and FDI improved more rapidly in a grooved pegboard task (GPT). Sorinola et al. (2012) described that subjects which received two hours of median and ulnar nerve (The improvement in the Jebson-Taylor hand function test was also found after synchronous median, ulnar and radial nerve stimulation) synchronous stimulation performed quicker in the Jebson-Taylor hand function test (JTHFT) test. Schabrun et al. (2009) found significant improvements in cyclic drawings in dystonia patients after asynchronous (non-associative) stimulation of APB and FDI. Rosenkranz et al. (2009) reported a task-specific improvements for

musician's dystonia patients after asynchronous vibro-tactile stimulation of APB, FDI and ADM using a musical instrument digital interface (MIDI) piano.

Since the synchronous and asynchronous peripheral nerve (or motor point) stimulation are considered to affect predominantly the corticospinal projection to the target sites stimulated during the intervention (muscles or nerves, cf. McDonnell and Ridding, 2006; Pyndt and Ridding, 2004; Ridding and Uy, 2003; Schabrun and Ridding, 2007), it would be of interest to assess the task performance by studying selective movements differentially involving stimulated target sites. I hypothesise that the synchronous stimulation of the median and ulnar nerve will reduce the monkeys' capability being able to move the relevant (by these nerves innervated) muscles selectively. On the contrary, the asynchronous intervention should lead to more selective finger movements.

To test this hypothesis, I trained two macaque monkeys to perform a finger abduction task involving the independent and selective movement of the thumb and the index finger. Each of these finger movements was cued by a vibro-tactile stimulus (for details about the behavioural training process see Chapter 2). The finger abduction task performance was compared before and after one hour of synchronous (associative) or asynchronous (non-associative) median (predominantly innervating APB) and ulnar (predominantly innervating FDI) nerve stimulation (intervention).

Both monkeys showed an increased number of errors after the synchronous and decreased number of errors after the asynchronous intervention. Similarly, both monkeys' performances decreased after the synchronous, and increased after the asynchronous intervention. The difference of the performance change between the synchronous and asynchronous interventions was significant for both monkeys. The monkeys' performance speed decreased for both monkeys.

These results suggest that stimulation-site specific effects of the peripheral nerve stimulation intervention selectively interferes with the task performance.

The synchronous intervention (considered to increase motor-cortical extend and overlap of target muscles) might make the independent and selective movement of the thumb and index finger more difficult, while the asynchronous intervention makes it easier.

These findings imply that paired synchronous and asynchronous peripheral nerve (and motor point) stimulation can be used to selectively target task-relevant muscles. This might lead to improved stimulation paradigms connecting (or disconnecting) the concurrent (or disjoint) muscle activity of adjacent motor points leading to novel therapeutic interventions for a variety of neurological conditions.

3.3. Materials and methods

3.3.1 *Subjects and experimental design*

Two female macaque monkeys (monkey S: six years old with an average weight of $6.3\,\mathrm{kg}$; and monkey U: four years old with an average weight of $4.76\,\mathrm{kg}$) were trained to perform a finger abduction task involving the independent movement of either the thumb or the index finger.

For details about the subjects, housing, behavioural training (intermediate and final training stages) and training concepts and procedures see Chapter 2.

In brief, the monkeys were conditioned to position their hand in a custom made manipulandum (Figure 2.1G). The movement of either the thumb or the index finger was then instructed by a vibrational cue delivered through vibration disks (Precision Microdrives Ltd, Catalogue Number: 308-100) positioned underneath each plastic shaft of the manipulandum (Figure 2.1G). A clear abduction movement of the instructed finger crossing a pre-defined positional threshold had to be performed while keeping all non-instructed finger below a maximal-motion

threshold. Both of these thresholds (the lower limit for the instructed and the upper limit for the non-instructed finger movement) were gradually refined during the training process.

Both monkey S and U were able to perform distinct abduction movements of the instructed (in comparison to the non-instructed) finger after the behavioural training. Monkey S and U were able to move their thumb more selectively than their index finger. This enabled a continuous reduction of the aforementioned maximal-motion threshold level of the index finger (whenever the thumb was instructed).

However, whenever the index finger was instructed by the vibrational cue, the animals performed less selective movements. Therefore, the maximal-motion threshold for the thumb had to be kept higher than in the previous condition (cf. maximal-motion threshold of the index finger when the thumb was instructed). Nevertheless, the maximal-motion threshold for the thumb was below the pre-defined minimal-motion threshold in conditions where the thumb motion was instructed. Acknowledging this discrepancy in non-instructed maximal-motion thresholds between thumb and index finger, it can be expected that errors (crossing the maximal-motion threshold with the non-instructed finger) are more likely to occur with the index finger (more difficult condition because of the lower threshold) than with the thumb (easier because the thumb is allowed to move more whenever the index finger is instructed).

Several task specific parameters including the number of errors with the thumb $e_{\mathtt{thumb}}$ and index finger $e_{\mathtt{index}}$, the total time performing the task $t_{\mathtt{task}}$, and the total number of correct trials $n_{\mathtt{ctrials}}$ were measured before and after $1\,\mathrm{h}$ of two different interventions. The first intervention consisted of $1\,\mathrm{h}$ of synchronous (associative) median and ulnar nerve stimulation. The second intervention was one hour of asynchronous (non-associative) median and ulnar nerve stimulation.

The stimulation frequency was randomised between 8.3 and 12.5 Hz (10 Hz average stimulation frequency). During the synchronous (associative) intervention both the median and ulnar nerve were stimulated simultaneously approximately 10 times per second. During the asynchronous (non-associative) intervention both median and ulnar nerve stimulation were stimulated alternatingly out-of-phase with an average frequency of 10 Hz each. The stimulation was applied through implanted bipolar nerve cuff electrodes (see Chapter 4 e.g. Figure 4.1A). The stimulation intensity was 2 times above the motor threshold (clear twitch in the muscles innervated by the nerves). Biphasic stimulation pulses (1 ms width) were applied by two isolated pulse stimulator (stimulator 1: AM Systems, Catalogue Number: 720005; stimulator 2: DS4, Digitimer Ltd).

The synchronous and asynchronous interventions were applied alternatingly from one experimental session to the next. Sessions were interleaved by at least 36 h to avoid potentially lasting effects to affect subsequent sessions.

The behavioural assessment was performed immediately before and approximately 15 min after the end of the stimulation (interventions).

3.3.2 *Data acquisition and analysis*

All task relevant (i.e. position signals, force traces, task marker) and electrophysiological (see Chapter 4) data were captured with a high speed data acquisition (DAQ) card (National Instruments) and saved to hard disk.

The total error number e_{total} was expressed as the sum of the number of errors for thumb and index finger. In principle it would have been possible to register errors from the little finger as well. This finger however never moved (presumably because it was never task relevant) by any of monkeys.

The error difference $\Delta e = e^{\mathtt{aft}} - e^{\mathtt{bef}}$ was calculated for the total number of errors $\Delta e_{\mathtt{total}}$, and the errors for the index finger $\Delta e_{\mathtt{index}}$ and thumb $\Delta e_{\mathtt{thumb}}$, respectively.

The performance p was expressed as

$$\rho = -\left(\frac{\left(\frac{e_{\mathtt{total}}}{t_{\mathtt{task}}}\right)}{\left(\frac{e_{\mathtt{total}}}{t_{\mathtt{task}}}\right) + \left(\frac{n_{\mathtt{ctrials}}}{t_{\mathtt{task}}}\right)}\right) \tag{3.1}$$

with the total number of errors $e_{\mathtt{total}}$, task length $t_{\mathtt{task}}$ (in $\min$) and the total number of correct trials $n_{\mathtt{ctrials}}$.

The performance difference $\Delta\rho$ was defined as the difference in performance between after and before the intervention:

$$\Delta\rho = \rho^{\mathtt{aft}} - \rho^{\mathtt{bef}} \tag{3.2}$$

The performance speed s was defined as the ratio between the number of correct trials $n_{\mathtt{ctrials}}$ divided by the task length $t_{\mathtt{task}}$. The speed difference Δs was compared between the two different stimulation protocols.

Data analysis and visualisation was performed with custom written Python (Rossum, 1995) scripts (Python, Version 2.7.9, `https://www.python.org`).

Statistical comparisons of task-specific parameter were performed by calculating a two-sample t-test with a significance level of $p < 0.01$. Significant differences will be highlighted with an asterisk in the following sections.

3.4. Results

Before investigating the influence of the type of intervention on the number of errors (threshold crossings with the non-instructed finger), the influence of other task dependent parameters were assessed (data not shown). Preliminary analysis for example revealed that a particular reward type (see fruit hierarchy, Figure 2.2) did not affect the number of errors but the reaction time (RT).

3.4.1 *The number of errors increased after the synchronous and decreased after the asynchronous stimulation intervention*

Comparing the number of errors (threshold crossings with the non-instructed finger) before and after both the synchronous (associative) and asynchronous (non-associative) interventions, revealed intervention-type dependent error differences (Figure 3.1A and 3.1B).

The total number of errors $e_{\texttt{total}}$ was higher after the synchronous intervention in most of the sessions for monkey S (Figure 3.1A) and monkey U (Figure 3.1B). The total number of errors $e_{\texttt{total}}$ after the asynchronous interventions on the other hand was decreased (Figure 3.1A and 3.1B) for both monkeys.

The difference of this total error change between the synchronous and asynchronous intervention was significant for monkey U ($p < 0.01$, two-sample t-test) and not significant for monkey S ($p = 0.06$, two-sample t-test).

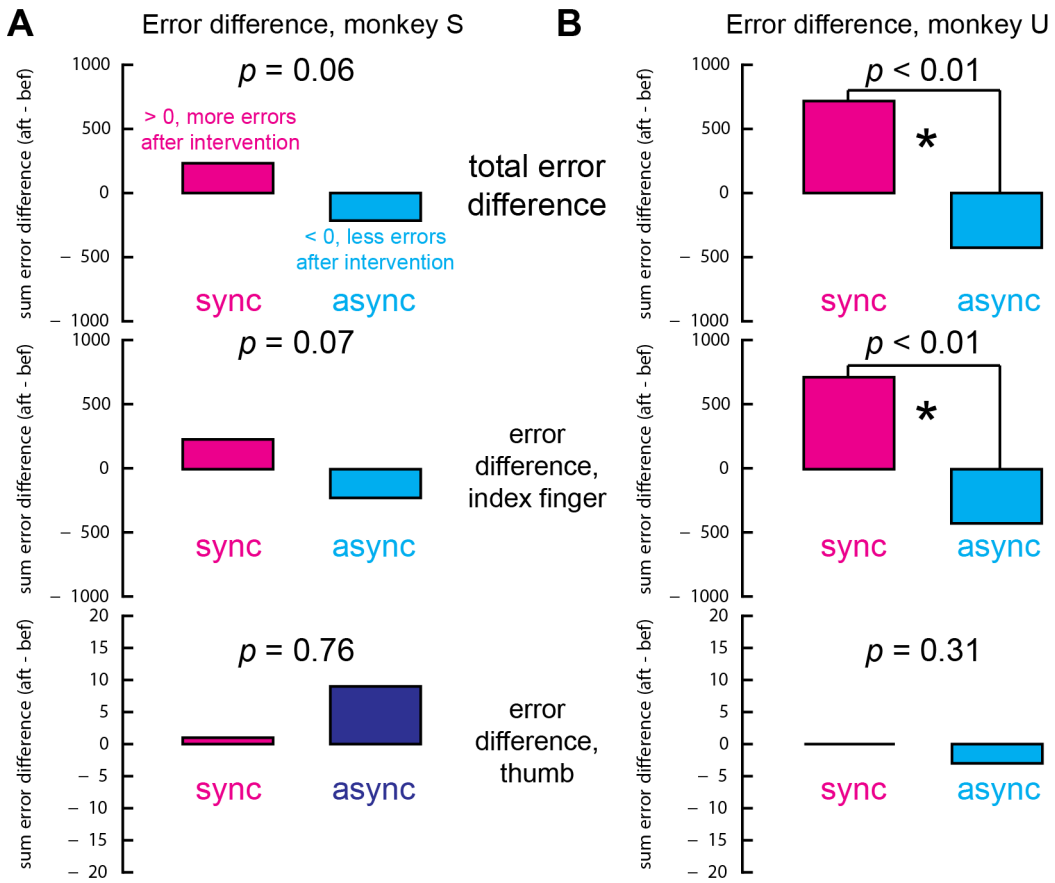

Figure 3.1: *Error difference after synchronous and asynchronous stimulation.* (A) Error difference $\Delta e = e^{\mathtt{aft}} - e^{\mathtt{bef}}$ after one hour of synchronous (sync) and asynchronous (async) stimulation for monkey S. (B) Error difference for monkey U. Significant difference between the stimulation protocols are indicated with an asterisk ($p < 0.01$, two sample t-test). The error difference Δe is shown for the total number of errors (first row), number of errors with the index finger (second row), and for the number of errors with the thumb (third row). Note that values > 0 indicate a higher number of errors after and values < 0 a lower number of errors after the intervention.

Analysing the error difference Δe for the index finger revealed a similar effect for monkey S (Figure 3.1A) and monkey U (Figure 3.1B). The number of errors for the index finger $e_{\mathtt{index}}$ did increase in most of the sessions after the synchronous intervention for both monkeys (Figure 3.1A and 3.1B, middle row). The number of errors for the index finger $e_{\mathtt{index}}$ after the asynchronous intervention did decrease for most of the sessions for monkey S (Figure 3.1A, middle row) and monkey U (Figure 3.1B, middle row). The difference in error change Δe between

the synchronous and asynchronous intervention was significant for monkey U ($p < 0.01$, two-sample t-test) and not significant for monkey S ($p = 0.07$, two-sample t-test).

The errors for the thumb $e_{\texttt{thumb}}$ did slightly increase for monkey S (Figure 3.1A, bottom row) after both the synchronous and asynchronous intervention. For monkey U, the number of errors after the synchronous intervention marginally increased and decreased after the asynchronous intervention (Figure 3.1B, bottom row). The differences between the error change comparing the effects after the synchronous and asynchronous intervention showed no significant differences for monkey S ($p = 0.76$, two-sample t-test) and monkey U ($p = 0.31$, two-sample t-test). It should be stressed that the error differences $\Delta e_{\texttt{thumb}}$ were much smaller compared to the error difference $\Delta e_{\texttt{index}}$.

3.4.2 *The monkey's performance increased after the asynchronous and decreased after the synchronous intervention*

Comparing the performance p (see Equation 3.1) before and after the synchronous (associative) and asynchronous (non-associative) intervention also revealed intervention-dependent effects for monkey S (Figure 3.2A) and monkey U (Figure 3.2B). The performance p was reduced after the synchronous intervention for monkey S (Figure 3.2A) and monkey U (Figure 3.2B). After the asynchronous intervention however, the performance was increased for both monkeys (Figure 3.2A and 3.2B).

The difference of the performance change Δp between the synchronous and asynchronous intervention was significant for monkey S ($p < 0.01$, two-sample t-test) and monkey U ($p < 0.01$, two-sample t-test).

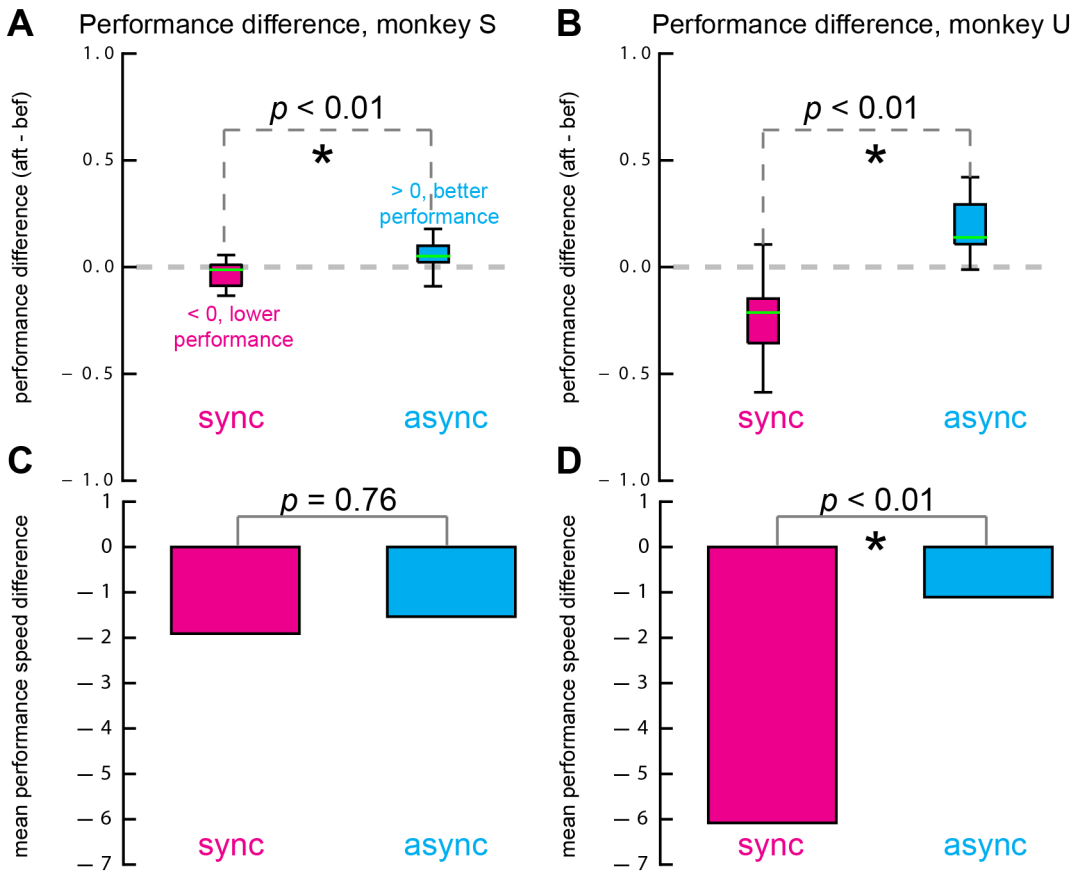

Figure 3.2: *Performance and speed difference after synchronous and asynchronous stimulation*. (A) Performance difference after synchronous and asynchronous stimulation for monkey S. (B) Performance difference for monkey U. (C) Performance speed difference after synchronous and asynchronous stimulation for monkey S. (D) Performance speed difference for monkey U. Significant differences are indicated with an asterisk ($p < 0.01$, two sample t-test).

Comparing the performance speed before and after the interventions revealed that the speed (number of correct trials per task length) reduced for both monkeys after both interventions (Figure 3.2C and 3.2D). The difference of the speed change Δs was significant for monkey U ($p < 0.01$, two-sample t-test) and not significant for monkey S ($p = 0.76$, two-sample t-test).

3.5. Discussion

3.5.1 *Errors caused by non-selective finger movements are differentially affected by type of intervention*

The analysis of the behavioural task performance revealed a intervention-specific modulation of the total number of errors after the synchronous (associative) and asynchronous (non-associative) intervention for monkey S (Figure 3.1A) and monkey U (Figure 3.1B). The total number of errors increased after one hour of synchronous and decreased after asynchronous repetitive median and ulnar nerve stimulation (Figure 3.1A and 3.1B). The total number of errors was predominantly influenced by the number of errors with the index finger (cf. Figure 3.1A and 3.1B, top and middle row). For details about thumb and index movement selectivity see Section 3.3.1.

The observation of an increased number of errors after the synchronous and decreased number of errors after the asynchronous intervention is in line with the working hypothesis.

Studies on human subjects using TMS stated that the motor-cortical excitability as indicated by changes in MEPs is increased after prolonged synchronous (associative) motor point or peripheral nerve stimulation (McDonnell and Ridding, 2006; McKay et al., 2002; Pyndt and Ridding, 2004; Ridding and Uy, 2003; Schabrun and Ridding, 2007). This increase in MEPs is accompanied by an increase of motor-cortical representation and overlap of the during the intervention stimulated muscles (or by the peripheral nerve innervated muscles). These and other findings led to the assumption that an increased neuronal excitability and overlap of the motor-cortical representations of two separate task-relevant muscles would make independent finger movements more difficult. Since the median nerve is innervating mainly APB and the ulnar nerve FDI, I hypothesised that synchronous (associative) stimulation of the median and ulnar

nerve would constrain an independent thumb and index finger. And indeed, the number of total errors increased for both monkeys (Figure 3.1A and 3.1B) implying that both monkeys' ability to perform selective thumb and index movements was restricted. This is in agreement with behavioural assessments of patients with focal hand dystonia (FHD). Subjects with musician's dystonia for example were unable to move their fingers as selective as healthy subjects during a piano task (Rosenkranz et al., 2009).

Studies using asynchronous (non-associative) stimulation protocols were able to temporarily reverse the motor-cortical overlap (Schabrun et al., 2009) and increase the independent finger movement performance (Rosenkranz et al., 2009) of patients with FHD. This is similar to the results of the present study. The instructed thumb and index finger movements were performed more selectively by both monkeys as indicated by decreased error numbers after the asynchronous intervention (Figure 3.1A and 3.1B). This decrease in total error number was however not significantly different compared to the number of errors before the intervention. Studies on healthy human subjects did not find significant task-specific effects after prolonged asynchronous stimulation (Rosenkranz et al., 2009; Schabrun et al., 2009). This has a number of potential reasons. Some of these studies are not explicitly assessing selective finger movements (Schabrun et al., 2009). Furthermore, these studies compare task differences after the asynchronous intervention to a control condition. Here I presented the performance differences after asynchronous as opposed to synchronous interventions.

3.5.2 *The monkeys' task performance decreased after the synchronous and increased after the asynchronous intervention*

Although the change in total number of errors already gives a good estimate of the monkeys' selective finger abduction performance, slight variations in the total

number of trials would affect the likelihood of error occurrences. Therefore, the performance measure was introduced normalising the error rate in dependence on the total number of trials (see Equation 3.1).

The performance decreased after synchronous (associative) and increased after asynchronous (non-associative) prolonged median and ulnar nerve stimulation for monkey S (Figure 3.2A) and monkey U (Figure 3.2B). The synchronous intervention led to a significantly different performance compared to the asynchronous intervention ($p < 0.01$, two-sample t-test). This is also in correspondence with the aforementioned hypothesis that synchronous peripheral nerve stimulation would make an independent finger movement of the relevant muscles innervated by the nerves more difficult.

The performance speed was decreased for monkey S (Figure 3.2A) and monkey U (Figure 3.2D) after both the synchronous and asynchronous interventions. McDonnell and Ridding (2006) described a more rapid improvement in subjects performing a grooved pegboard task (GPT) after receiving synchronous (associative) stimulation of APB and FDI. Furthermore, subjects receiving either synchronous or asynchronous motor point stimulation performed better than those who did not receive any stimulation at all. But what made the subjects improve more rapidly after the synchronous (associative) intervention in this study? Since the coordinated movement of the index finger and the thumb is essential for handling a peg, it would be conceivable that this task-dependent co-activation of the thumb and index finger muscles was improved after the associative APB and FDI stimulation. Schabrun et al. (2009) found improvements in cyclic drawings in patients with FHD. Other measures of focal hand function were not significantly improved. This might lead to the question whether other assessments of focal hand function (e.g. grip force, handwriting) provide a sufficiently sensitive and selective measure to register representational changes of affected muscle groups. Rosenkranz et al. (2009) used a MIDI piano keyboard task. This task more selectively assessed the individual finger movements

involving (relatively) distinct muscle groups. In the present study, performance was assessed based on individual thumb and index finger abduction movements. This might have provided a sensitive measure of behavioral effects induced by prolonged activation (indirectly by nerve stimulation) of the task-relevant muscles. Involving an increased number of stimulation sites (particularity on intrinsic hand muscles) along with increasing the number of instructed (task-relevant) fingers could reveal the validity of the previously stated hypothesis and potentially identify interactions between stimulated and non-stimulated sites.

Chapter 4. Neuronal responses to peripheral nerve stimulation

4.1. Summary and key findings

- Non-invasive methods have been developed to induce plastic changes in the sensorimotor cortex. These rely on the electrical or tactile stimulation of pairs of digits, muscles or peripheral afferent nerves (McKay et al., 2002).
- Synchronous and asynchronous muscle and peripheral afferent nerve stimulation might be of therapeutic use for the treatment of a number of neurological conditions associated with a dysfunction of sensory (afferent) processing leading to maladaptive cortical and sub-cortical plasticity including stroke, spinal cord injury, pain and dystonias.
- In the present study, I recorded the single (SU) and multi unit (MU) activity of 456 stable neurons in the primary motor cortex (M1) of two macaque monkeys before and after one hour of synchronous and asynchronous median and ulnar nerve stimulation (interventions).
- The M1 cell responses to afferent inputs were assessed following single-site stimulation to EDC, median and ulnar nerve.
- Evoked M1 cell responses showed a significant suppression in neuronal firing after the synchronous and asynchronous interventions. After the synchronous intervention the M1 population response difference was characterised by a significant suppression at $\sim$20 ms followed by a rebound at 30-40 ms. After the asynchronous intervention, the suppression profile was broader and temporally less precise. Dividing neuronal data based on which nerve gave significant responses, revealed that M1 units receiving inputs from both median and ulnar nerve were mediating these changes. Timing analysis implied that a STDP-like mechanisms might be involved in plastic changes induced by the synchronous peripheral nerve stimulation.

4.2. Introduction

Non-invasive methods have been developed to induce plastic changes in the sensorimotor cortex. These rely on the electrical (Kowalewski et al., 2012; McDonnell and Ridding, 2006; Ridding and Uy, 2003; Schabrun and Ridding, 2007) or tactile (Godde et al., 1996; Hoffken et al., 2007; Vidyasagar et al., 2014) stimulation of pairs of digits (Kowalewski et al., 2012; Schabrun and Ridding, 2007), muscles (motor point stimulation, Barsi et al., 2008; McDonnell and Ridding, 2006; Pyndt and Ridding, 2004; Ridding and Uy, 2003) or peripheral afferent nerves (Charlton et al., 2003; McKay et al., 2002; Ridding et al., 2000, 2001).

The plastic changes in motor and somatosensory areas have been investigated with a variety of neurophysiological stimulation and imaging techniques (Chipchase et al., 2011; Veldman et al., 2014; Ziemann et al., 2008).

Godde et al. (1996) reported an extension and increased overlap of the rat's somatosensory paw representation in the primary somatosensory cortex (S1) after synchronous (associative) tactile stimulation of two paw digits. This was accompanied by an increased firing of extracellularly recorded S1 neurons. Furthermore, the authors of this study used the same intervention (paired associative stimulation of two digits) with human subjects and found improvements in a spatial discrimination task (Bliem et al., 2007; Godde et al., 1996; Hoffken et al., 2007). Hoffken et al. (2007) found changes in somatosensory evoked potentials (SEPs) after the synchronous (associative) tactile intervention implying an enhanced cortical excitability in S1.

Studies with similar interventions using synchronous (associative) stimulation of two peripheral nerves or muscles report changes in the excitability of the motor cortex (McDonnell and Ridding, 2006; McKay et al., 2002; Ridding and Uy, 2003; Ridding et al., 2001; Schabrun and Ridding, 2007) as indicated by

changes in motor evoked potentials (MEPs) elicited by transcranial magnetic stimulation (TMS). The change of the MEP (and thereby the change in motor-cortical excitability, see e.g. Stagg et al., 2011) is either assessed by using a consistent TMS stimulation intensity (expressed as percentage of the maximum stimulator output) or by testing a range of TMS intensities to study the MEP input-output relation (recruitment curve, see Ridding and Rothwell, 1997).

The excitability changes in the motor cortex are accompanied by an increased overlap of motor-cortical representations. In a number of studies on human subjects utilising synchronous (associative) peripheral nerve or motor point stimulation as an intervention, an extension of motor-cortical maps and an increased overlap of areas in the motor cortex projecting to the muscles stimulated in the intervention have been identified (Ridding et al., 2001; Schabrun and Ridding, 2007). The increased overlap of motor-cortical maps has been determined by calculating the centre of gravities (CoGs) (Wassermann et al., 1992) of these muscles. The CoGs move towards each other indicating a stronger overlap (Ridding et al., 2001; Schabrun and Ridding, 2007). This increase in overlap after three hours of synchronous co-activation of two digits has been confirmed in studies using functional magnetic resonance imaging (Vidyasagar et al., 2014).

Stimulating one peripheral nerve or motor point after the other (alternatingly, for example in a randomised fashion) has been termed asynchronous or non-associative stimulation (NAS). This kind of stimulation did not lead to excitability changes in the sensorimotor cortex (Ridding and Uy, 2003; Schabrun and Ridding, 2007). Obviously, other excitability changes might have occurred, which were not picked up by measuring MEPs induced by TMS. Applying vibrational or electrical NAS to two (or more) sites led to improved behavioural performance, reduced volume of motor cortical representations and increased CoGs distances of the muscles stimulated in the intervention (Rosenkranz et al., 2008, 2009; Schabrun et al., 2009) in patients with focal hand dystonia (FHD).

4.2 Introduction

The pathophysiology of FHD has been hypothesised to be linked to maladaptive (co-) activation of sensory afferents which lead to aberrant motor-cortical plasticity (Quartarone et al., 2008, 2014; Schabrun et al., 2009; Tinazzi et al., 2000). These characteristics are similar to those artificially induced in healthy human subjects using paired synchronous (associative) motor point or nerve stimulation (McKay et al., 2002; Ridding and Uy, 2003; Schabrun and Ridding, 2007; Schabrun et al., 2009).

Synchronous and asynchronous muscle and peripheral afferent nerve stimulation might be of therapeutic use for the treatment of a number of neurological conditions associated with a dysfunction of sensory (afferent) processing leading to maladaptive cortical and sub-cortical plasticity including stroke (Di Pino et al., 2014; Dos Santos-Fontes et al., 2013; Liao et al., 2014), spinal cord injury (Gomes-Osman and Field-Fote, 2015; Lala et al., 2015; Ragnarsson, 2008; Yiannikas et al., 1986), neuropathic pain (Kadrie et al., 1976; McGowan, 2006; Mobbs et al., 2007; Nashold et al., 1982; Picaza et al., 1977; Schabrun et al., 2013, 2014), and dystonias (Furuya and Altenmuller, 2013, 2015; Hallett, 2006, 2011; Marsden and Sheehy, 1990; Rosenkranz et al., 2005, 2008, 2009; Schabrun et al., 2009; Sussman, 2015).

There are several lines of evidence suggesting that plastic changes induced by paired synchronous (associative) motor point or peripheral nerve stimulation occur within M1. Ridding et al. (2001) did not find excitability changes in spinal motoneurons assessed by F-waves after two hours of synchronous ulnar and radial nerve stimulation. Additionally, a number of studies using paired pulse TMS found an increase of intracortical facilitation (ICF) in M1 in line with increased MEP excitability (Pyndt and Ridding, 2004). Therefore, M1 is a likely site of plasticity but other sub-cortical locations including brainstem pathways would be feasible candidates as well.

McDonnell and Ridding (2006) suggested a mechanism similar to long-term

potentiation (LTP) underlying the plastic changes in M1 in response to the synchronous intervention. The similarities in the motor-cortical excitability (Pyndt and Ridding, 2004) and time course (McKay et al., 2002) support this hypothesis. Furthermore, neuropharmacological studies investigating the influence of N-methyl-D-aspartate (NMDA) receptor antagonists on MEPs and other intracortical (TMS-induces) phenomena reinforce this view (Butefisch, 2004; Butefisch et al., 2000; Hallett, 2007; Paulus et al., 2008; Stefan et al., 2002; Ziemann, 2004; Ziemann et al., 1998). The importance of spatial and temporal coincidence of associative (afferent) inputs on synaptic strengthening (Brown and Milner, 2003; Hebb, 1949) makes a LTP-like mechanism reasonable.

In the present study, I seek to answer the following questions: Do changes occur on a single cell level in M1 after one hour of synchronous (associative) and asynchronous (non-associative) median and ulnar nerve stimulation? Do these changes differ? Are these changes affecting selectively particular types of neurons, i.e. identified pyramidal tract neurons (PTNs)? Is there any evidence for a putative mechanism leading to changes in M1 neurons? And finally, is the non-human primate model appropriate to study neuroplasticity and are the present findings consistent with human studies utilising associative (and non-associative) peripheral afferent stimulation as an intervention?

I hypothesis that changes in M1 neurons in response to prolonged peripheral nerve stimulation should occur. Especially in those cells relevant to mediating effects as seen in excitability changes of the corticospinal system as expressed by modulations of MEPs induced by TMS. Thus, PTNs should show a modulation as well. Furthermore, the activation of identified and unidentified neurons in M1 by individual or multiple peripheral stimulation sites should matter. If changes on a single cell level could be identified, these should take place in neurons, which have access to intra- and inter-cortical circuitry processing the relevant peripheral stimuli. Therefore, it would be feasable that after the prolonged synchronous stimulation of two peripheral nerves a modulation of the neuronal

4.2 Introduction

M1 cell discharges could involve STDP-like mechanisms. In the case of the asynchronous intervention, where there is no consistent timing between the two peripheral stimuli, potential changes of M1 neurons should (if these exist) rely on other network phenomena rather than STDP.

For the purpose of testing these assumptions, I recorded the single (SU) and multi unit (MU) activity of 456 *stable* neurons in the primary motor cortex (M1) of two macaque monkeys before and after one hour of synchronous and asynchronous median and ulnar nerve stimulation (interventions). Stable neurons in the present dissertation refer to either a single unit (SU) which is clean (no inter-spike intervals smaller than 1 ms) and fulfils the stability criteria (Figure 4.3A-4.3D), or to a multi unit (MU) which was recorded on a *stable* single unit channel (therefore it is assumed that the electrode did not move and the same contribution of units is picked up by this channel). The discharges of M1 neurons to afferent inputs were assessed in response to single-site stimulation to a finger extensor muscle serving as a control stimulation site (EDC) and to two peripheral nerves (median and ulnar nerve). Stimulation to EDC refers to applying electrical stimulation through the implanted EMG wire throughout the present text. Stimulating median and ulnar nerve means stimulating through the implanted bipolar nerve cuff electrodes (Figure 4.1A). M1 cell responses evoked by the relevant stimulation sites (median and ulnar nerve) changed after both interventions whereas responses evoked by EDC stimulation (non-stimulated control site) did not change. The majority of those M1 units showed a significant suppression in neuronal firing after the synchronous and asynchronous intervention. Analysing the time profile of these differences revealed differential temporal effects of the M1 population response after the synchronous in contrast to the asynchronous intervention. After the synchronous intervention the M1 population response difference was characterised by a significant suppression at approximately 20 ms followed by a significant facilitation or *rebound* between 30 and 40 ms after the single stimulation-site evoked response. *Rebound* in the

context of the neuronal population response difference describes qualitatively the transition from a clear significant suppression to a subsequent facilitation or change towards the baseline level (cf. Figure 4.8A). After the asynchronous intervention, the suppression profile was broader and temporally less precise. Dividing the neurons into classes based on the afferent input that they received, revealed that M1 units which receive both inputs from median and ulnar nerve were mediating the change in neuronal firing after the intervention. Investigating the timing of these inputs to M1 neurons implied that STDP-like mechanisms might be involved in the changes after the synchronous intervention.

Using the non-human primate model to investigate the modulation and the mechanism underpinning the (plastic) neuronal changes after synchronous (and asynchronous) peripheral nerve stimulation might help to develop novel afferent stimulation protocols and advanced non-invasive therapeutic interventions.

4.3. Materials and methods

4.3.1 *Subjects*

Two female macaque monkeys (monkeys S was six years old and monkey U four years) were used for the present stimulation experiment. The average weight for monkey S was $6.30\,\mathrm{kg}$ and $4.76\,\mathrm{kg}$ for monkey U. Both monkeys were housed in facilities of the CBC and participated in behavioural training and enrichment routines (for more details about the subjects and the training see Section 2.3.1).

All experimental procedures were carried out under authority of licenses issued by the UK Home Office under the Animals (Scientific Procedures) Act 1986 and were approved by the Animal Welfare and Ethical Review Board of Newcastle University.

4.3.2 *Surgical preparation*

After the behavioural training (see Chapter 2) was completed, the monkeys received EMG, nerve cuff and a headpiece implant within a surgical procedure. All surgical interventions were performed under aseptic conditions and under deep general anesthesia. The general anaesthesia was induced by $10\,\mathrm{mg\,kg^{-1}}$ ketamine (intramuscular) and maintained with $2.0\text{-}3.5\,\%$ sevoflurane in $100\,\%$ O_2. Analgesia was provided by intravenous infusion of alfentanil ($0.025\,\mathrm{mg\,kg^{-1}\,h^{-1}}$) throughout the surgical procedure.

Custom-made flexible bipolar median and ulnar nerve cuffs (supplying forearm flexors and intrinsic hand muscles including muscles of all digits) were implanted under aseptic conditions. Two 7 strand Teflon-insulated stainless steel EMG wires were positioned in the extensor digitorum muscle (EDC). The wires of the EMG and nerve cuff implants were routed subcutaneously to the back of the monkey (Figure 4.1A and 4.1B). Subsequently, the monkeys received a $30\,\%$ carbon fiber reinforced PEEK (Engineering & Design Plastics Ltd, Catalogue Number: Kentron CA30 PEEK) headpiece enabling atraumatic head fixation (Baker et al., 1999; Lemon and Prochazka, 1984).

The EMG and nerve cuff wires came together in the monkey's back and led along the neck to a crimp connector on top of the headpiece (Figure 4.2B, top image). Next, a recording chamber (Figure 4.2B, top and bottom image) was positioned over the hand area of the right primary motor cortex (M1). The orientation of the central sulcus was determined by pre-surgery acquired structural magnetic resonance imaging (MRI) scans.

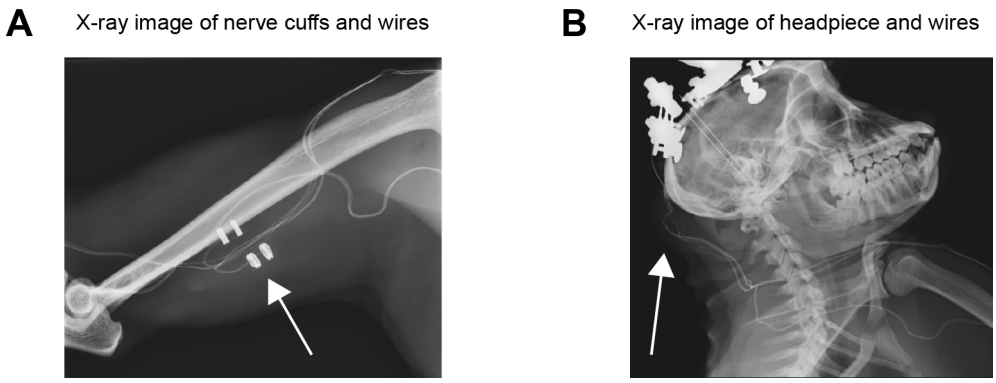

Figure 4.1: *X-ray images of nerve cuffs, wires and headpiece*. (A) X-ray image of nerve cuffs and stainless steel (Teflon insulated) wires. The white arrow shows one bipolar nerve cuff (see platinum contacts). Nerve cuffs were implanted around the median and ulnar nerve. (B) X-ray image of stainless steel wires and headpiece. The white arrow indicates the position where the joint bundle of EMG and nerve cuff wires was routed along the monkey's neck before it led to the EMG and nerve cuff connector (cf. Figure 4.2B). X-rays were taken from monkey S under sedation.

In another surgery the monkeys were implanted with pyramidal tract (PT) electrodes. Two fine varnish-insulated tungsten electrodes were positioned in the medullary pyramid (Baker et al., 1999). The location was confirmed during the surgery by measuring antidromic field potentials recorded with *ball-shaped* electrodes on the surface of the dura mater overlying M1.

After the surgery, the monkeys received treatment with antibiotics ($6\,\mathrm{mg\,kg^{-1}}$ Ceftiofur, Pfizer, Catalogue Number: MSDS 087 and $8\,\mathrm{mg\,kg^{-1}}$ Dexamethasone, Hameln Pharmaceuticals Ltd) and analgesics ($6\,\mathrm{mg\,kg^{-1}}$ Meloxicam, Boehringer Ingelheim Limited). All surgical and experimental procedures were carried out under authority of licenses issued by the UK Home Office under the Animals (Scientific Procedures) Act 1986 and were approved by the Animal Welfare Ethical Review Board (AWERB) of Newcastle University.

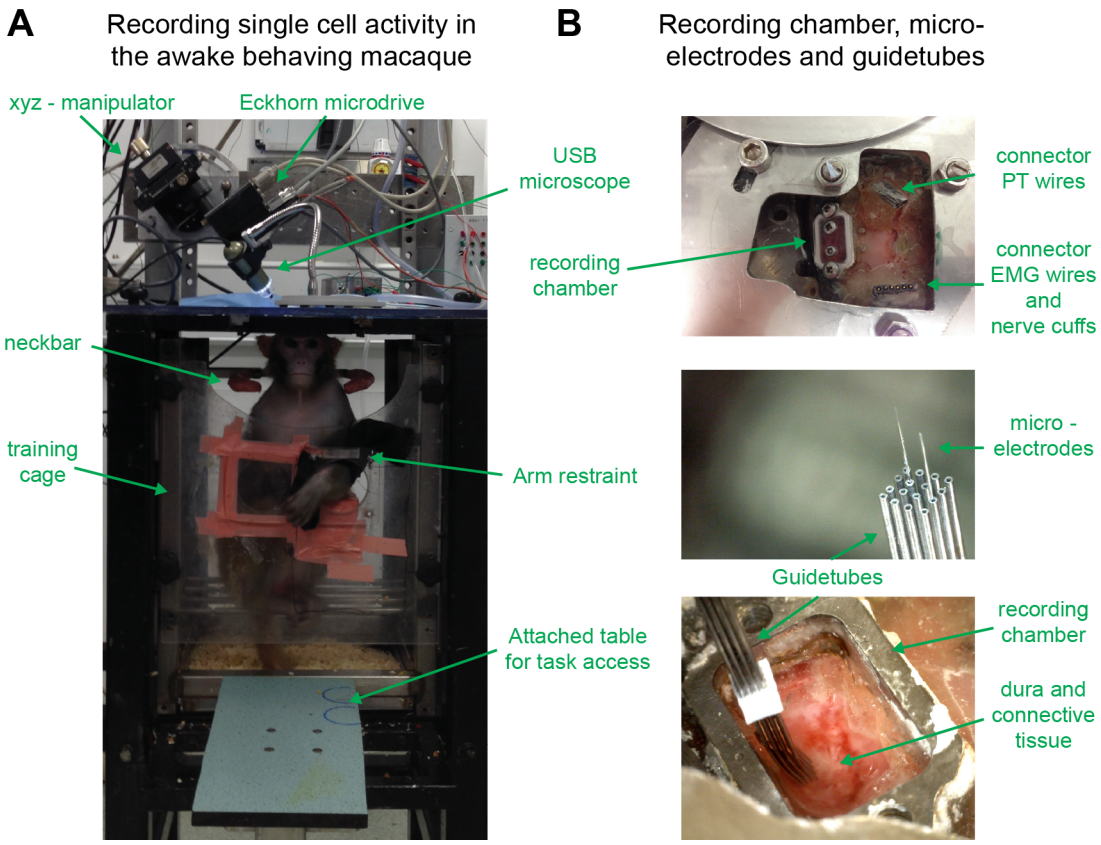

Figure 4.2: *Setup and equipment for electrophysiological recordings in the awake behaving monkey*. (A) Recording setup for electrophysiological recordings. The monkey was head-fixed and sitting in its training cage. The head was positioned with a head-plate and fixed with screws (see Figure B, top image, cf. implanted bolts as part of the monkey's headpiece, Figure 4.1B) to the recording rig. An Eckhorn microdrive was positioned over the recording chamber with the xyz-manipulator. Electrode position and recording chamber were monitored using an universal serial bus (USB) microscope. (B) Recording chamber, guidetubes and micro-electrodes. The implanted EMG wires and nerve cuff electrodes (Figure 4.1A and 4.1B) were connected via crimp-plugs to a relay box (capable of switching between recording and stimulation mode) which itself was connected to the EMG amplifier and the stimulator, respectively. Extracellular single cell recordings were performed using motor-controlled micro-electrodes routed through guidetubes (middle image). The guidetubes were positioned on top of the dura within the saline filled recording chamber (bottom image). Electrodes were subsequently advanced in small increments of a few micrometer before reaching the desired recording depth.

4.3.3 *Experimental design and stimulation sequences*

After the monkeys fully recovered and resumed their training, the motor thresholds (MTs) of EDC, median and ulnar nerve were determined. MTs were measured while the monkey relaxed the arm containing the relevant muscles and nerves. The MT for EDC was $2.2\,\mathrm{mA}$ for monkey S and $1.2\,\mathrm{mA}$ for monkey U. The motor threshold (MT) for median nerve was $600\,\mu\mathrm{A}$ for monkey S and $400\,\mu\mathrm{A}$ for monkey U. The MT for ulnar nerve was $500\,\mu\mathrm{A}$ for monkey S and monkey U. Stimulation throughout the experiment was performed at $2\times$ MT. Biphasic stimulation pulses were used, duration $1\,\mathrm{ms}$ per phase.

The experiment consisted of four parts: During the first *assessment* phase EDC, median and ulnar nerve were stimulated individually. The order of stimulating any of these three stimulation sites was randomised. The stimulation frequency during the *assessment* phase was also randomised between 0.8 and $3\,\mathrm{Hz}$.

During the subsequent *intervention* phase, median and ulnar nerve were either stimulated synchronously (associative nerve stimulation) or asynchronously (non-associative nerve stimulation). Associative and synchronous nerve stimulation are used interchangeably throughout the present study. Both terms refer to the simultaneous stimulation of the median and ulnar nerve. Non-associative or asynchronous nerve stimulation on the other side refers to non-simultaneous (one stimulation site after the other) stimulation of median and ulnar nerve. The stimulation frequency during the *intervention* phase was randomised between 8.3 and $12.5\,\mathrm{Hz}$ with an average stimulation frequency of $10\,\mathrm{Hz}$. Thus, during the synchronous stimulation *intervention*, on average 10 times per second, both median and ulnar nerve were stimulated simultaneously. During the asynchronous (non-associative) intervention both median and ulnar nerve stimulation were stimulated alternatingly out-of-phase with a frequency of $10\,\mathrm{Hz}$ each. Both *interventions* were applied for $1\,\mathrm{h}$ per recording session.

Immediately after the *intervention*, the aforementioned *assessment* of individually stimulating either EDC, median or ulnar nerve was repeated. Each assessment consisted of 300 stimuli per stimulation site.

Subsequently, a behavioural assessment was performed (data not shown in this chapter, see Chapter 3). Since the length of the behavioural assessment varied, the second stimulation *assessment* was performed approximately $1\,\mathrm{h}$ (depending on the task length) after the end of the *intervention* (± 0.5 hours).

The order of these four parts (assessment, intervention, and two assessments after the intervention) was kept constant for every recording session. The synchronous and asynchronous stimulation interventions were applied alternatingly from one session to the next. Recording sessions were interleaved by at least $36\,\mathrm{h}$ to avoid potential long lasting effects of the intervention which might affect the subsequent recording session.

Median nerve stimulation was applied by an isolated pulse stimulator (AM Systems, Catalogue Number: 720005). EDC and ulnar nerve stimulation were applied with another stimulator (DS4, Digitimer Ltd).

Stimulation was controlled by sequencer scripts using Spike2 software (Cambridge Electronic Design). Trigger were send to the stimulator via a Micro 1401 (Cambridge Electronic Design).

4.3.4 *Data acquisition and extracellular recordings*

Extracellular single and multi unit recordings were performed using a 16-channel microdrive (Figure 4.2A) loaded with glass-insulated platinum electrodes (Figure 4.2B, middle image) advanced in small increments via guidetubes (Eckhorn and Thomas, 1993). The micro-electrodes possessed a tip impedance of approximately $1\text{-}2\,\mathrm{M}\Omega$. Spike containing raw data ($300\,\mathrm{Hz}$-$10\,\mathrm{kHz}$ bandpass,

Figure 4.3A) was constantly sampled at $25\,\mathrm{kHz}$ with a high speed data acquisition (DAQ) card (National Instruments) and saved to hard disk.

The spike event occurrence times were then discriminated using custom written Python (Rossum, 1995) scripts (Python, Version 2.7.9, `https://www.python.org`). In brief, the spike discrimination process consisted of a threshold determination, defining spike discrimination widows, calculating the principal components and manually defining data cluster.

The threshold for spike events was determined manually and usually very close to an automatically determined one (Quiroga et al., 2004, 2007):

$$\Theta = 5 \cdot \mathtt{median}\left\{\frac{|x|}{0.6745}\right\} \tag{4.1}$$

Subsequently, the spike waveforms which crossed the threshold $\pm\Theta$ were aligned, visualised and spike discrimination windows defined.

Using the remaining (filtered) spike waveforms, a dimensionality reduction was performed using principal component analysis (PCA). Clusters of principal components (i.e. similar spike waveforms) were chosen manually by defining a closed polygon around the cluster of interest. Thereby, clearly distinct cells could be identified on the same recording channel (Figure 4.3A-4.3D). Only clean single units (SUs) with stable spike waveform shapes (Figure 4.3C) and no inter-spike times $< 1\,\mathrm{ms}$ (cf. inter-spike interval histogram, Figure 4.3D) were used for subsequent analysis.

In addition to the analysis of SUs, *stable* multi units (MUs) were analysed separately (MU group). *Stable* MUs were defined as multiple unit (MU) activity recorded on an electrode which also contained a stable single unit (SU) on the above criteria.

Extracellular recordings were performed while the monkeys were at rest or during EDC, median or ulnar nerve stimulation.

4.3.5 *Stability assessment of identified and unidentified single units*

The stability of each recorded single unit (SU) was carefully monitored throughout the recording session. Once a stable SU was found, an oscilloscope (Tektronix, Catalogue Number: TDS2001C), was used to display the spike waveform. The average spike waveform of a particular cell was saved and compared to subsequently triggered spikes. In one recording session up to 6 cells could be monitored on different oscilloscopes. In principle more cells (e.g. 12 in total, 2 channel inputs of oscilloscope) could have been monitored. However, it was rarely the case that more than 6 stable cells were found in one recording session.

In addition to unidentified (UID) single units, recordings of identified pyramidal tract neurons (PTNs) were performed. PTNs were identified by searching for latency-invariant antidromic spike responses to stimulating the pyramidal tract (PT) with a single pulse of 50-400 µA (biphasic pulse, each phase 0.2 ms). Once a cell was clearly activated by the pyramidal tract (PT) stimulation, the antidromic latency was determined. The antidromically activated neuron was then discriminated on-line using custom written on-line spike discrimination software (This on-line spike discrimination software was written by SN Baker) and a collision test (Baker et al., 1999; Lemon and Prochazka, 1984) was performed. Additionally, the interval between the spontaneous spike and the PT stimulation which led to a collision was determined. This collision test was carried out at the beginning and the end of the recording session which allowed for an additional single unit stability assessment.

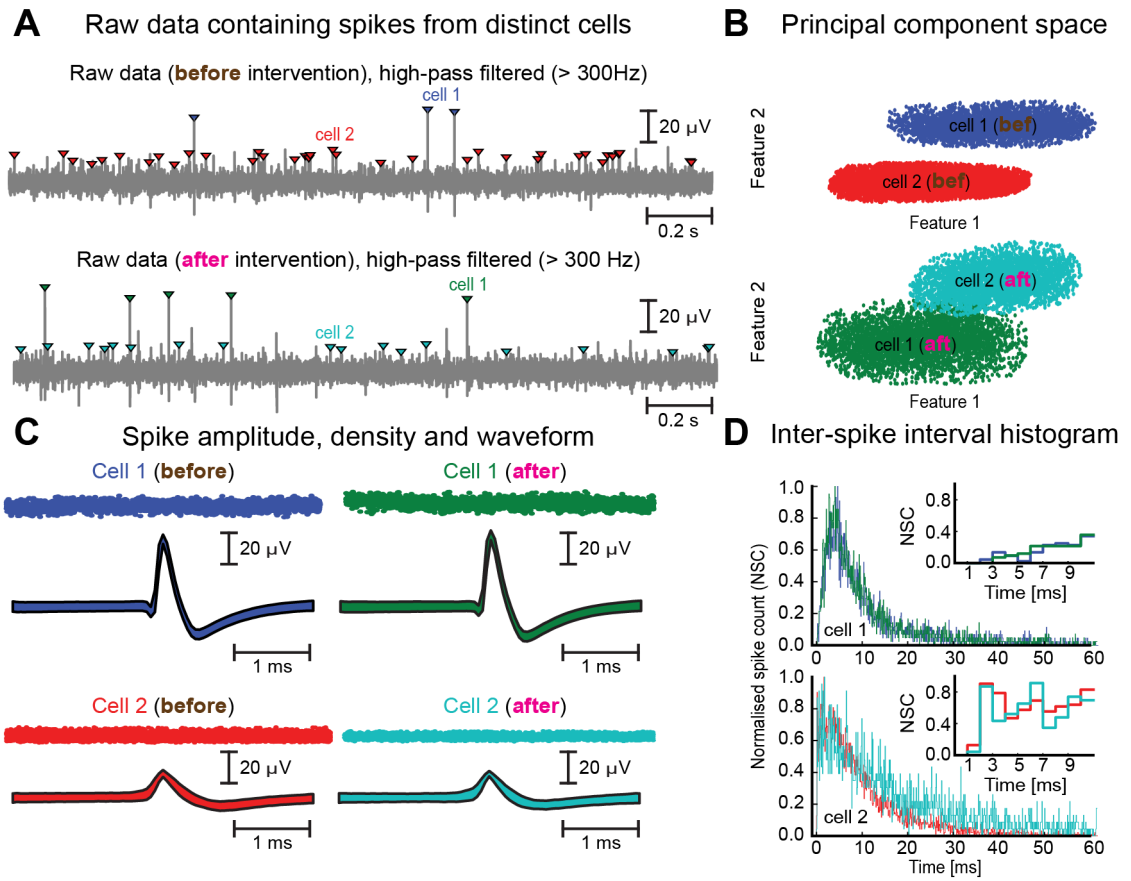

Figure 4.3: *Simplified illustration of post-recording stability assessment of single units in M1.* (A) High-pass filtered raw data containing spikes before (top trace) and after (bottom trace) the intervention (either one hour of synchronous or asynchronous median and ulnar nerve stimulation). Two distinct cells (cell 1 and 2) were detected on these traces. (B) Post-hoc spike discrimination consisted of threshold detection, spike-waveform alignment and distinguishing neurons by manually selecting data cluster in the principal component space. (C) The spike waveform, the density of the spike events, and the amplitude were compared before and after the intervention for all cells on a given channel. The spike waveforms were compared to notes and drawings within the contemporaneous lab note book (spike waveforms were monitored on oscilloscopes throughout the recording session). (D) The timing of successive spikes are shown as inter-spike interval histograms (ISIHs). ISIHs were compared before and after the intervention. Neurons with stable metrics (spike waveform, amplitude and ISIH) in which successive action potentials (APs) were separated by an interval of at least 1 ms were categorised as single units (SUs).

Recorded cells were further characterised by their motor responses to intracortical micro-stimulation (ICMS) with 13 to 18 biphasic pulses (80 µA) at 300 Hz (Boudrias et al., 2010; Park et al., 2001) with a phase of 0.2 ms each.

The approximate spike waveform and all available characteristics (e.g. motor responses, size of spike waveform) were recorded in a research diary.

In addition to these on-line (measured and monitored during the recording session) stability assessments, SU stability was carefully assessed off-line (Figure 4.3A-4.3D). The spike characteristics (spike waveform, responses to stimulation) were compared to the notes in the research diary. The spike waveform (Figure 4.3C) was compared before and after the intervention. Additionally, the time progression of the spike waveform, the density of spike events and the amplitude (Figure 4.3A) were assessed. Furthermore, the inter-spike interval histogram (ISIH) of a particular neuron was compared before and after the intervention (Figure 4.3D).

4.3.6 *Data analysis*

All data analysis was performed with custom written Python (Rossum, 1995) scripts (Python, Version 2.7.9, `https://www.python.org`). For numerical time series analysis and matrix operations I used some of the functionality of the numpy (Numpy, Version 1.9.2, `http://www.numpy.org`) for statistical distributions and tests the scipy (Scipy, Version 0.15.1, `http://www.scipy.org`) and for data visualisation the matplotlib (Matplotlib, Version 1.4.3, `http://matplotlib.org`) library. The spike off-line discrimination software was also written in Python and graphical user interfaces (GUIs) were implemented using the Tkinter (Tkinter, Version 2.4, `https://wiki.python.org/moin/TkInter`) library.

Peristimulus time histograms (PSTHs) were estimated by binning the spikes, $\tilde{r}(t) = \frac{\Delta n(t)}{\Delta t}$, where $\Delta n(t)$ was the number of spikes in the interval $[t, t + \Delta t]$. The bin size Δt was set to $1\,\mathrm{ms}$ to be able to capture fine temporal changes in response to the stimulation.

The difference of the individual stimulation site evoked peristimulus time histogram (PSTH) response profiles was determined for each bin Δt_i by calculating the z-score with

$$z\left(\Delta t_i\right) = \frac{\left(N_{\mathtt{aft}} - B_{\mathtt{aft}}\right) - \left(N_{\mathtt{bef}} - B_{\mathtt{bef}}\right)}{\sqrt{N_{\mathtt{aft}} + N_{\mathtt{bef}} + \frac{B_{\mathtt{bef}}}{n_{\mathtt{bef}}} + \frac{B_{\mathtt{aft}}}{n_{\mathtt{aft}}}}} \tag{4.2}$$

with the number of spikes in a particular bin before $N_{\mathtt{bef}}$ and after $N_{\mathtt{aft}}$ the intervention, the number of spikes in the baseline period before $B_{\mathtt{bef}}$ and after $B_{\mathtt{aft}}$ the intervention, and the number of bins of the baseline period before $n_{\mathtt{bef}}$ and after $n_{\mathtt{aft}}$ the intervention.

The population difference was expressed as the population $\bar{z}$ score

$$\bar{z} = \frac{1}{\sqrt{N}} \cdot \sum_{i=1}^{N} z_i\left(\Delta t_i\right) \tag{4.3}$$

with the total number of cells N and all z_i for each bin Δt_i. These normalised z-scores allow for statistical comparison and significance tests (Larsen and Marx, 2012). Significant differences were determined with a significance level of $p < 0.05$.

	MUs	SUs		$\sum$
Category	$\sum$	PTNs	UIDs	total
All clustered cells	274	52	292	618
Stable cells	185	39	232	456
Neurons responding to EDC stimulation	87	15	56	158
Neurons responding to median stimulation	130	22	130	282
Neurons responding to ulnar stimulation	121	20	107	248

Table 4.1: *Summary of recorded single and multi units*. Only stable (for stability assessment see Figure 4.3A-4.3D) single and multi units (MUs) were used for subsequent analysis. Out of these neurons only a fraction of cells responded to individual EDC, median and ulnar nerve stimulation.

4.4. Results

In total, I recorded from 618 single (SU) and multi units (MUs) in primary motor cortex (M1). 73 % of all units were stable (Table 4.1). Out of all stable SUs, 131 stable units were recorded from monkey S and 140 from monkey U. The majority of these stable SUs responded to median stimulation (56 % of all stable single units), followed by ulnar nerve stimulation (46 %) and EDC (26 %). 97 SUs responded to both median and ulnar nerve stimulation, whereas 55 to median and 30 to ulnar only. Out of all stable pyramidal tract neurons (39 PTNs), 38 % were activated by EDC, 56 % by median, and 51 % by ulnar stimulation.

The single unit PSTH response profile (Figure 4.4A-4.4C) varied depending on the stimulation side (EDC, median or ulnar nerve) and was heterogeneous between responding M1 units in terms of the response onset latency and the shape of the response (Figure 4.4A-4.4C).

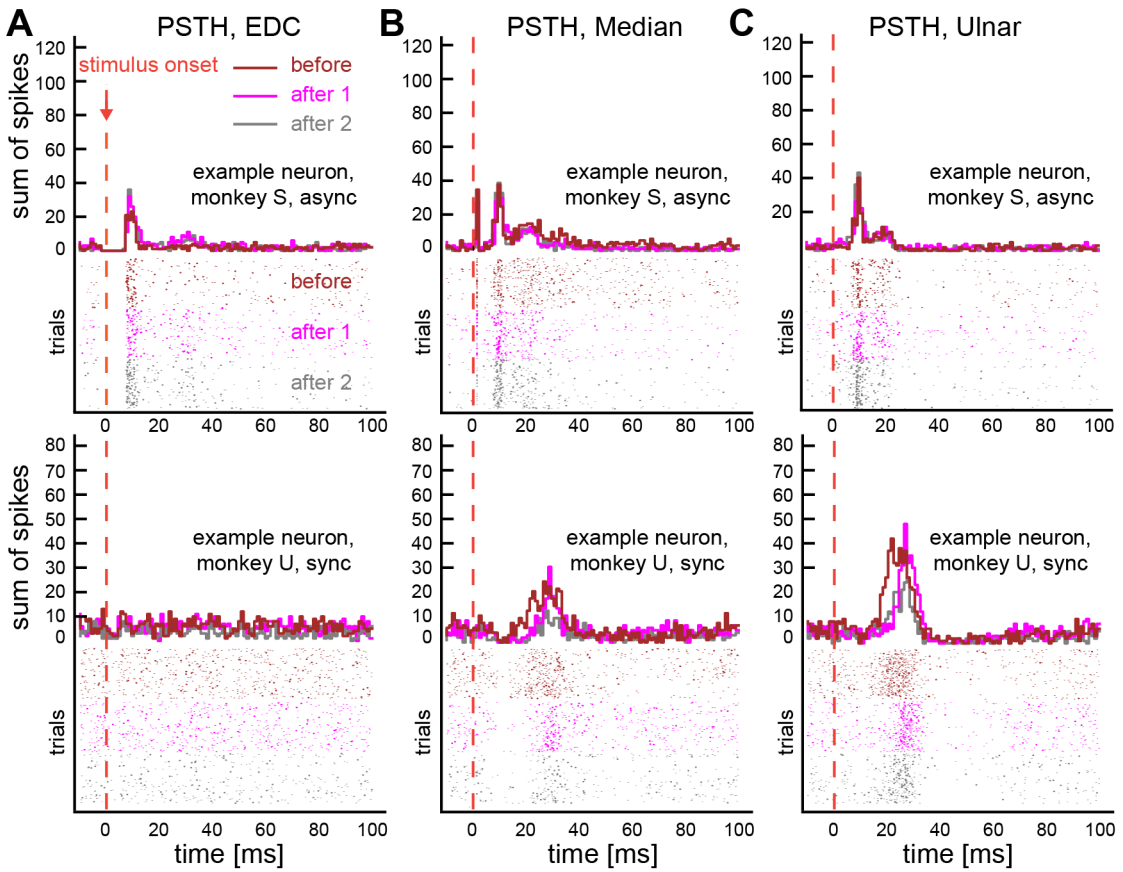

Figure 4.4: *Single unit responses to EDC, median and ulnar nerve stimulation.* (A) Peristimulus time histograms (PSTHs) of one example M1 neuron for monkey S (top row) and monkey U (bottom row) relative to EDC stimulation. (B) PSTHs for those example neurons relative to median nerve stimulation. (C) PSTHs relative to ulnar nerve stimulation. Each sub-plot contains the response profile (sum of all events in one bin, binsize $\Delta = 1\text{ms}$) before (brown), immediately after (after 1, magenta), and approximately one hour (after 2, gray) after asynchronous (A-C, top row) or synchronous (A-C, bottom row) median and ulnar nerve stimulation. Below the response profiles, raster-plots are shown. The colour of the dots corresponds to the aforementioned time of assessment. Each assessment consisted of 300 trials. Stimulation onset at 0 ms is highlighted with an orange dashed line.

For some SUs there was no apparent change in the response profile comparing before and after the intervention (example neuron top row, Figure 4.4A-4.4C). For other single units there was a clear change in the magnitude (and occasionally the width) of the stimulation-evoked responses after the intervention (example neuron bottom row, Figure 4.4A-4.4C).

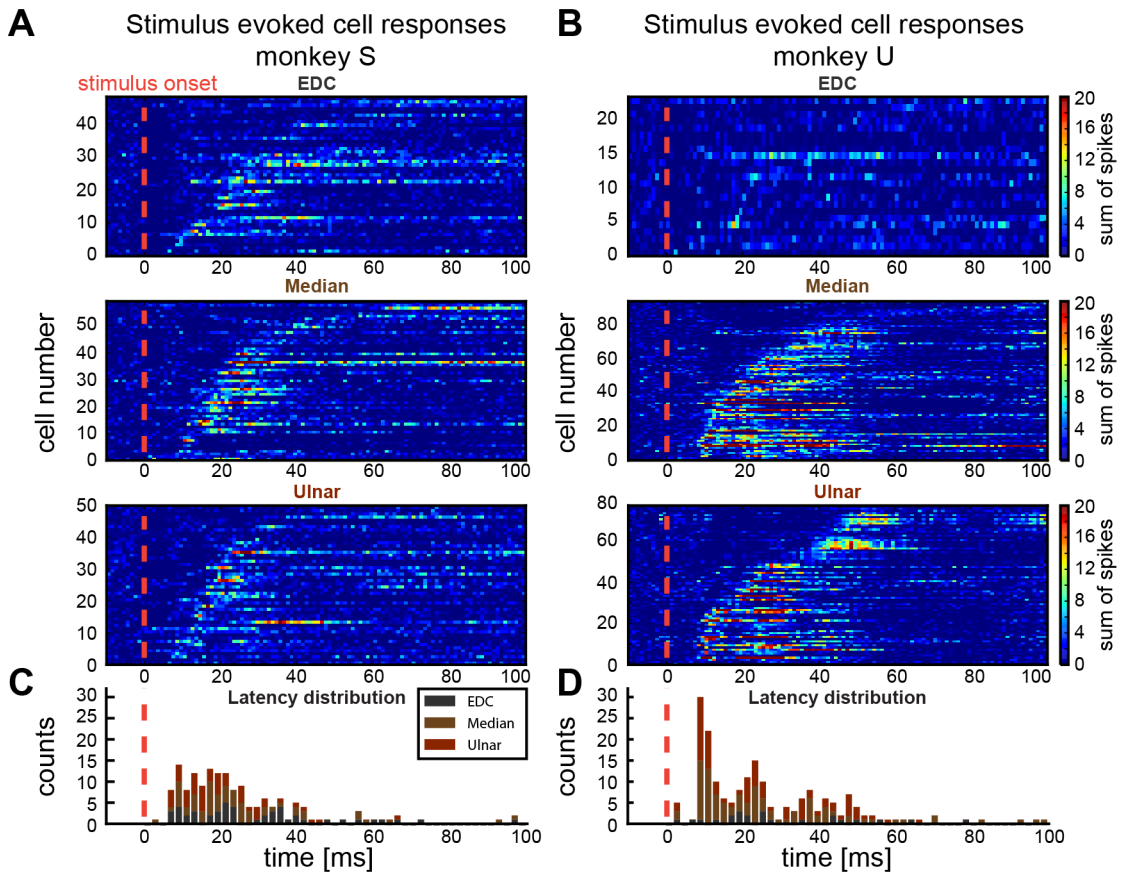

Figure 4.5: *Single unit responses to EDC, median and ulnar nerve stimulation for all responding neurons ordered by response onset latency.* (A) EDC, median and ulnar nerve stimulation evoked single unit responses for monkey S. (B) Stimulation evoked single unit responses for monkey U. (C) Response onset latency distribution for monkey S. (D) Response onset latency distribution for monkey U. The stimulation onset at $0\,\mathrm{ms}$ is highlighted with an orange dashed line. The binned (binwidth $\Delta = 1\mathrm{ms}$) sum of spikes profile for each responding neuron is colour coded. The response onset latency distribution histograms (C and D) illustrate a broad variety of response onset latencies for both monkeys. SU response profiles are shown before the intervention.

Because of the divergent nature of these SU responses (Figure 4.4A-4.4C), the response onset latencies and the PSTH response onset profiles were analysed for the whole single unit population for monkey S and monkey U separately (Figure 4.5A-4.5D).

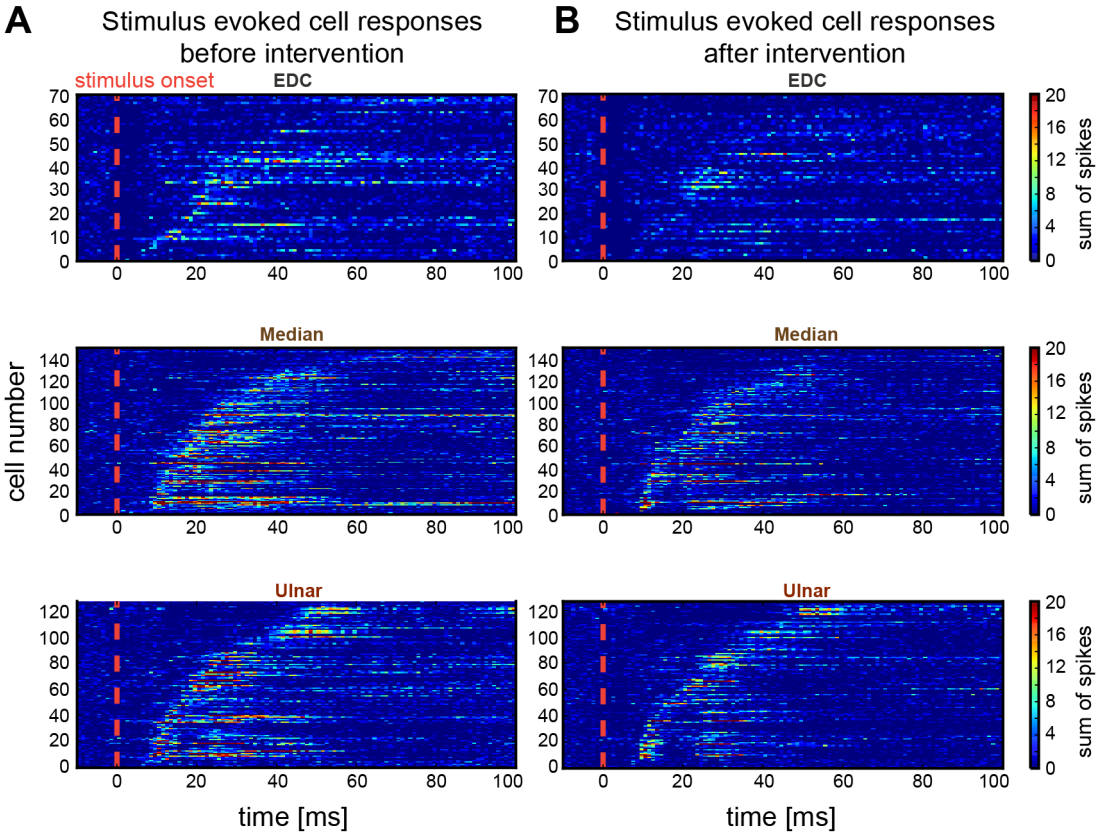

Figure 4.6: *Single unit responses to EDC, median and ulnar nerve stimulation for all responding neurons before and after the intervention.* (A) EDC, median and ulnar stimulation evoked single unit responses for all responding neurons of both monkeys before the intervention. (B) Stimulation evoked SU responses for both monkeys after the intervention. The stimulation onset at $0\,\mathrm{ms}$ is highlighted with an orange dashed line. The binned (binwidth $\Delta = 1\mathrm{ms}$) sum of spikes profile for each responding neuron is colour coded.

4.4.1 *Single units in M1 fire in response to EDC, median and ulnar nerve stimulation at various latencies*

The response onset latencies varied substantially for monkey S (Figure 4.5A and 4.5C) and monkey U (Figure 4.5B and 4.5D). Furthermore, the time profile of the stimulation evoked single unit discharges were divergent among the neuronal population with respect to the width and the shape of the overall evoked responses for both monkey S (Figure 4.5A) and monkey U (Figure 4.5B).

The stimulation evoked PSTH profiles were comparable between monkeys and therefore combined for subsequent analysis (Figure 4.6A-4.6B).

4.4.2 *Stimulation evoked single unit response profiles changed after the intervention*

Comparing the overall SU response profile distribution for all responding neurons before (Figure 4.6A) and immediately after (Figure 4.6B) the intervention (cells for both the asynchronous and synchronous stimulation intervention are shown), revealed some differences in both earlier and later spike responses.

Inspecting the response density across the SU population, indicated a general reduction of the sum of spikes per bin after the intervention (Figure 4.6A and 4.6B).

To investigate this difference in more detail, difference response profiles are examined by dividing the SU data into difference plots after synchronous (Figure 4.7A) and asynchronous (Figure 4.7B) median and ulnar nerve stimulation.

4.4.3 *Both synchronous and asynchronous median and ulnar nerve stimulation led to predominantly suppression of stimulation evoked responses after the intervention*

The PSTH response profile difference (z-score, see Equation 4.2) after synchronous (Figure 4.7A) and asynchronous (Figure 4.7A) median and ulnar nerve stimulation, showed both facilitation (red colour range, Figure 4.7A and 4.7B) and suppression (blue colour range, Figure 4.7A and 4.7B).

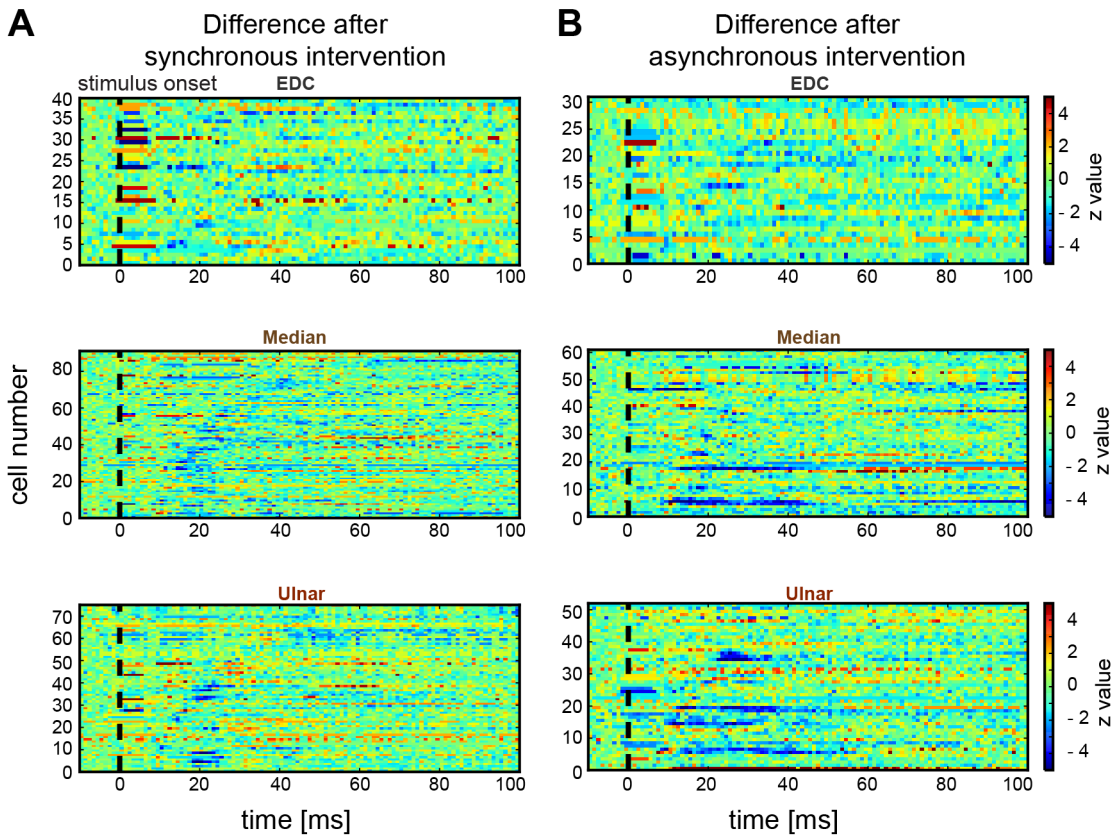

Figure 4.7: *Differences in the stimulation evoked single unit response profiles after synchronous and asynchronous median and ulnar nerve stimulation.* (A) Differences in the EDC, median and ulnar stimulation evoked single unit response profiles after 1 h of synchronous median and ulnar nerve stimulation (intervention). (B) Differences in stimulation evoked SU responses after 1 h of asynchronous median and ulnar nerve stimulation. The difference was expressed as z values (see Equation 4.2). A facilitation after the intervention is shown in red and a suppression in blue colour ranges.

The majority of neurons however showed a suppression of the individual stimulation site evoked SU responses for both interventions (Figure 4.7A and 4.7B). This suppression followed the time-response onset latency profile in the case of the synchronous (Figure 4.7A) intervention. After asynchronous median and ulnar nerve stimulation the suppression profile was broader and temporally less precise (Figure 4.7B). To study the response (time) profile difference for the whole SU population in more detail, the population difference profile ($\bar{z}$-score, see Equation 4.3) was examined (Figure 4.8A and 4.8B).

4.4.4 *Different single unit population response difference characteristics after synchronous in comparison to asynchronous stimulation*

The one hour of median and ulnar nerve stimulation (intervention) led to distinct population difference profiles ($\bar{z}$-score, see Equation 4.3) evoked by individual EDC, median and ulnar nerve stimulation for synchronous (Figure 4.8A) and asynchronous (Figure 4.8B) stimulation.

The population difference profile in response to individual EDC stimulation (control stimulation site) led to only a very few significant facilitations (see number of bins greater than the $95\,\%$ confidence interval and number of cells per bin with significant facilitations, cf. binomial distribution, Figure 4.8A and 4.8B, top row) and suppressions (number of bins in population $\bar{z}$ score $< 95\,\%$ CI and number of significantly suppressed neurons per bin, Figure 4.8A and 4.8B). Very close to the onset of the individual stimulation for bins smaller than approximately $7\,\mathrm{ms}$, a few significant facilitations and suppressions occurred. There was no difference between the individual EDC stimulation evoked population difference response profile after the synchronous in comparison to the asynchronous intervention. Also in response to repetitive electrical peripheral nerve stimulation both M1 and S1 show increased excitability (Schabrun et al., 2012) as expressed by increased MEPs and SEPs.

The PSTH response population difference profile evoked by individual median and ulnar stimulation however, led to clear significant effects (predominantly suppressions) after the intervention (Figure 4.8A and 4.8B). Furthermore, there was a distinct temporal profile after the synchronous (Figure 4.8A) in comparison to the asynchronous (Figure 4.8B) intervention.

After the synchronous median and ulnar nerve stimulation, the population of SUs showed a clear and consistent significant ($p < 0.01$, binomial distribution test) suppression around $20\,\mathrm{ms}$ post-stimulation.

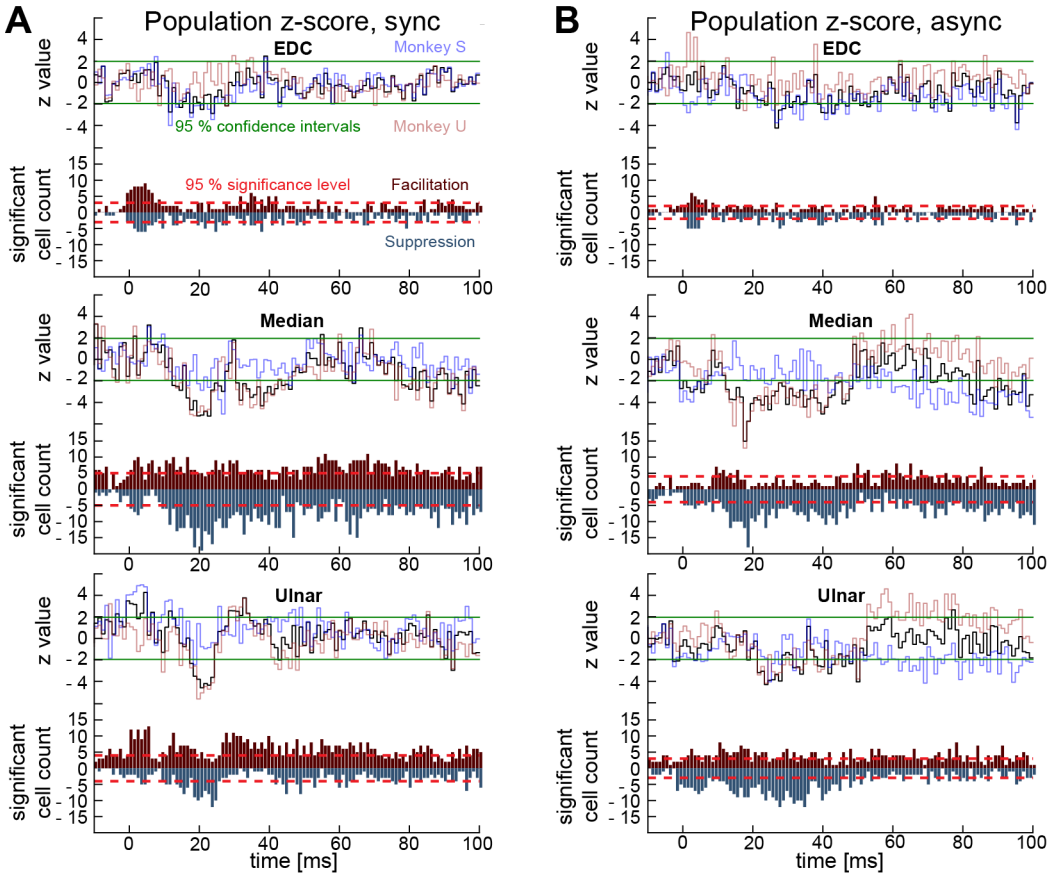

Figure 4.8: *Population z-score comparing single unit differences after synchronous and asynchronous stimulation.* (A) Population z-score $\bar{z}$ (Equation 4.3) and significant cell profile after $1\,\mathrm{h}$ of synchronous median and ulnar nerve stimulation. (B) Population z-score $\bar{z}$ and significant cell profile after $1\,\mathrm{h}$ of asynchronous median and ulnar nerve stimulation. Each sub-plot consists of the population $\bar{z}$ profile on the top and the number of significantly different cells per bin below. The $95\,\%$ confidence intervals are shown in green. The dashed red line indicates the number of cells with a significant ($p < 0.05$) facilitation (or suppression) by chance which would be expected based on a binomial probability distribution.

This suppression was followed by a facilitation with a peak around 30-$40\,\mathrm{ms}$ post-stimulation onset. This facilitation was only significant ($p < 0.01$, binomial distribution test) in response to individual ulnar nerve stimulation where the number of significantly suppressed neurons critically reduced and at the same time interval the number of significantly facilitated cells increased (Figure 4.8A, bottom row). A similar facilitation evoked individual median nerve stimulation occurred slightly earlier (Figure 4.8A, middle row).

In this case the number of significantly suppressed neurons reduced in a slower way whereas the number of significant facilitated cells remained fairly constant. Therefore, the facilitation after median nerve stimulation was not significant and could rather be described as a return to baseline (Figure 4.8A, middle row). This was previously denoted as rebound effect and refers to the return to the response difference baseline.

The population response difference profiles evoked by individual median and ulnar nerve stimulation after the asynchronous intervention showed different response characteristics (Figure 4.8B). Rather than a localised suppression around 20 ms after the stimulation onset as seen in response to the synchronous intervention, the onset of the suppression effect occurred slightly later (Figure 4.8B, middle and bottom row). This suppression remained until approximately 50 ms (sustained suppression period).

Although the overall characteristics of the population response difference profile were comparable between monkey S and U in terms of local facilitations and suppressions, some of these were heterogeneous between monkeys and depending on the time of the assessment post-intervention (Figure 4.9A and 4.9B).

4.4.5 *Heterogeneity between population response difference profiles between subjects and dependence on post-intervention time*

Comparing the SU population $\bar{z}$-score profile evoked by median and ulnar nerve stimulation for monkey S and U after the synchronous (Figure 4.9A) and asynchronous (Figure 4.9B) intervention revealed some subject-specific divergence whereas some characteristic features remained comparable.

The aforementioned temporal response profile after the synchronous intervention consisting of a significant suppression around 20 ms post individual median and ulnar stimulation followed by a facilitation or rebound at approximately 30-40 ms was present in both monkey S and U (Figure 4.9A). The shape and the amount of this differential effect however was heterogeneous between monkeys and depending on the post-intervention time.

Monkey S for example showed a stronger suppression at $\sim$20 ms post-stimulation onset approximately one hour after the end of the synchronous intervention (gray trace, Figure 4.9A) as opposed to immediately after the end of the intervention (magenta trace, Figure 4.9A).

For monkey U on the other side the suppression at $\sim$20 ms was stronger immediately after (magenta trace, Figure 4.9A) as opposed to approximately one hour after (gray trace, Figure 4.9A) the synchronous intervention. Nevertheless, the suppression at $\sim$20 ms followed by the facilitation or rebound around 30-40 ms was consistent for responses evoked by individual median and ulnar stimulation.

The population $\bar{z}$-score after asynchronous median and ulnar nerve stimulation however was more divergent between monkey U and S (Figure 4.9B).

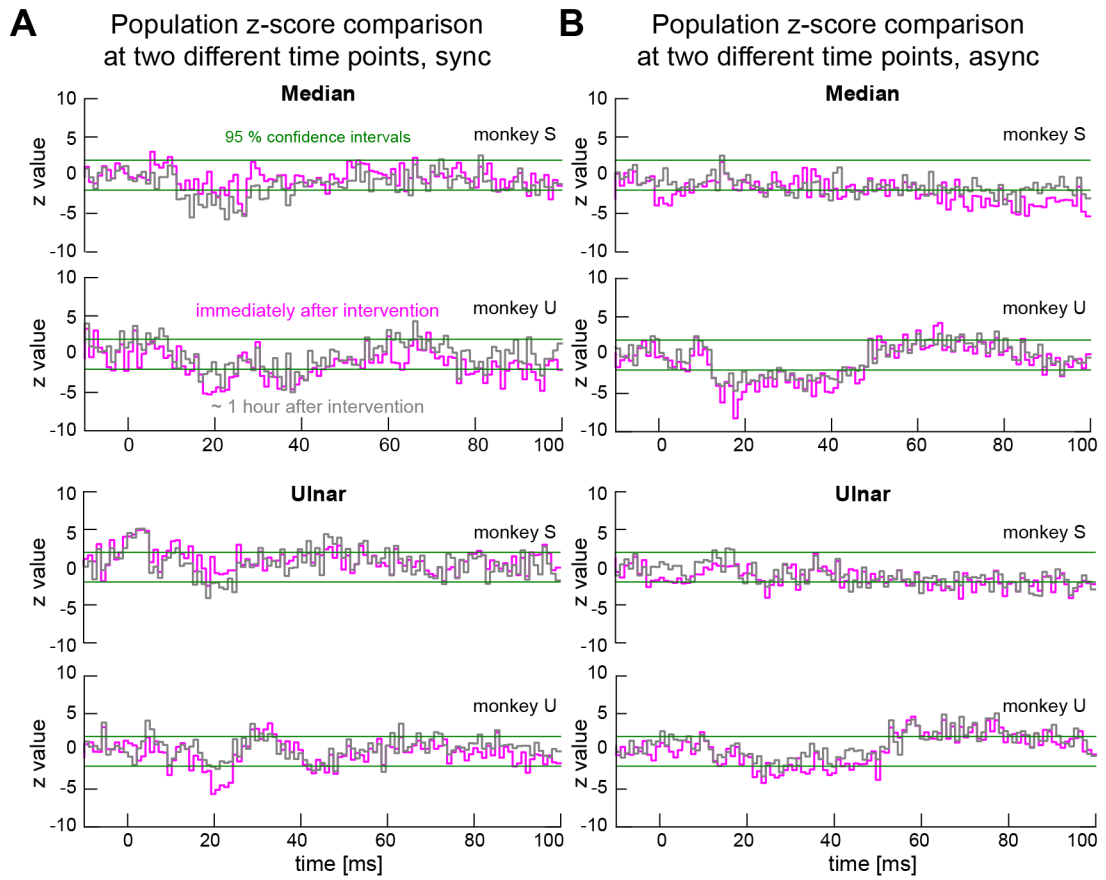

Figure 4.9: *Heterogeneity of single unit responses differences evoked by independent median and ulnar nerve stimulation after the synchronous and asynchronous intervention*. (A) SU response differences immediately after (magenta trace) and approximately one hour after synchronous stimulation (gray trace) for monkey S and U. (B) SU response differences immediately after (magenta trace) and approximately one hour after asynchronous stimulation for monkey S and U. The 95 % confidence intervals are shown in green.

Whereas there was a broad sustained suppression from around 20 ms to 30-40 ms for monkey U, this suppression was less pronounced and with a later suppression onset for monkey S (Figure 4.9B).

4.4.6 *Stable multi unit population response difference profiles indicate a similar temporal profile than those of the single units*

Investigating the effects induced by synchronous and asynchronous median and ulnar nerve stimulation on stable multi units (MUs) revealed a similar

temporal population $\bar{z}$ response difference profile than for the SUs (Figure 4.10A and 4.10B). Responses to individual EDC stimulation led to a few significant facilitations and suppressions (Figure 4.10A and 4.10B) like the ones previously observed in the responses profiles of SUs (cf. Figure 4.8A and 4.8B).

The temporal characteristics of the MU population response difference profiles after the synchronous intervention (Figure 4.10A and 4.10B) were very similar to those of the SUs. After the synchronous intervention MUs response difference profiles showed a significant suppression at around $20\,\mathrm{ms}$ after the stimulation onset of individual median and ulnar stimulation (Figure 4.10A and 4.10B, middle and bottom row). This suppression was followed by a facilitation or rebound effect at approximately $30\text{-}40\,\mathrm{ms}$ induced by individual median and ulnar stimulation. This is also in-line with the results regarding the population $\bar{z}$-scores of the SUs (Figure 4.8A and 4.8B).

The MU response difference profile after the asynchronous intervention (Figure 4.10A and 4.10B) however differed slightly from those of the SUs (Figure 4.8A and 4.8B). Although there was a weak sustained suppression after the asynchronous intervention evoked by individual median and ulnar nerve stimulation, this suppression was not as pronounced as on the SU level.

4.4.7 *Identified pyramidal tract neurons were changed by synchronous and asynchronous peripheral nerve stimulation*

The population response difference profiles of identified pyramidal tract neurons (PTNs) were also influenced by synchronous and asynchronous median and ulnar nerve stimulation (Figure 4.11A and 4.11B). The population $\bar{z}$-score profile of identified PTNs evoked by individual EDC stimulation was not significantly affected after either the synchronous or asynchronous intervention (data not shown). After the synchronous intervention, population response difference

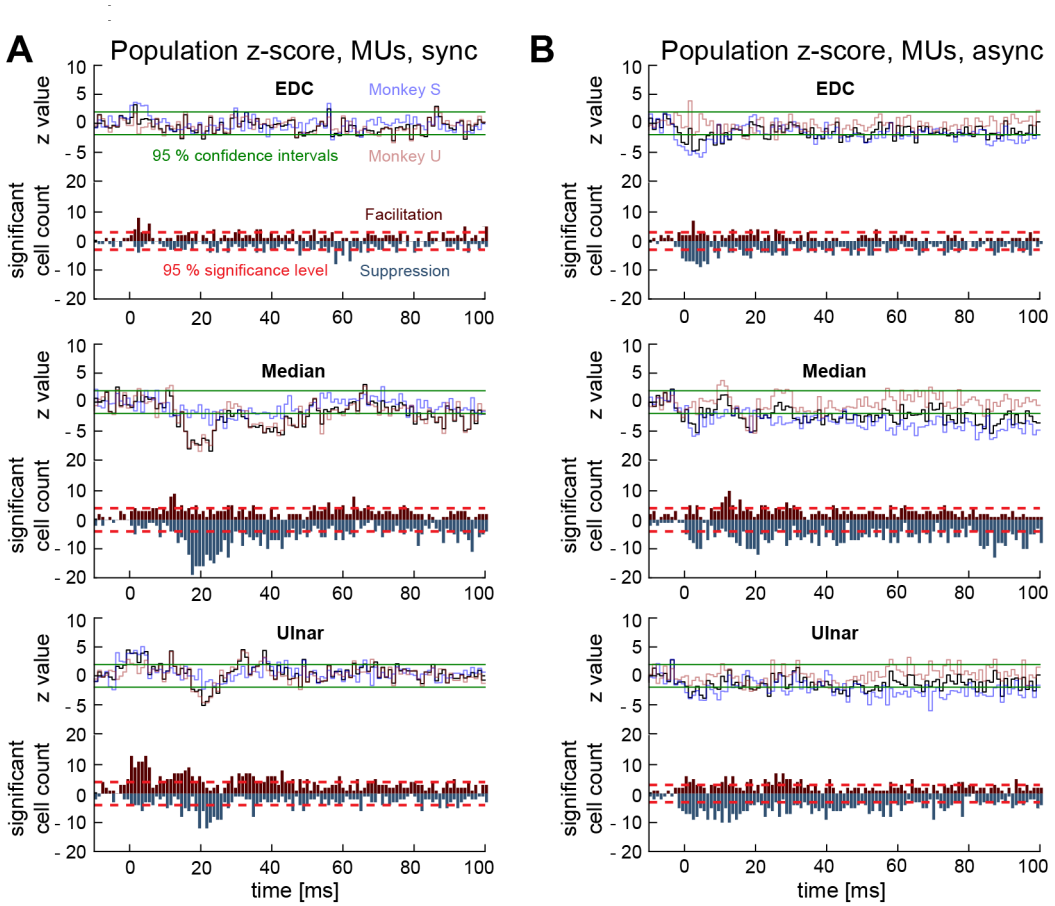

Figure 4.10: *Population z-score comparing multi unit differences after synchronous and asynchronous stimulation.* (A) MU population z-score $\bar{z}$ (Equation 4.3) and significant cell profile after $1\,\mathrm{h}$ of synchronous median and ulnar nerve stimulation. (B) MU population z-score $\bar{z}$ and significant cell profile after $1\,\mathrm{h}$ of asynchronous median and ulnar nerve stimulation. Each sub-plot consists of the population $\bar{z}$ profile on the top and the number of significantly different cells per bin below. The $95\,\%$ confidence intervals are shown in green. The dashed red line indicates the number of cells with a significant ($p < 0.05$) facilitation (or suppression) by chance which would be expected based on a binomial probability distribution.

profiles were characterised by a significant suppression around $20\,\mathrm{ms}$ after individual median and ulnar nerve stimulation (Figure 4.11A). This was followed by a return to baseline around 30-40 ms. No clear significant facilitation (cf. Figure 4.8A) around this time interval could be observed for the PTNs. After the asynchronous intervention, population $\bar{z}$-score profiles showed some indication of a sustained suppression from $20\,\mathrm{ms}$-40 ms (Figure 4.11B).

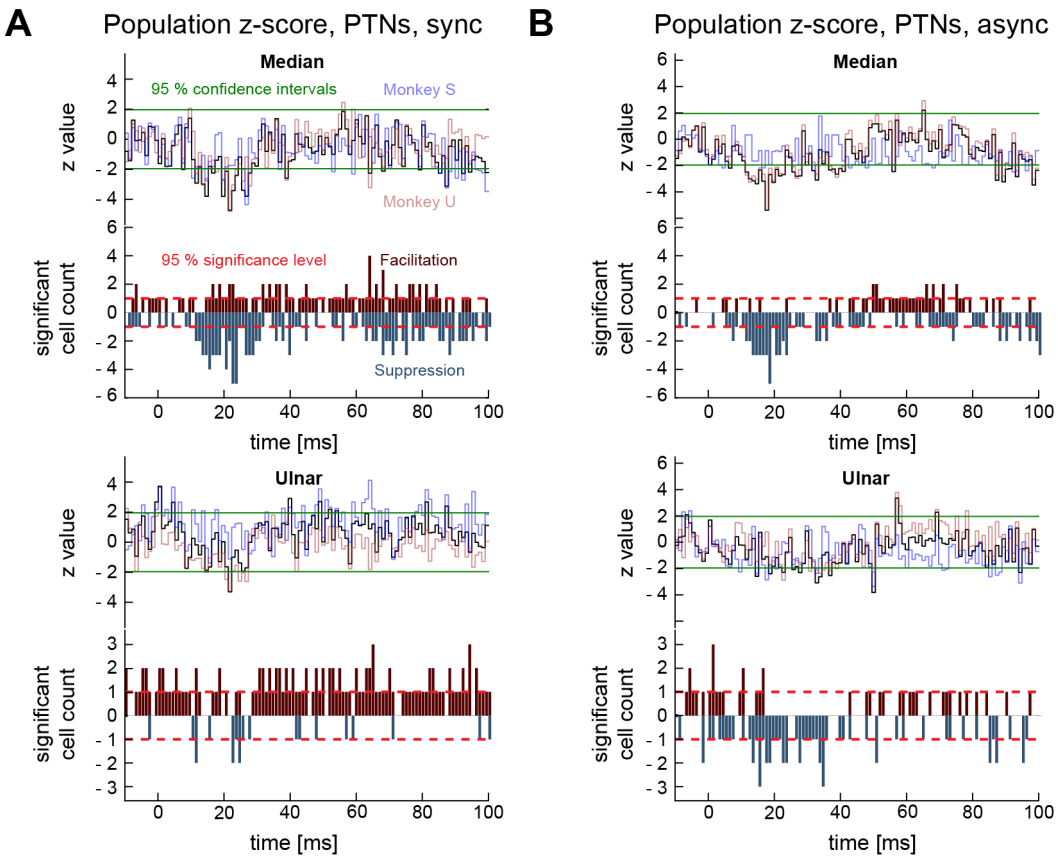

Figure 4.11: *Population z-score comparing pyramidal tract neuron differences after synchronous and asynchronous stimulation.* (A) PTN population z-score $\bar{z}$ (Equation 4.3) and significant cell profile after $1\,\mathrm{h}$ of synchronous median and ulnar nerve stimulation. (B) PTN population z-score $\bar{z}$ and significant cell profile after $1\,\mathrm{h}$ of asynchronous median and ulnar nerve stimulation. Each sub-plot consists of the population $\bar{z}$ profile on the top and the number of significantly different cells per bin below. The $95\,\%$ confidence intervals are shown in green. The dashed red line indicates the number of cells with a significant ($p < 0.05$) facilitation (or suppression) by chance which would be expected based on a binomial probability distribution.

Since the population $\bar{z}$-score profiles for the PTNs relied only on a small number of stable cells which responded to individual median and ulnar nerve stimulation (Table 4.1), the temporal response profile was more noisy.

Similar tendencies like in the case of the single unit profiles were observed (Figure 4.11A and 4.11B).

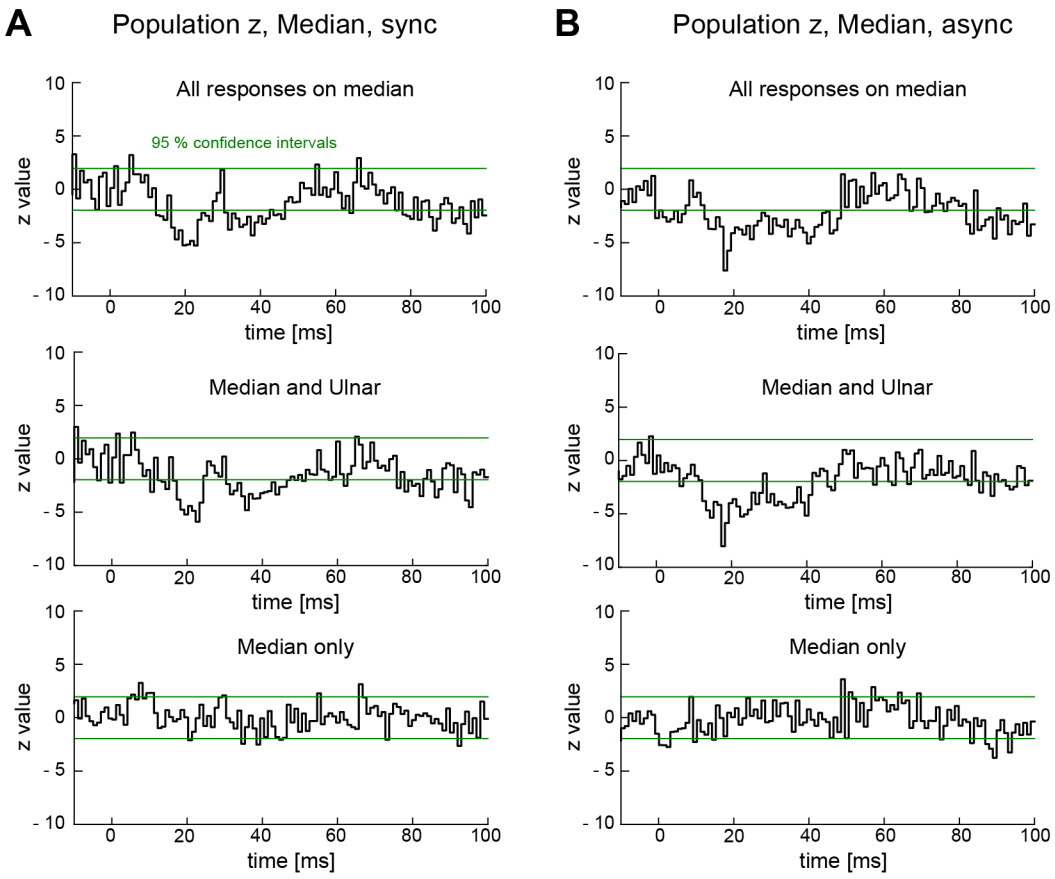

Figure 4.12: *Population response differences of single units evoked by median nerve stimulation grouped by responses to median only and both median and ulnar nerve*. (A) SU responses to individual median nerve stimulation grouped by cells responding to median stimulation only (bottom row) or both median and ulnar stimulation (middle row). The population difference $\bar{z}$ profile is shown for each of these conditions after 1 h of synchronous median and ulnar nerve stimulation (intervention). (B) SU response profiles after 1 h of asynchronous median and ulnar nerve stimulation.

4.4.8 *The intervention effects on single units were mediated by cells which receive inputs from both median and ulnar nerve*

Dividing all of the SUs responding to individual median nerve stimulation into those neurons which responded to both median and ulnar nerve stimulation (Figure 4.12A and 4.12B, middle row) versus those responding to median nerve stimulation alone (Figure 4.12A and 4.12B, bottom row), revealed distinct population response difference profiles for these groups.

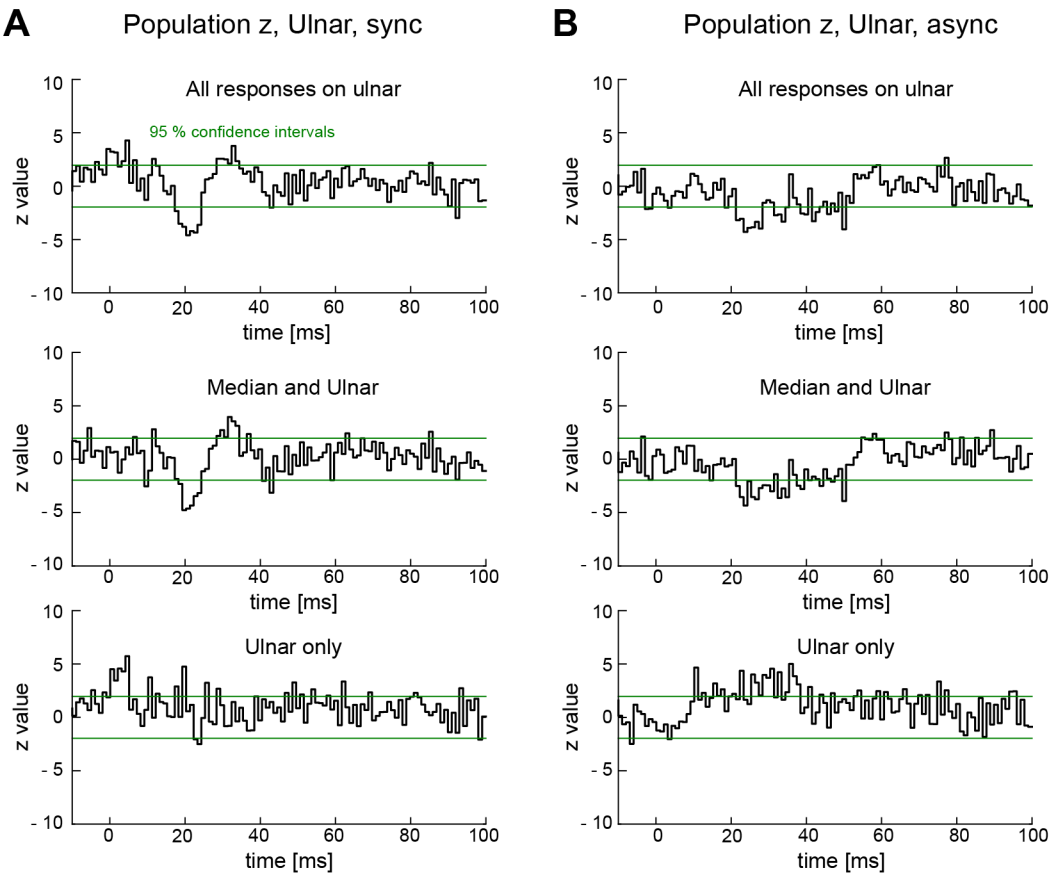

Figure 4.13: *Population response differences of single units evoked by ulnar nerve stimulation grouped by responses to ulnar only and both median and ulnar nerve*. (A) SU responses to individual ulnar nerve stimulation grouped by cells responding to ulnar stimulation only (bottom row) or both median and ulnar stimulation (middle row). The population difference $\bar{z}$ profile is shown for each of these conditions after 1 h of synchronous median and ulnar nerve stimulation (intervention). (B) SU response profiles after 1 h of asynchronous median and ulnar nerve stimulation.

There was almost no significant difference after both interventions on SUs responding to median nerve stimulation alone (Figure 4.12A and 4.12B).

SUs which responded to both individual median and ulnar stimulation possessed the aforementioned temporal response difference profile similar to the overall SU population response (Figure 4.12A and 4.12B).

A similar tendency could be identified for the SU $\bar{z}$-score profiles evoked by individual ulnar stimulation (Figure 4.13A and 4.13B).

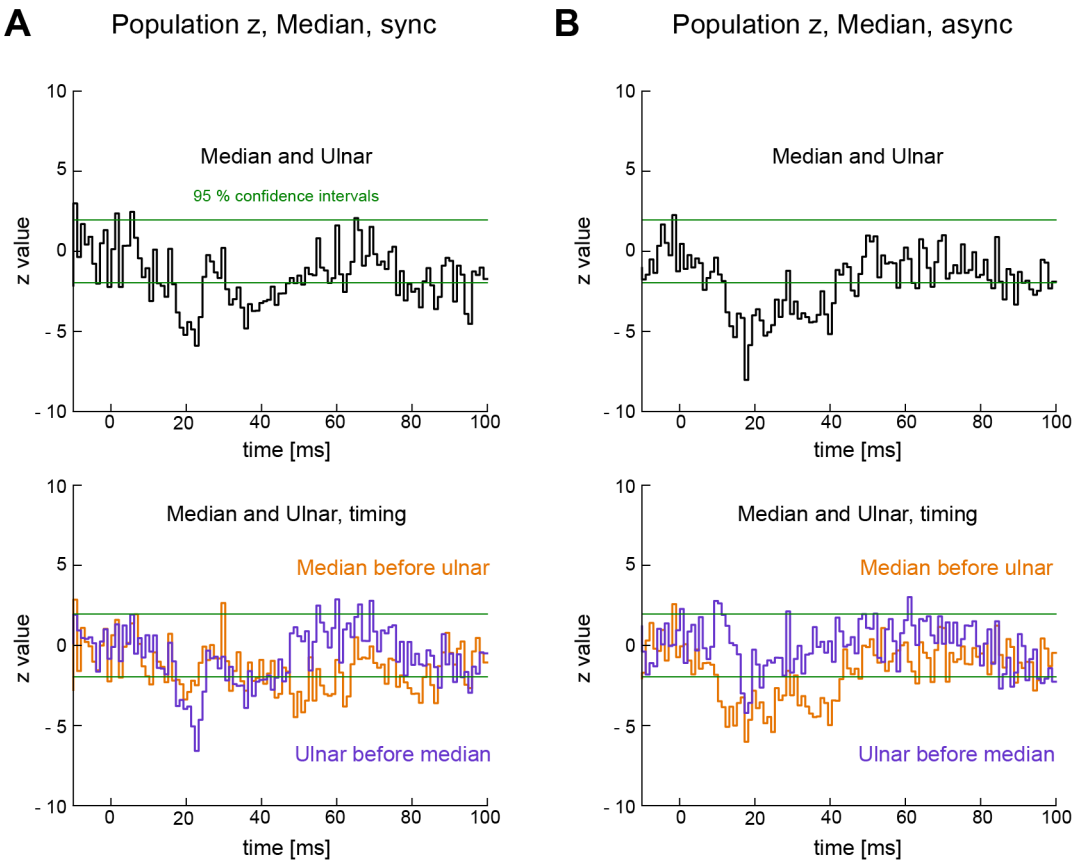

Figure 4.14: *Population response differences of single units evoked by median nerve stimulation responding to both median and ulnar nerve stimulation grouped by response onset time.* (A) SU population response difference of single units responding to median and ulnar nerve stimulation after 1 h of synchronous median and ulnar nerve stimulation. (B) SU population response differences after 1 h of asynchronous median and ulnar nerve stimulation. The top row shows the total (population $\bar{z}$ score, Equation 4.3) and the bottom row shows the response difference profiles grouped by neurons which receive input from the median nerve first and then the ulnar nerve (orange traces) and vice versa (purple traces).

Population response difference profiles after individual ulnar nerve stimulation which were activated by only individual ulnar nerve stimulation however, showed small significant facilitations after the intervention (Figure 4.13A and 4.13B, bottom row). This effect nonetheless did not resemble the characteristic temporal response difference profile observed in cells with median and ulnar nerve input.

4.4.9 *Intervention effects depend on median and ulnar nerve input timing*

The variation in response onset latency to median and ulnar nerve stimulation gave the opportunity to determine whether the effects of the intervention depended on the relative timing of responses to each nerve individually.

Therefore, all SUs responding to both individual median and ulnar nerve stimulation (Figure 4.14A and 4.14B, top row) were divided into cell responses which received activations by median first followed by ulnar nerve stimulation (orange trace, Figure 4.14A and 4.14B) and vice-versa (purple traces, Figure 4.14A and 4.14B).

These sub-divided datasets possessed different population response difference profiles.

After the synchronous median and ulnar nerve stimulation intervention, both sub-divided groups of SUs (SUs activated by median before ulnar and the other way around) showed the previously described population response differences consisting of a suppression around 20 ms followed by a rebound or facilitation at approximately 30-40 ms (Figure 4.14A).

The SU response around 20 ms (and the subsequent rebound response) after individual median nerve stimulation was facilitated in comparison to the overall population response difference profile (Figure 4.14A, top row) in SUs which were activated by median first followed by ulnar nerve stimulation (orange trace, Figure 4.14A).

The opposite was the case in SUs which were activated by ulnar first followed by median nerve stimulation (purple trace, Figure 4.14A). Response differences indicated more suppression in neurons with shorter input latencies to ulnar than median nerve.

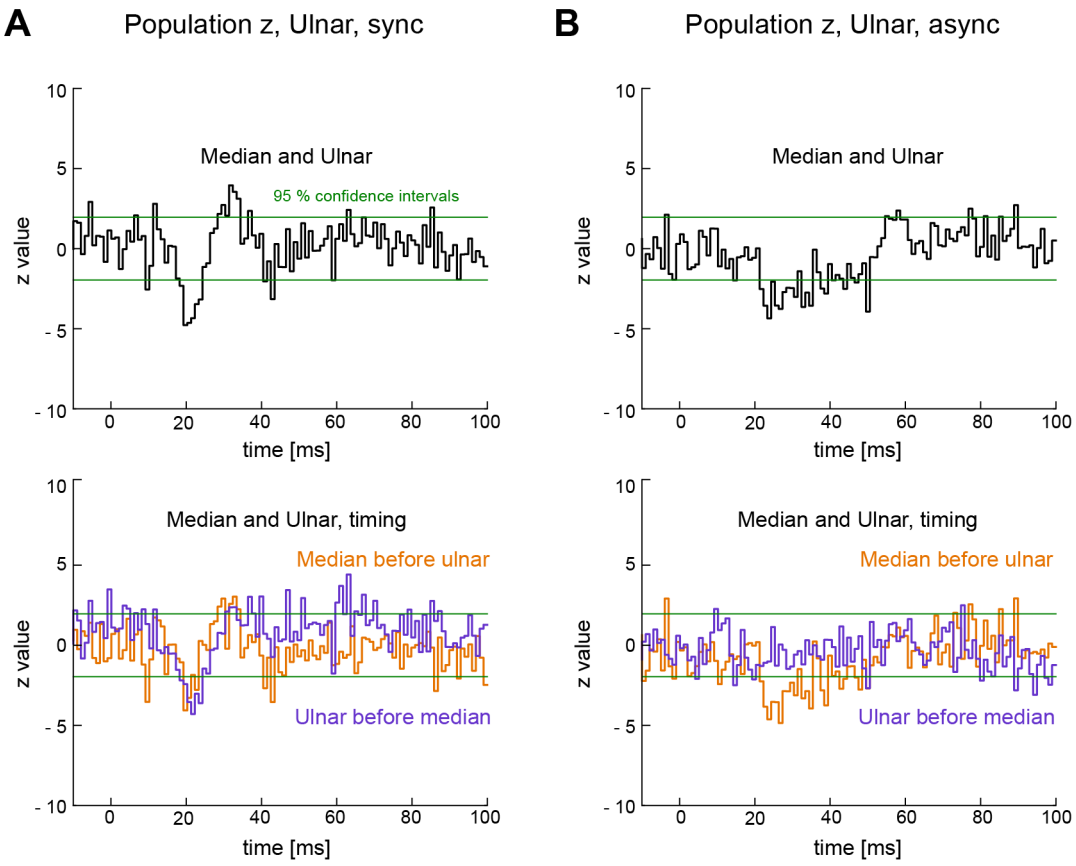

Figure 4.15: *Population response differences of single units evoked by ulnar nerve stimulation responding to both median and ulnar nerve stimulation grouped by response onset time.* (A) SU population response difference of single units responding to median and ulnar nerve stimulation after 1 h of synchronous median and ulnar nerve stimulation. (B) SU population response differences after 1 h of asynchronous median and ulnar nerve stimulation. The top row shows the total (population $\bar{z}$ score, Equation 4.3) and the bottom row shows the response difference profiles grouped by neurons which receive input from the median nerve first and then the ulnar nerve (orange traces) and vice versa (purple traces).

The population $\bar{z}$-score profiles after the asynchronous intervention on the contrary showed a decreased response from around 20-40 ms in response to individual median nerve stimulation in SUs which received activation from median first followed by ulnar nerve stimulation (orange trace, Figure 4.14B).

In cells receiving activation from ulnar first followed by median nerve stimulation the response was facilitated (purple trace, Figure 4.14B) in comparison to the general population response difference profile (Figure 4.14B, top row).

Applying the same subdivision to the population SUs activated by individual ulnar nerve stimulation did not lead to different effects for the population response profiles in these conditions (median before ulnar and vice versa) after the synchronous intervention (cf. orange and purple trace, Figure 4.15A).

The nerve stimulation timing dependent effects after the asynchronous intervention however were similar to those reported for response difference profiles induced by individual median nerve stimulation (Figure 4.14B). M1 neurons which were activated by median first followed by ulnar nerve stimulation showed a suppression of the characteristic population $\bar{z}$-score profile (orange trace, Figure 4.15B). SUs which elicited action potentials to ulnar first followed by median nerve stimulation showed a facilitation of the population response difference profile (purple trace, Figure 4.15B).

4.5. Discussion

4.5.1 *Neurons in M1 are differentially modulated by synchronous and asynchronous peripheral nerve interventions*

Neurons in M1 changed their pattern of neuronal firing after synchronous and asynchronous interventions (Figure 4.7A, 4.7B, 4.8A, and 4.8B). The M1 population response difference revealed a differential temporal profile of all responding and stable units after the synchronous in contrast to the asynchronous intervention (Figure 4.8A and 4.8B). After one hour of synchronous median and ulnar nerve stimulation, the population difference profile was characterised by a significant suppression at approximately $20\,\mathrm{ms}$ for the relevant (single median or ulnar nerve stimulation as an assessment) single stimulation-site evoked responses (Figure 4.8A). This significant suppression was followed by a facilitation or rebound between 30 and $40\,\mathrm{ms}$ for both the single median and ulnar evoked responses (Figure 4.8A, middle and bottom row). The M1 cell

responses evoked by EDC stimulation did not change after both the synchronous and asynchronous intervention (Figure 4.8A and 4.8B, top row). This was expected since EDC served as a control stimulation site. These findings are in line with studies investigating the motor-cortical excitability by means of changes in motor evoked potentials (MEPs) and changes in motor cortical representations conducted on human subjects using similar peripheral nerve interventions (McKay et al., 2002; Ridding et al., 2001). In these and other studies performing synchronous interventions on two motor points (McDonnell and Ridding, 2006; Ridding and Uy, 2003; Schabrun and Ridding, 2007) MEP changes were predominantly reported in the target muscles (the during the intervention stimulated sites). Also in the present study, changes in M1 neurons' discharge mainly occurred in responses evoked by the target peripheral nerves (median and ulnar nerve) and not by the control site (EDC).

However, there are a number of studies reporting that some effects such as the increase in the number of active sites or the MEP amplitude assessed by TMS did also increase (although to a weaker extend compared to the target sites) for the control muscle (e.g. Schabrun and Ridding, 2007).

This minor difference to the results of the present study could be due to a number of reasons. First, the type of assessment measuring excitability of MEPs as opposed to responses of single M1 neurons to afferent stimulation is qualitatively different. TMS is considered to activate corticospinal neurons in M1 both directly and indirectly (Amassian et al., 1990; Baker and Lemon, 1995; Baker et al., 1994; Di Lazzaro et al., 2008; Edgley et al., 1990, 1997) and provides thereby a measure of corticofugal descending output (and corticospinal excitability) of projections to the target muscles. The assessment in the present study on the other hand measured changes of single identified and unidentified neurons in M1 activated by either EDC, median or ulnar nerve stimulation. Therefore, it measures the ascending afferent input to and the modulation of these M1 neurons. Interestingly, identified pyramidal tract neurons (PTNs) changed

their firing rates evoked by single ulnar or median nerve stimulation after both interventions (Figure 4.11A and 4.11B). This is important because PTNs are involved in the generation of the motor evoked potential (MEP).

Secondly, the sites stimulated during the intervention and the control sites were different to other studies. Schabrun and Ridding (2007) stimulated the first dorsal interosseous (FDI) and the abductor digiti minimi (ADM) muscles during the synchronous (and asynchronous) intervention. The abductor pollicis brevis (APB) muscle served as the control site in that study. Considering that one of the muscles stimulated during the intervention was adjacent to the control muscle (FDI and APB), and taking the stimulation intensity ($3\times$ perceptual threshold) into account, it is possible that the adjacent muscle was also activated. Adjacent muscles might have been activated by inter-muscular connections via gap junctions, increased spread of activity at higher electrical currents and due to the fact that some motoneurons provide more divergent innervation of muscles than others. In another study, Ridding et al. (2001) stimulated the radial (innervates e.g. the abductor pollicis longus (APL) muscle) and the ulnar (innervates e.g. the FDI muscle) nerve. ADM and APB served as control sites. Since APB is adjacent to one of the by the stimulation activated muscles (FDI) and ADM are innervated by the ulnar nerve directly, although to a weaker extend than FDI) an effect on these muscles after the intervention would also be feasible. Another explanation would be that a single corticomotoneuronal (CM) neuron can innervate a variety of muscles (cf. divergent CM output, Bennett and Lemon, 1996; Cheney and Fetz, 1980; Lemon, 2008; Ridding et al., 2001) and could therefore activate (adjacent) control muscles. Here, EDC served as a control (stimulation) site whereas median and ulnar nerves were used for the intervention. Since EDC is innervated by the radial nerve, it can be hypothesised that the effects induced by the intervention might be more spatially distinct.

Finally, the stimulation applied in the present study can be assumed to be more focal and spatially precise than in the aforementioned studies on human subjects.

The stimulation was applied through implanted wires (EDC), and bipolar nerve cuffs (median and ulnar nerve, see Figure 4.1A) allowing a selective activation of the target sites. In human subjects however the stimulation was applied through surface electrodes thereby being less spatially accurate.

Nevertheless, the plastic changes induced in M1 neurons projecting to (and receiving input from) the stimulated target muscles are predominantly affected by the intervention. Here, I show for the first time a direct modulation of the stable M1 unit firing induced by repetitive co-activation of the stimulated peripheral afferent nerves.

4.5.2 *M1 neurons fired with various latencies to single-site EDC, median and ulnar nerve stimulation*

Single (SU) and multi units (MUs) in M1 responded to individual EDC, median and ulnar nerve stimulation with a broad variety of response onset latencies (Figure 4.5A, 4.5B, 4.6A, and 4.6B). These ranged from early response onset latencies between 5 and 7 ms up to late response onset latencies between 60 and 80 ms post single-site stimulation (Figure 4.5A and 4.5B). This variety of onset response latencies has been reported in a number of studies performing extracellular recordings in M1 of the macaque monkey in response to peripheral nerve stimulation (Jones, 1982; Kozelj and Baker, 2014). However, late response onset latencies between 60 and 80 ms are only rarely reported in studies conducting extracellular M1 recordings. This is probably the case because the majority of studies focusses on early responses rather than later ones.

Studies measuring the response onset latencies of somatosensory evoked potentials (SEPs) in monkeys after median nerve stimulation however found a range of early and late response onsets in agreement with the range of response onset latencies measured in the present study (Allison et al., 1991; Raymond et al., 2000).

Which pathways are likely to convey the sensory information to M1 neurons? What would be a possible ascending pathways leading to early and late responses in M1? In principle, peripheral afferent inputs to M1 can be either mediated through the dorsal column system directly via the thalamic ventrolateral (VL) or ventral posterolateral (VPL) nuclei or indirectly via S1 over cortico-cortical connections (Asanuma et al., 1980; Butler et al., 1992; Jones, 1982, 2002; Kosar et al., 1985). Neurons in VPL respond with latencies between 4 and 8 ms driven by median and radial nerve stimulation (Jones, 1982; Lemon, 1979). Adding latencies between 0.5 and 1.5 ms from the thalamus to M1 would result in total response latencies between 4.5 and 9.5 ms (Jones, 1982; Lemon, 1979). This would explain a possible pathway leading to early response onset latencies as observed in this study. Later response onset latencies were measured in response to wrist perturbations in caudal VPL ranging between 10 and 80 ms (Butler et al., 1992). Later response onsets to perturbations were also directly measured within M1 (Omrani et al., 2014). Peripheral afferent signals are also relayed in the cuneate nucleus before the thalamic nuclei. In the cuneate nucleus response latencies to median or ulnar nerve stimulation at approximately 5 ms were measured (Witham and Baker, 2011). Later responses might arise via slow conducting fibres or polysynaptic cortico-cortical pathways relayed in S1 or M1. This pathway might be the most likely one to account for the majority of the later responses. Late SEP components are usually considered to reflect cortical processing of the input via reentrant loops within the cortical circuitry.

Jones et al. (1986) stated that afferent inputs to M1 have to be relayed via area 1 or 2. A number of more recent studies however did find direct connections from area 3a of S1 to M1 (DeFelipe et al., 1986; Huerta and Pons, 1990). Since area 3a of S1 also receives in addition to cutaneous (Pons et al., 1992) inputs from muscle afferents (e.g. group I afferents) mediated via the dorsal column system (Huffman and Krubitzer, 2001a,b) with response onset latencies between 5 and 10 ms (measured in response to radial and ulnar nerve stimulation Phillips et al.,

1971), an indirect activation of M1 units via area 3a would be feasible as well.

4.5.3 *Evidence for plasticity mechanism in M1*

Studies investigating the effects of synchronous (associative) or asynchronous (non-associative) motor point or peripheral nerve stimulation on motor-cortical excitability and reorganisation argue that changes occur in M1 for a number of reasons: Ridding et al. (2000) did not find any changes in spinal motor neuron excitability indicated by a lack of modulation of the F-wave. Furthermore, changes in MEPs elicited by TMS but not by TES (which is considered to activate PTNs directly) led to changes after peripheral nerve stimulation (Schabrun and Ridding, 2007). Furthermore, intracortical phenomena like intracortical facilitations increased in M1 after the intervention (Pyndt and Ridding, 2004). Also in the present study, changes in response to synchronous and asynchronous median and ulnar nerve stimulation occur in M1. Identified and unidentified neurons in M1 change their firing in response to single-site stimulation after the intervention.

M1 is however not the only possible site where changes induced by (paired) repetitive nerve stimulation might exist. Experiments studying synchronous (associative) tactile stimulation of two digits find an increase in somatosensory receptive field (RF) extent, overlap and somatosensory excitability (Godde et al., 1996; Vidyasagar et al., 2014). Within S1 these changes might occur depending on the sensory modality in area 1, 2, 3a or 3b.

Plastic changes might arise on any stage of the aforementioned dorsal column system. Changes might take place in VPL (Butler et al., 1992) before being relayed to M1. Another site of plasticity would be the cuneate nucleus. Witham and Baker (2011) for example did find changes in neurons recorded from the cuneate nucleus after conditioning experiments. Although Witham and Baker

(2011) did not directly study effects induced by plasticity, interactions of different stimuli sweep-by-sweep, stressed the *modifiable* nature of the cuneate nucleus.

4.5.4 *M1 neurons with inputs arising from both median and ulnar nerve mediate plastic changes induced by the intervention*

Dividing the M1 single unit responses depending on its activation by EDC, median and ulnar nerve, revealed that neurons which receive inputs from both median and ulnar nerve (stimulated during the intervention) exclusively mediate the population response differences (Figure 4.12A, 4.12B, 4.13A, and 4.13B). This finding provides important evidence that the plasticity may be occurring in the sensorimotor cortex, possibly at the last order synapse onto the recorded cell (because only cells which have convergent input show the plasticity effects).

Therefore, a M1 neuron needs to receive inputs from all relevant (during the intervention stimulated) muscles or nerves to participate in the population changes as implied by the population difference profile (Figure 4.8A and 4.8B). Although this is the case for the population response differences after both (synchronous and asynchronous median and ulnar nerve stimulation) interventions, this constraint is slightly more obvious after the synchronous (associative) stimulation (cf. some effects of cells activated by ulnar nerve stimulation only after the asynchronous intervention, Figure 4.13B, bottom row). Considering the nature of stimulation patterns applied during the asynchronous (non-associative) intervention, in which stimuli were randomly applied to either median or ulnar nerve, it may be concluded that both of these inputs to M1 neurons are crucial to mediate the plastic changes (sustained suppression after the intervention, see e.g. Figure 4.12A, top and middle row), but that the timing between peripheral stimuli (which was randomised in this condition) did not play an important role (see Figure 4.14B and 4.15B). Furthermore, parts of these

changes might be mediated by neurons which receive input to either median or ulnar nerve (Figure 4.15B).

After the synchronous (associative) intervention however, the timing of the concurrent median and ulnar nerve inputs to M1 neurons was crucial (Figure 4.14A). Interestingly, the response population difference profile (mediated by neurons which were activated by both median and ulnar nerve stimuli) after the associative intervention possessed a characteristic time profile for responses elicited by both relevant peripheral stimulation sites (Figure 4.12A and 4.13A). This consisted of a significant suppression at 20 ms followed by a facilitation or rebound between 30 and 40 ms post stimulation onset for both individual median and ulnar nerve stimulation evoked responses (Figure 4.14A and 4.15A). Especially the significant suppression at approximately 20 ms was consistent in response to both individual median and ulnar nerve stimulation and was influenced by the timing of afferent inputs which led to firing of median nerve evoked M1 discharges (see Figure 4.14A, bottom row).

What could be the functional significance of this suppression at approximately 20 ms after individual median and ulnar nerve stimulation induced by the synchronous (associative) intervention?

There are a number of studies which investigated the somatosensory evoked potentials (SEPs) in the frontal and sensorimotor cortex in response to peripheral nerve stimulation (Allison et al., 1991; Barba et al., 2008; Cebolla and Cheron, 2015; Cebolla et al., 2011). Allison et al. (1991) did make a direct comparison between SEPs in the human and non-human primate. Several studies debate the origin of early SEPs (Allison et al., 1991; Baumgartner et al., 2010; Cebolla and Cheron, 2015) which might be within M1 or S1. One characteristic feature of the SEPs is an early response with a latency of 10 ms in monkeys. This response is characterised by a positive deflection (P10) in the precentral gyrus (M1), which decreases in amplitude towards the central sulcus (Papazachariadis

et al., 2013), before it becomes a negative deflection (N10) in the postcentral gyrus (Allison et al., 1991; Papazachariadis et al., 2013). More interestingly, in monkeys' M1 there is a negative deflection at approximately 20 ms (N20) after median nerve stimulation (Allison et al., 1991). This component has directly been linked to the human N30 component which is part of the P20-N30 complex (Allison et al., 1991; Cebolla and Cheron, 2015). This component is of interest for a number of reasons. The human N30 is decreased in patients with Parkinson's disease (Cheron et al., 1994; Rossini et al., 1993). In contrast, the human N30 is increased in patients with dystonia (Kanovsky et al., 1997; Reilly et al., 1992). N30 increases after motor training with the thumb or middle three fingers (Andrew et al., 2015; Dancey et al., 2014).

Although the generator and physiological mechanism underlying the N30 SEP component is not very well known (Barba et al., 2008; Cebolla and Cheron, 2015), it has been hypothesised that the N30 might reflect somatosensory integration (Cebolla and Cheron, 2015; Haavik and Murphy, 2011).

Using event related synchronisation (ERS) and inter-trial coherence (ITC) in the beta-gamma frequency range in human EEG experiments made it possible to identify possible sources of the N30 component (Cebolla and Cheron, 2015; Cebolla et al., 2011). These sources were in Brodmann area (BA) 4 and 6. Therefore a possible source for the N30 component might be in the primary motor cortex (M1).

Witham et al. (2007) showed that there exists an oscillatory coupling in the beta frequency range between M1 and S1. This coupling might play an important role in the sensorimotor integration of proprioceptive and cutaneous signals (Witham et al., 2007).

Invasive recordings in human subjects did reveal that there are somatosensory inputs from the hand projection from S1 to M1 in its medial portion (Balzamo et al., 2004). These were responding to median nerve stimulation with similar

latencies compatible with the aforementioned P20-N30 complex. Therefore, this might be another indication that the N30 SEP component might be a marker of sensorimotor integration.

Considering the analogy between the N30 component in humans and N20 in monkeys (Allison et al., 1991), one might hypothesise that the M1 population response suppression at 20 ms evoked by individual median and ulnar nerve stimulation after the synchronous (associative) intervention, reflects a change of the sensorimotor integration (of median and ulnar nerve inputs) within M1.

Indeed, there is evidence that the stimulation evoked neuronal population firing might underly some high-frequency oscillatory (HFO) components of SEPs (Baker et al., 2003b; Curio, 2000; Papazachariadis et al., 2013).

Considering that the pathophysiology of (focal hand) dystonia has been hypothesised to be linked to maladaptive (co-)activation of sensory afferents which lead to aberrant motor-cortical plasticity (Quartarone et al., 2008, 2014; Schabrun et al., 2009; Tinazzi et al., 2000), and assuming that repetitive synchronous (associative) stimulation of (two or more) peripheral nerves *artificially* induces a similar condition qualitatively (Schabrun and Ridding, 2007; Schabrun et al., 2009), it is reasonable that a spatial-temporal component in the evoked population response in identified and unidentified M1 neurons linked to sensorimotor integration is affected.

In fact, changes in the motor-cortical excitability indicated by changes in the excitability of MEPs after prolonged synchronous (associative) motor point or peripheral nerve stimulation (McDonnell and Ridding, 2006; Ridding and Uy, 2003; Ridding et al., 2000; Schabrun and Ridding, 2007), might reflect the (excitability) changes of the neuronal M1 population (receiving inputs from the relevant afferents) caused by altered sensorimotor integration. This modified sensorimotor integration might also become apparent in studies on dystonia patients reporting an increased N30 (analog to the monkeys' N20)

SEP component (Kanovsky et al., 1997; Reilly et al., 1992), increased MEPs (Schabrun and Ridding, 2007; Schabrun et al., 2009), increased motor-cortical representation of muscles (Schabrun et al., 2009), and increased overlap of cortical representations of the relevant (during the intervention stimulated) muscles or peripheral nerves (Schabrun et al., 2009).

Schabrun et al. (2012) investigated SEPs and MEPs after repetitive peripheral electrical stimulation (PES) and found a co-modulation in excitability between S1 and M1. The authors of this study concluded that excitability changes in M1 might be mediated by cortico-cortical connections from S1 to M1. Considering that changes in this study occurred in an earlier SEP component would make a relayed processing of (e.g. weighted) peripheral inputs from S1 to M1 plausible.

4.5.5 *Underlying mechanism of M1 population response differences after synchronous and asynchronous peripheral nerve stimulation*

The sustained suppression after the asynchronous (non-associative) intervention is predominantly mediated by M1 neurons which receive afferent inputs from both median and ulnar nerve (Figure 4.12B and 4.13B). This effect does not consistently depend on the timing of those inputs (Figure 4.14B and 4.15B) which is not surprising taking the asynchronous (randomised) nature of those inputs into account.

The synchronous (associative) intervention however is characterised by consistent timed co-activation of the median and ulnar nerves. Since the changes observed on the M1 population level are exclusively mediated by M1 neurons receiving inputs from both of these peripheral afferents, it is conceivable that the timing of those inputs does matter. Investigating the timing of median and ulnar nerve evoked responses in M1 neurons, displayed no differences for individual ulnar nerve evoked responses (Figure 4.15A). However, median nerve evoked M1 response differences depend on the timing of the peripheral inputs (Figure 4.14A).

Cells in M1 which received inputs from median first and subsequently from ulnar nerve led to a facilitation of the suppression at approximately 20 ms post stimulation onset. This increase of the evoked population response is compatible with long term potentiation (Malenka and Bear, 2004). Neurons in M1 which were activated by ulnar first and subsequently by median nerve led to a suppression of the individual median-evoked population differences response at approximately 20 ms. This decrease of the evoked population response is compatible with long term depression (Malenka and Bear, 2004).

A number of studies using synchronous (associative) motor point or peripheral nerve stimulation did hypothesise that due to the importance of concurrent inputs for synaptic modifications (Hebb, 1949), a LTP-like mechanism might underly the changes seen in motor-cortical excitability in human subjects (McDonnell and Ridding, 2006; Pyndt and Ridding, 2004; Ridding and Uy, 2003; Schabrun and Ridding, 2007). Butefisch et al. (2000) for example found that a GABA type A receptor agonist and NMDA receptor antagonist substantially reduced use-dependent plasticity. The authors of this study stressed the similarity between the use-dependent plasticity and LTP.

The dependence on concurrent peripheral inputs of M1 units (median and ulnar nerve), the time course of changes observed on the M1 population level, and the dependence on the timing of peripheral nerve inputs to these neurons, might provide evidence for a STDP mechanism underlying the changes induced by the synchronous (associative) median and ulnar nerve intervention in the present study.

With respect to the initially mentioned hypothesis and assumptions (see Section 4.2), this study revealed a number of facts regarding changes of identified and unidentified neurons in M1 after prolonged peripheral nerve stimulation. The initial assumption that modulation in response to PNS should occur in M1 neurons can be verified. Furthermore, the current study provides direct evidence that

modulation of the neuronal activity is present in units with convergent inputs from relevant peripheral stimuli. Thus, M1 neurons must receive activation by the median and ulnar nerves to be able to modulate changes as seen after the synchronous (median and ulnar nerve) intervention. PTNs showed modified evoked cell discharges after both interventions. Therefore, it can be concluded that PTNs are also affected by the plasticity-inducing interventions. Finally, the present study provides evidence for a STDP-like mechanism responsible for the changes in the evoked M1 responses after the synchronous intervention.

4.5.6 *Studying invasive and non-invasive stimulation techniques in the non-human primate*

The present study was designed to investigate single stimulation-site induced responses in identified and unidentified neurons in M1 before and after one hour of synchronous (associative) and asynchronous (non-associative) peripheral nerve stimulation.

Changes in stimulation site evoked neuronal discharges could be identified in response to stimulation of the relevant (during the intervention stimulated) in contrast to the control (EDC) stimulation site. This is in line with previous studies implying that projections to stimulated muscles are particularly affected (McDonnell and Ridding, 2006; Ridding and Uy, 2003; Schabrun and Ridding, 2007; Schabrun et al., 2009).

Although studies on human subjects revealed a number of plastic changes in the sensorimotor cortex in response to non-invasive interventions relying on synchronous or asynchronous stimulation of motor points or peripheral nerves, the exact underlying mechanism leading to those plastic changes remains elusive despite some indirect evidence (e.g. for LTP-like mechanisms and neuropharmacological evidence) (Schabrun and Ridding, 2007). Furthermore, some plasticity-inducing interventions like the pairing of peripheral and cortical

stimuli (Stefan et al., 2000), lack focal specificity for the peripheral (e.g. electrical stimulation applied via surface electrodes leading to mixed nerve activation) and cortical (e.g. TMS induced current) stimulus.

Applying the electrical stimulation via implanted wires and nerve cuffs in the present study enabled increased precision regarding focal motor point and peripheral nerve stimulation.

A number of studies used invasive electrophysiological recordings performed in the monkey's sensorimotor cortex to study peripheral nerve stimulation evoked firing and potentials (Allison et al., 1991; Baker et al., 2003b; Papazachariadis et al., 2013; Raymond et al., 2000). The study of (non-invasively induced) neuroplasticity in the non-human primate enables the use of a range of additional techniques studying plasticity underpinning mechanisms.

The non-human primate has been used to study the motor-cortical representation after paired associative stimulation (PAS) of the median nerve and TMS induced motor cortex activation (Amaya et al., 2010). The authors found similar effects like increased MEPs and changes in CoGs to those reported in studies on human subjects (Stefan et al., 2000). Papazachariadis et al. (2013) investigated the effect of repetitive median nerve stimulation on multi unit (MU) activity and SEPs and found adaptation effects.

The non-human primate model is an excellent model to study non-invasively induced neuroplasticity because of its neuroanatomical similarities to humans (e.g. increase in size of neocortex). Furthermore, the monkey possesses similarities in the motor system for example with respect to the existence of direct corticomotoneuronal (CM) connections (Lemon, 2008).

In addition, the monkey can be trained to perform skilled motor tasks (see Section 2) involving independent dexterous finger movements (Schieber, 1991).

Non-invasively induced neuroplasticity can be assessed in non-human primates by means of non-invasive stimulation techniques (e.g. TMS, Amaya et al., 2010) or by a combination of non-invasive and invasive ones (e.g. simultaneous TMS and single neuron recordings, cf. Mueller et al., 2014).

The present study shed light on the underlying mechanism, namely the change in cellular discharges of identified and unidentified M1 neurons, affected by prolonged peripheral nerve stimulation. Changes in PTNs stress the relevance of the primate model to be able to understand changes in MEPs as seen in human subjects after prolonged non-invasive peripheral nerve stimulation.

These and other neurophysiological measurements can help us understanding the underlying neuroplasticity mechanisms induced by a variety of non-invasive stimulation techniques. This might lead to improved plasticity inducing stimulation paradigms defining *optimal* parameter and novel therapeutic interventions.

Chapter 5. Structural changes induced by long-term median and ulnar nerve stimulation.

5.1. Summary and key findings

- Plastic changes can be induced by associative peripheral nerve stimulation (Ridding et al., 2001) and structural plastic modifications affect excitatory (glutamatergic) and inhibitory (GABAergic) neuronal populations (Flores and Mendez, 2014).
- Parvalbumin-positive neurons and perineuronal nets (PNNs) are modified by neuroplasticity (Donato et al., 2015).
- In the present study, I investigated the effects of long-term associative median and ulnar stimulation on inducing structural changes in sensorimotor cortices of two macaque monkeys.
- I used a novel automated cell and perineuronal net detection algorithm to quantify laminar specific changes as well as colocalisation.
- I found differential laminar specific expression of parvalbumin and Wisteria floribunda agglutinin (perineuronal nets): The expression was significantly reduced in layer I, II and VI. In layer III-V, PNNs were significantly increased whereas parvalbumin expression was divergently changed on the stimulated (contralateral) hemisphere.
- Colocalisation was significantly decreased on the stimulated hemisphere predominantly in layer III-V.
- I conclude that these alterations in GABAergic interneurons, perineuronal nets and a reduction in colocalisation in layer III-V reflect structural plastic changes induced by PNS.

5.2. Introduction

Activity dependent plasticity induced by non-invasive stimulation can lead to several functional and physiological manifestations (see Chapter 3 and 4).

In addition to physiological modifications, plasticity has been associated with structural differences in excitatory (Cichon and Gan, 2015; Holtmaat and Svoboda, 2009; Honkura et al., 2008; Johansen et al., 2014; Matus, 2000; Xu et al., 2009; Yang et al., 2009), and inhibitory neuronal populations (Chu et al., 2015; Flores and Mendez, 2014; Fritschy et al., 2012; Kullmann et al., 2012; Schuemann et al., 2013; Scimemi, 2014).

These structural changes are characterised by changes in neuronal distributions (Balmer et al., 2009; Benali et al., 2008; Chen and Nedivi, 2013; Donato et al., 2015; Flores and Mendez, 2014; Han et al., 2013; King et al., 2014; Mix et al., 2015; Miyata et al., 2012; Ueno et al., 2013; Urakawa et al., 2013), morphological synapse structures (Caillard et al., 2000; Caroni et al., 2012; Cichon and Gan, 2015; Matsuzaki et al., 2004; Mayford et al., 2012; Spruston, 2008; Xu et al., 2009; Yuste and Bonhoeffer, 2001), dendritic spines (Borges et al., 2010; Brudvig and Cain, 2015; Dias et al., 2015; Joachimsthaler et al., 2015; Reiner and Dunaevsky, 2015; Wiegert and Oertner, 2015), receptors trafficking (Constals et al., 2015; Zhang et al., 2015), neuronal transporter (Diamond et al., 1998; Larsen et al., 2014; Luscher et al., 1998, 2000; Park et al., 2014; Pulido et al., 2015) and diversification in the microtubule stabilising protein tau (Deak, 2014; Levenga et al., 2013; Yang et al., 2015)

During the last decade, the role of inhibitory GABAergic circuits in experience and activity-dependent plasticity has been elucidated intensively (Flores and Mendez, 2014; Kullmann et al., 2012). A subtype of those GABAergic interneurons, which express parvalbumin (Parv), a calcium-binding protein, is especially involved and affected by neural plasticity (Bartley et al., 2008; Donato et al., 2013; Pieraut

et al., 2014; Tang et al., 2014). Parvalbumin-containing neurons are implicated in learning and memory (Allen and Monyer, 2013) by tuning the spike-timing of pyramidal cells in local circuits (Orduz et al., 2013). Another structure linked to neuronal plasticity is the extracellular matrix complex called perineuronal nets (Balmer et al., 2009; Celio and Blumcke, 1994; Mix et al., 2015; Miyata et al., 2012; Romberg et al., 2013; Wang and Fawcett, 2012). Perineuronal nets (PNNs) are associated with the lack of cortical (Nowicka et al., 2009; Sonntag et al., 2015; Vivo et al., 2013) and spinal (Burnside and Bradbury, 2014; Miao et al., 2014) plasticity. PNNs are considered to play an important part in axonal growth (Rhodes and Fawcett, 2004), regeneration after neuronal injuries (Silver and Miller, 2004) and regulating excitation and inhibition (Benali et al., 2008; Hensch, 2005).

Plastic modifications in parvalbumin-positive interneurons and perineuronal nets are correlated to a number of neurological conditions including epilepsy (Huusko et al., 2015; McRae and Porter, 2012) and schizophrenia (Bitanihirwe and Woo, 2014; Cabungcal et al., 2014).

Yamada et al. (2015) have recently shown that the vast majority of parvalbumin-positive basket cells are surrounded by PNNs. The absence of PNNs affects parvalbumin expression (Yamada et al., 2015) indicating a substantial coupling between these entities (Balmer et al., 2009; Donato et al., 2015; Mix et al., 2015).

In the somatosensory neocortex, parvalbumin-positive interneurons are predominantly located in the internal granular layer (Defelipe and Jones, 1991). This location (layer IV) has further been characterised by morphological changes in dendritic spines and parvalbumin-positive interneurons (Miquelajauregui et al., 2015). The effects of neuroplasticity on these parvalbumin-positive interneurons and PNNs in the primary motor cortex (M1) however has not been studied extensively, although other structural differences like diversification of dendritic

spines in response to motor learning have been reported (Xu et al., 2009; Yang et al., 2009).

In the present study, I ask whether long-term associative peripheral stimulation of the median and ulnar nerve can induce structural changes in the sensorimotor cortex. Specifically, I investigated discrepancies in parvalbumin (Parv)-positive and perineuronal net (PNN) distributions in the primary motor and somatosensory cortex. I hypothesis that long term application of synchronous PNS should lead to structural changes in both motor- and somatosensory cortices. These should be quantifiable in changes in laminar-specific distributions, cell and perineuronal net densities on the contralateral (stimulated hemisphere) as opposed to the ipsilateral sensorimotor cortices. Finally, the colocalisation of Parvalbumin-positive interneurons and perineuronal nets (PNNs) should be altered on the stimulated hemisphere.

I developed an automated Parv-positive cell and PNN detection algorithm to quantify laminar specific changes as well as colocalisation of cells and nets.

I found differential laminar specific expression of parvalbumin and Wisteria floribunda agglutinin (perineuronal nets): The expression was significantly reduced in layer I, II and VI. In layer III-V, PNNs were significantly increased whereas Parv expression was divergently changed on the stimulated (contralateral) hemisphere. Colocalisation was significantly decreased on the stimulated hemisphere predominantly in layer III-V.

I conclude that these alterations in GABAergic interneurons, perineuronal nets and a reduction in colocalisation in layer III-V reflect structural plastic changes induced by PNS.

5.3. Materials and methods

5.3.1 *Subjects and surgical preparation*

Two adult female macaque monkeys (age between four and six years, weight between five and six kilograms) were implanted with flexible bipolar median and ulnar nerve (supplying forearm flexors and intrinsic hand muscles including muscles of all digits) cuffs. The monkeys received a $30\,\%$ carbon fiber reinforced PEEK (Engineering & Design Plastics Ltd, Catalogue Number: Kentron CA30 PEEK) headpiece enabling atraumatic head fixation (Baker et al., 1999; Lemon and Prochazka, 1984) and providing the base to attach a custom-made hat including an electronic wearable device used for recording and delivering stimulation.

All surgical interventions were performed under aseptic conditions and under deep general anesthesia. General anesthesia was induced by $10\,\mathrm{mg\,kg^{-1}}$ ketamine (intramuscular) and maintained with $2.0\text{-}3.5\,\%$ sevoflurane in $100\,\%$ O_2. Analgesic care was provided by intravenous infusion of alfentanil ($0.025\,\mathrm{mg\,kg^{-1}\,h^{-1}}$) throughout the surgical procedure.

After the surgery, the monkeys received treatment with antibiotics ($6\,\mathrm{mg\,kg^{-1}}$ Ceftiofur, Pfizer, Catalogue Number: MSDS 087 and $8\,\mathrm{mg\,kg^{-1}}$ Dexamethasone, Hameln Pharmaceuticals Ltd) and analgesics ($6\,\mathrm{mg\,kg^{-1}}$ Meloxicam, Boehringer Ingelheim Limited). All surgical and experimental procedures were carried out under authority of licenses issued by the UK Home Office under the Animals (Scientific Procedures) Act 1986 and were approved by the Local Research Ethics Committee of Newcastle University.

An electronic wearable device (Brown et al., 2012) was positioned within a custom-made hat (Figure 5.1A-5.1B) fabricated from Acetal Copolymer (RS Components Ltd, Catalogue Number: 680–498).

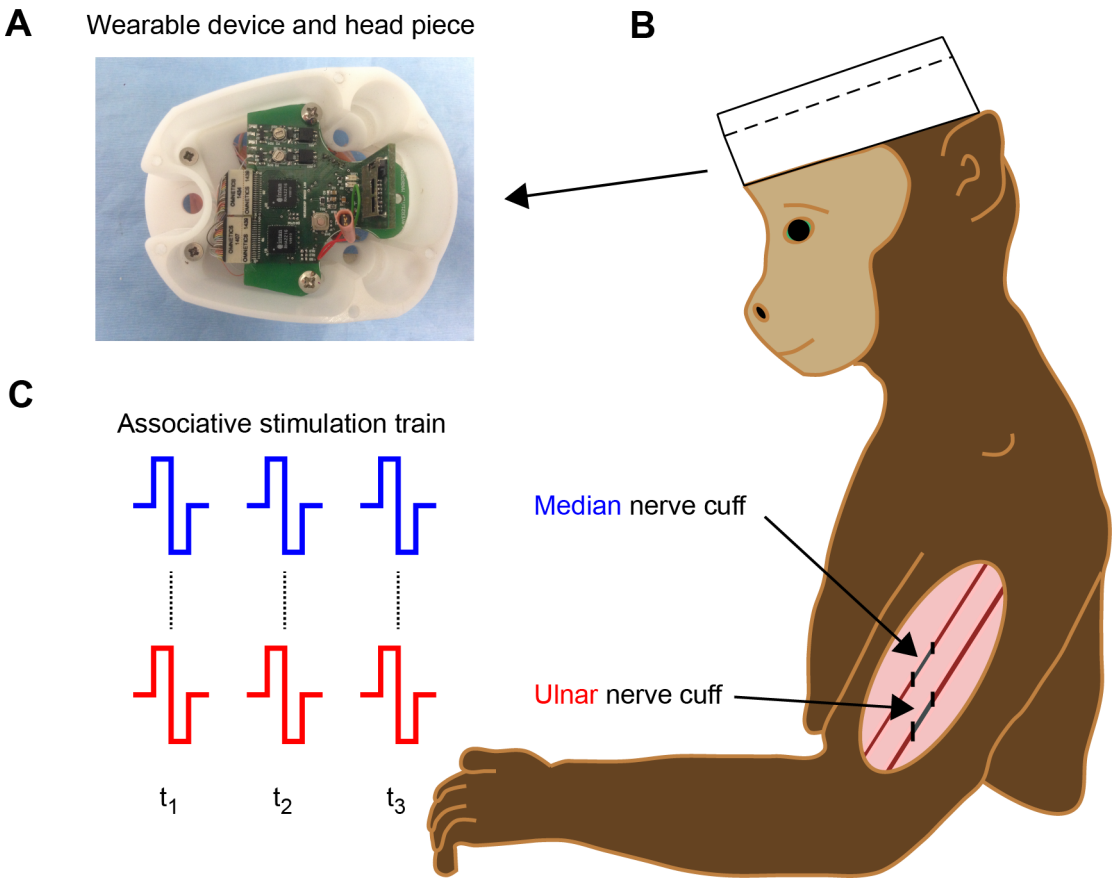

Figure 5.1: *Experimental design and wearable device stimulation.* (A) The wearable device was positioned within a custom-made head piece. This electronic device is capable of acquiring data as well as delivering stimuli. (B) Monkeys were implanted with bipolar median and ulnar nerve cuffs. (C) Associative stimulation trains consisted of biphasic stimuli (0.2 ms pulse-width) were applied to median and ulnar nerve with a frequency of 10 Hz.

5.3.2 *Stimulation procedure*

The miniature circuit of the electronic wearable device (Figure 5.1A) is capable of delivering paired associative stimulation of the median and ulnar nerve (Figure 5.1B) while the monkeys were in their homecages. The stimulation consisted of approximately 55 min of paired associative median and ulnar nerve stimulation. The inter-stimulus interval was chosen from an uniform distribution between 90 and 110 ms. The stimulation intensity was adjusted to elicit a twitch and therefore just above the resting motor threshold (RMT). To avoid charge

imbalance (Hofer et al., 2002; Woodcock et al., 1999), a biphasic pulse with a pulse width of $0.2\,\mathrm{ms}$ was used for stimulation (Figure 5.1C). Once every $55\,\mathrm{min}$, a short assessment stimulation period for $5\,\mathrm{min}$ was performed. This period consisted of a series of single pulse stimulation of median and ulnar nerve alone, followed by a paired stimulus of both. These were presented alternatingly at an average frequency of $10\,\mathrm{Hz} \pm 1\,\mathrm{Hz}$. The associative stimulation was applied for $7\,\mathrm{d}$, each day for approximately $14\,\mathrm{h}$ to induce long-term changes. The stimulation was only interrupted for one hour at a consistent time each day to acquire behavioural assessments. Usually the monkeys woke up around 6 am (when the light in the animal unit was turned on), the stimulation was turned on at 7 am (the wearable electronic device possesses timer functionality), and the stimulation was interrupted for training sessions usually around 3 pm. The stimulation, was switched off at 10 pm and monkeys would fall asleep between 10 and 11 pm (the light intensity of the animal facilities does gradually decrease between these times).

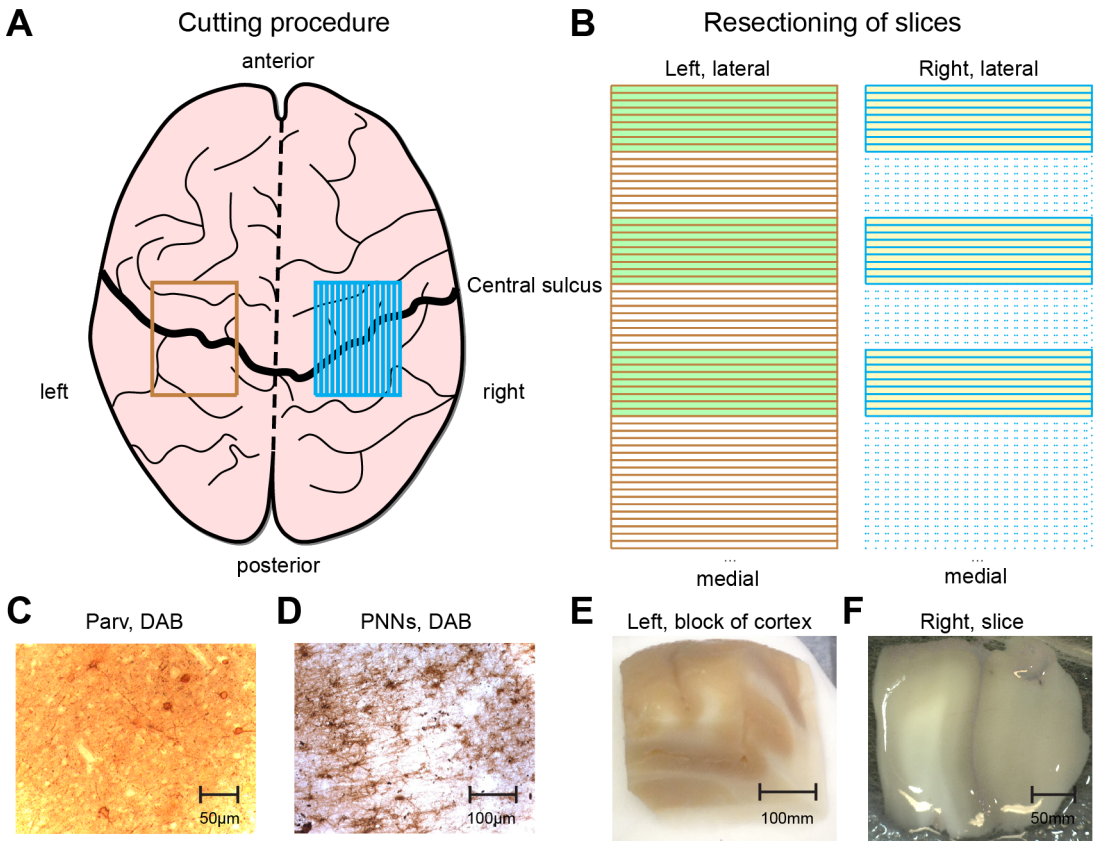

Figure 5.2: *Preparation of slices for immunohistochemical processing.* (A) Sagittal slices with a thickness of 450 µm (blue rectangles) were dissected from the right hemisphere (contralateral to the stimulation) and a block of cortex (brown rectangle) was taken from the left hemisphere (ipsilateral to the stimulation side). These samples contained both pre- and post- central gyri for monkey S. For monkey U, a block of cortex was taken from either hemispheres. (B) Both the block of cortex and the 450 µm thick slices were resectioned to 50 µm section for monkey S and 60 µm for monkey U for further immunohistochemical processing. For every slice of the contralateral hemisphere (yellow sections) corresponding sections of the ipsilateral side (green sections) were chosen and processed under the same conditions. (C) Visualisation of parvalbumin Parv-positive neurons using DAB. (D) Visualisation of perineuronal nets (PNNs). (E) Example block of cortex before resectioning. (F) Example slice before resectioning.

5.3.3 *Immunohistochemical processing*

Immunohistochemical Preparation and Cutting Procedure

After the seven days of stimulation, the monkeys were deeply anesthetized and a trans-cardiac perfusion was performed using oxygenated (95 % O_2 / 5 % CO_2) ice-cold artificial cerebrospinal fluid (ACSF) consisting of (in nmol) 3 KCL, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose and 252 sucrose for monkey S. Monkey U, was perfused using 4 % paraformaldehyde (PFA). Sagittal slices with a thickness of 450 µm (Figure 5.2F) were taken from the contralateral side to the stimulation and a block of cortical tissue (Figure 5.2E) was taken from the ipsilateral side (for detailed cutting procedure see Figure 5.2A and 5.2B). Both the block of cortical tissue and the slices were immediately transferred to 4 % PFA. For monkey U, blocks of cortical tissue were taken from both the ipsilateral and contralateral side after the 7 d stimulation and also transferred to 4 % PFA. After the tissue was structurally fixed, slices were cryoprotected using 30 % sucrose (Ebbesson and Cheek, 1988; Mi et al., 2000; Rosene and Mesulam, 1978) in tris-buffered saline (TBS) (150 µmol NaCl, Sigma Aldrich, Catalogue Number S9888; and 38 µmol $NH_2C(CH_2OH)_3 \cdot HCl$, Sigma Aldrich, Catalogue Number T5941; and 11 µmol $NH_2C(CH_2OH)_3$, Sigma Aldrich, Catalogue Number T1503). The pH of the TBS was adjusted to 7.6 by adding 38 % HCl (Sigma Aldrich, Catalogue Number H1758). After cryoprotection, blocks of cortical tissue were resectioned to a thickness of 60 µm at $-20\,^{\circ}C$ using a freezing microtome (Shandon, Catalogue Number: 0200–001 with Shandon cooling unit AS 207) and stored at 4 °C in TBS no longer than 7 d.

Optimizing Antibody, Lectin Concentration and Mounting Procedure

To determine the optimal antibody (Parv) and biotinylated lectin (PNN) concentration, three different concentrations were tested: 1 in 1000, 1 in 2000

and 1 in 5000. These concentration tests were made on M1 and S1 slices using a 3,3'−−diaminobenzidine (DAB) reaction (Figure 5.2C and 5.2D). After resectioning, slices were washed 3 times for 10 min in TBS with 0.3 % hydrogen peroxide (H_2O_2, Sigma Aldrich, Catalogue Number 516813). Before applying the antibody and lectin in the specified concentration, the samples were washed another 3 times for 10 min in TBS. The primary Anti-Parv (monoclonal antibody produced in mouse, Sigma Aldrich, Catalogue Number: P3088) antibody was applied together with 3 % normal goat serum (Sigma Aldrich, Catalogue Number: G9023) to prevent non-specific staining. These were diluted in TBS with 0.3 % Triton (Triton x100, Sigma Aldrich, Catalogue Number: 93443) to increase membranous permeability. On other slices, the same steps except for the application of the primary antibody were carried out. To detect PNNs, slices were incubated with biotinylated lectin from Wisteria floribunda (WFA, Sigma Aldrich, Catalogue Number: L1516) together with 3 % normal goat serum in TBS with Triton. Samples were incubated for at least 24 h in a cold room at 4 °C and under constant motion (Stuart Scientific Gyro rocker, Catalogue Number: Z316520).

The following day, slices were exposed to biotinylated anti-mouse immunoglobulin G (IgG) antibody (raised in goat, Vector Laboratories Ltd., Catalogue Number: BA-9200) in TBS with 0.3 % Triton with a concentration of 1 in 200 for 2 h. After the samples were cleaned for 3 times 10 min with TBS, horseradish peroxidase (HRP) Streptavidin (Vector Laboratories Ltd., Catalogue Number: SA-5004) was applied in TBS with Triton for 1 h with a concentration of 1 in 200. The HRP is used to amplify the sensitivity of staining (Adams, 1992). After washing slices 3 times 10 minutes with TBS, the DAB reaction was initiated with DAB (3,3'-tetrahydrochloride, $(NH_2)_2C_6H_3C_6H_3(NH_2)_2 \cdot 4\,HCl$, Sigma Aldrich, Catalogue Number: D5905) diluted in distilled water (dH_2O). The strength of the DAB staining was assessed using a stereomicroscope (Zeiss Stemi 2000C, Fisher Scientific, Catalogue Number: 12-070-281). This took usually about 5 min. Finally, slices were washed for 10 min using TBS before they were positioned on subbed

slides (Fisher Scientific, Catalogue Number: 12-550-15).

In the following, three different approaches for mounting the slices were tested: Histomount (Fisher Scientific, Catalogue Number: 12825996) with dehydrated slices (for exact procedure see Appendix B.1), $70\,\%$ glycerol in TBS and using Fluoroshield mounting medium (with DAPI, Abcam, Catalogue Number: ab104139). The last and the first method turned out to be the best in terms of clarity and durability.

Fluorescent staining of parvalbumin-positive interneurons, perineuronal nets and cell nuclei

Since PNNs and Parv-positive interneurons are often co-localised (Brauer et al., 1993; Foster et al., 2014; Karetko and Skangiel-Kramska, 2009; Nowicka et al., 2009; Rossier et al., 2014; Vidal et al., 2006), I performed a triple-fluorescent staining for these two entities along with nuclei reference-staining using $4'$,$6-$diamidino$-2-$phenylindole (DAPI). Initially, slices were washed 3 times for $10\,\mathrm{min}$ using TBS to clean the slices from salts, sugars and proteins. Afterwards, the primary anti-Parv antibody (monoclonal anti-Parv antibody, raised in mouse, Sigma Aldrich, Catalogue Number: G9023), the biotinylated lectin from Wisteria floribunda (WFA, Sigma Aldrich, Catalogue Number: L1516) and normal goat serum (Sigma Aldrich, Catalogue Number: G9023) were diluted in TBS with $0.3\,\%$ Triton (Triton x100, Sigma Aldrich, Catalogue Number: 93443). Slices were incubated for at least $24\,\mathrm{h}$. The goat serum was used to prevent any unspecific goat antigen binding of the secondary antibody (which was raised in goat). The primary anti-Parv antibody binds specifically to the antigen of a subclass of GABAergic neurons: Interneurons containing the calcium-binding protein parvalbumin (see Figure 5.3D). The biotinylated Wisteria floribunda agglutinin binds to a group of extracellular matrix proteins, the chondroitin sulfate proteoglycan (CSPG). The perineuronal nets consist of these proteoglycans

which Wisteria floribunda (WFA) binds to (Bruckner et al., 1996; Deepa et al., 2006; Hartig et al., 1994). After washing samples 3 times for 10 min, the secondary antibody (goat anti-mouse IgG, Alexa Fluor 488, Abcam, Catalogue Number: ab150113) was applied with the Streptavidin with a fluorescent tag (Texas Red Streptavidin, Vector Laboratories, Catalogue Number: SA-5006) in TBS with 0.3 % Triton. The secondary anti-mouse antibody binds to the primary anti-Parv antibody (which was raised in mouse, Figure 5.3D). The Streptavidin possesses a high affinity to biotin due to formation of multiple hydrogen bounds and van der Waals interactions between the biotin and the protein (Weber et al., 1989). Therefore, the Streptavidin binds to the biotin part of the WFA (Diamandis and Christopoulos, 1991). After the samples were washed 3 times for 10 min, the slices were mounted on subbed slides (Fisher Scientific, Catalogue Number: 12–550–15) using mounting medium with DAPI (Abcam, Catalogue Number: ab104139). DAPI binds to the deoxyribonucleic acid (DNA) of the nucleus (Russell et al., 1975). In addition to endogenous molecules with fluorescent properties, it is possible to fuse particular exogenous fluorescent molecule with the complementary deoxyribonucleic acid (cDNA) of any target cell (Lippincott-Schwartz and Patterson, 2003; Stephens and Allan, 2003).

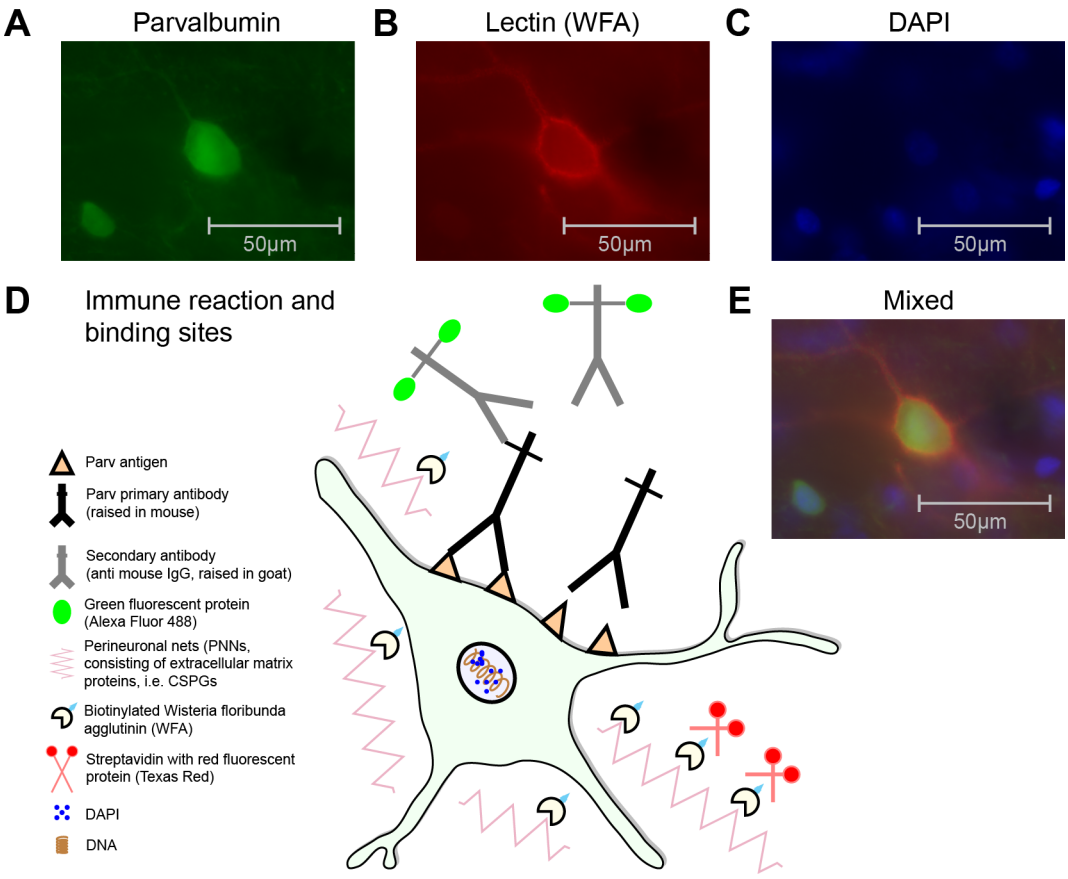

Figure 5.3: *Binding sites and target entities of antibodies, lectins and fluorescent molecules*. (A) Parvalbumin (Parv)–positive interneurons were tagged with a green fluorescent protein (GFP). (B) Perineuronal nets (PNNs) were targeted using biotinylated WFA. Texas Red conjugated streptavidin binds to the biotin. Therefore, PNNs appear red. (C) DAPI is staining cellular nuclei in blue. (D) Different binding sites for primary and secondary antibodies, lectins (WFA) and DAPI. (E) Multi-wavelength visualisation of Parv, PNNs and DAPI enables studying colocalisation. Images in A-C and E were taken with a 100 times magnification.

Using multiple fluorescent molecules with different excitation and emission wavelengths makes it possible to identify different molecular or cellular targets simultaneously (Nederlof et al., 1990; Stephens and Allan, 2003). In this case, a green fluorophore was conjugated with the secondary antibody (Figure 5.3D, anti-mouse IgG), a red with the streptavidin (Figure 5.3D, Texas Red) and DAPI possesses fluorescent properties by itself (Figure 5.3D). To increase the separability of these three different cellular entities of interest (i.e. the cellular targets these fluorophores are binding to: Parv, PNN, and DAPI), three distinct

fluorophores were chosen: Texas red (Lefevre et al., 1996), green fluorescent protein (GFP, Sumner and Kopelman, 2005; Zhu et al., 2014) and DAPI (Biancardi et al., 2013; Russell et al., 1975). The excitation wavelengths were (in nm) 595, 494, and 360 respectively. Thus, Parv appears green (Figure 5.3A), PNN red (Figure 5.3B), and DAPI blue (Figure 5.3C). The advantage of simultaneous fluorescent staining becomes immediately apparent when studying colocalisation of these entities (Figure 5.3D and 5.3E). To improve the signal to noise ratio of the staining and to remove artefacts caused by autofluorescent properties of lipids (Oliveira et al., 2010; Schnell et al., 1999), $0.01\,\%$ Sudan Black B (Sigma Aldrich, Catalogue Number: 199664) was applied in $70\,\%$ ethanol (Sigma Aldrich, Catalogue Number: 32221).

5.3.4 *Sampling of immunohistochemical processed cortical slices*

40 sagittal slices (20 monkey S and 20 monkey U; each containing 10 ipsilateral and 10 contralateral) were sampled using a fluorescent microscope (Axio Imager II, Zeiss, Catalogue Number: 490022). Image files were acquired with a $10\times$ magnification. This led to a resolution of $0.6498\,\mu\text{m pixel}^{-1}$. Images were sampled using the AxioVision (Zeiss, AxioVision 4.8) software. A multidimensional acquisition for PNN, Parv and DAPI was performed and merged to a single red, green and blue (RGB) matrix. The whole M1 / S1 slice was sampled using the *Mosaic* functionality of the AxioVision software. A focus and shading correction was applied to improve the quality of image data acquisition and to reduce artefacts. The resulting RGB matrix was saved in Zeiss AxioImager format as well as tagged image file format (TIFF) before defining each ROI for each sampled slice (cf. Section 5.3.5) and advanced image processing. All images were taken with the same settings for exposure and light.

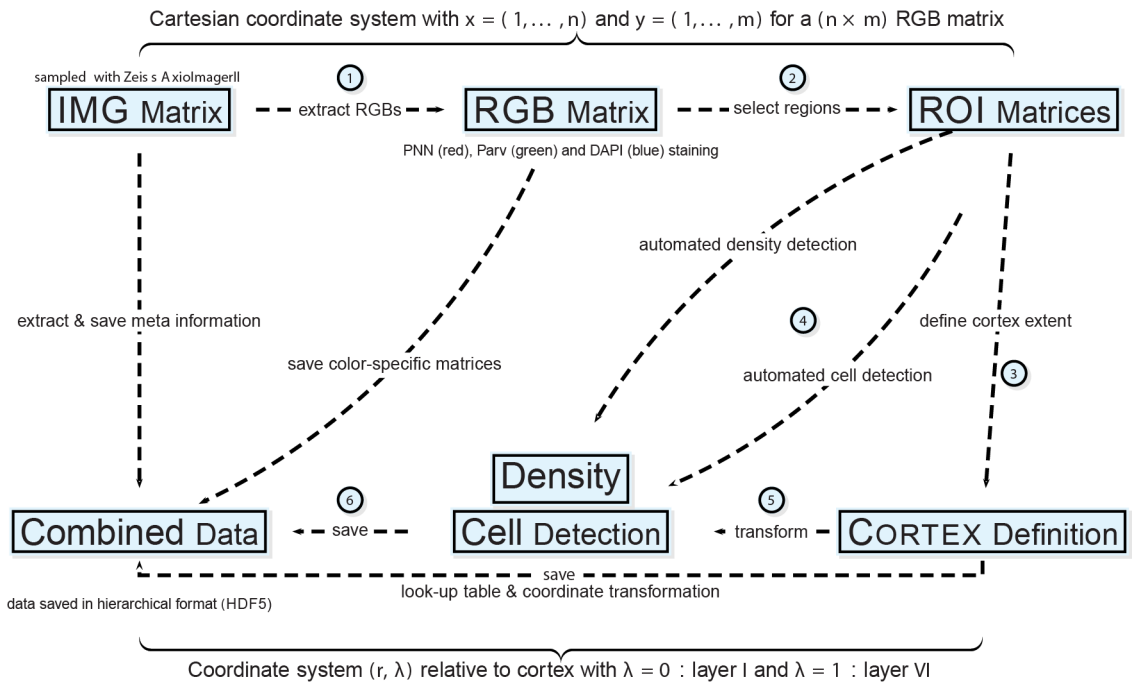

Figure 5.4: *Image processing and automated cell count.* The diagram illustrates the steps for image processing, determination of cell counts and density. The cortical M1 / S1 slices were sampled using a fluorescent microscope (Zeiss, Axio Imager II, Catalogue Number: 490022). The optimal excitation wavelength for PNN, Parv and DAPI were used (cf. Section 5.3.3) to capture colour-specific images of the corresponding stains. These were combined to one image matrix (IMG Matrix). Stain-specific information was extracted from each colour channel (1, RGB matrix). ROIs were selected by rotating and aligning sub-matrices (2, Figure 5.5). These ROIs were old M1, new M1, area 1 of S1, area 3a of S1 and area 3b of S1. Next, the cortical layer / laminar extend was defined by choosing an arbitrary number of characteristic points along layer I. A polynomial function was fitted through these points and the shift x_0, y_0 was defined (3, Figure 5.6). The luminance matrices of each ROI were determined, laminar specific densities and cell counts calculated (4, Figure 6.1). After these were calculated, each pixel and each cell were transformed from a Cartesian coordinate system ($x - y$ representation) to a new coordinate system ($r - \lambda$) relative to its laminar position (5, Figure 5.6E-5.6F). Finally, all data were saved using a hierarchical data format (6).

5.3.5 *Computer-assisted cell and perineuronal net count*

After capturing all slices (see Section 5.3.4) containing M1 and S1, images were further processed (Figure 5.4) for quantitative analysis. Advanced

image processing was performed using purpose-written Python (Rossum, 1995) scripts (Python, Version 2.7.9, `https://www.python.org`) and by including functionalities of the Python imaging library (PIL, Version 1.1.7, `http://www.pythonware.com/products/pil/`) and the scikit-image (Walt et al., 2014) library (Scikit-image, Version 0.11.3, `http://scikit-image.org`).

RGB matrices were separated by their colour channels thereby isolating information about PNN, Parv and DAPI, respectively (Figure 5.4, processing step number 1). Regions of interest (ROIs) were specified by choosing, rotating and aligning sub-matrices containing old M1, new M1, area 1 of S1, area 3a of S1 and area 3a of S1 (Figure 5.4, step 2). Next, the extent, shape, onset and offset (shift) of cortical laminar I to VI were specified by choosing characteristic data points (Figure 5.4, step 3). In the following, an automated Parv and DAPI cell and PNN count as well as density calculations were performed (Figure 5.4, step 4). Subsequently, all colour-specific luminance and cell count coordinates were transformed from a Cartesian $x - y$ representation to coordinates relative to laminar position (r, λ representation, Figure 5.4, step 5). Finally, all image data, meta information (for example subject name, date of processing) and automatically determined parameters were saved in a hierarchical data format (Hierarchical data format, `http://www.hdfgroup.org`) for optimal compression and accessibility (Figure 5.4, step 6).

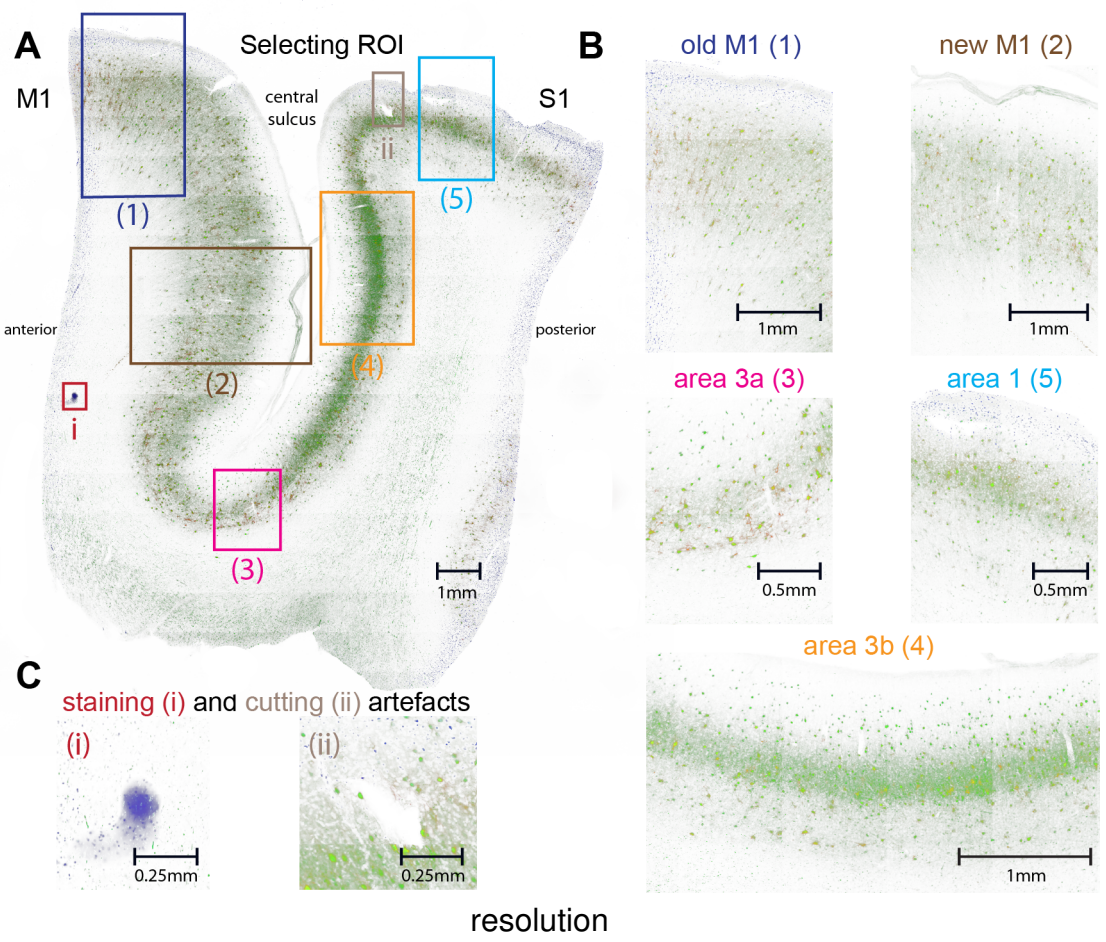

Figure 5.5: *Defining regions of interest.* (A) The whole sampled sagittal M1 / S1 slice showing the staining of PNN (red), Parv (green) and DAPI (blue). Regions of interest (ROI) were chosen in M1 (old and new M1; 1–2) and S1 (area 3a, 3b and 1; 3–5). (B) ROIs were then rotated, aligned and magnified. (C) Areas containing staining (i) or cutting / blade (ii) artefacts were avoided. Note: For illustration purposes the RGB values for black $(0, 0, 0)$ were replaced by white $(255, 255, 255)$.

Defining regions of interest (ROIs)

Regions of interest (ROIs) were defined by manually choosing rectangular sub-matrices of the overall RGB matrix (Figure 5.5A). This was accomplished by using the widget `RectangleSelector` of Matplotlib's (Matplotlib, Version 1.4.3, `http://matplotlib.org`) visualisation library (Hunter, 2007) in conjunction with the `rotate` method of Python imaging library (PIL). The primary motor cortex

(M1) was subdivided into a rostral part (old M1, Figure 5.5A-1, blue) and a caudal (new M1, Figure 5.5A-2, brown) part (Rathelot and Strick, 2009). The primary somatosensory cortex (S1) was partitioned (from caudal to rostral) into area 3a (Figure 5.5A-3, magenta), area 3b (Figure 5.5A-4, orange) and area 1 (Figure 5.5A-5, cyan). Area 3b was distinguished from old M1 by its prominent layer IV, old M1 by its lack of layer IV and a significant increase in cortical thickness, and area 3a as the transition zone between old M1 and area 3b by a weaker developed layer IV in comparison to area 3b (Krubitzer et al., 2004). The ROIs were then magnified for visual inspection (Figure 5.5B). Regions containing staining or blade / cutting artefacts were avoided when choosing the ROIs. In addition, fluorescent staining artefacts (Figure 5.5C) were removed from the RGB sub-matrices only if these were not on the cortical tissue itself. This improved the reliability of the automated staining process.

Determination of luminance and thresholding

Each colour specific matrix for PNN, Parv and DAPI was separated and the relative luminance L_r (Meyer and Greenberg, 1980) in the colorimetric space (see Figure 5.6B-5.6D) was calculated by

$$L_r = 0.2126 \cdot v_r + 0.7152 \cdot v_g + 0.0722 \cdot v_b \tag{5.1}$$

with v_r, r_g and $r_b \in \{0, 1, \ldots, 255\}$.

Afterwards, the threshold of the relative luminance θ_L was determined by the Ridler-Calvard method (Ridler and Calvard, 1978; Sezgin and Sankur, 2004). All RGB values below the threshold were set to 0 before automated cell and perineuronal net detection (Section 5.3.5).

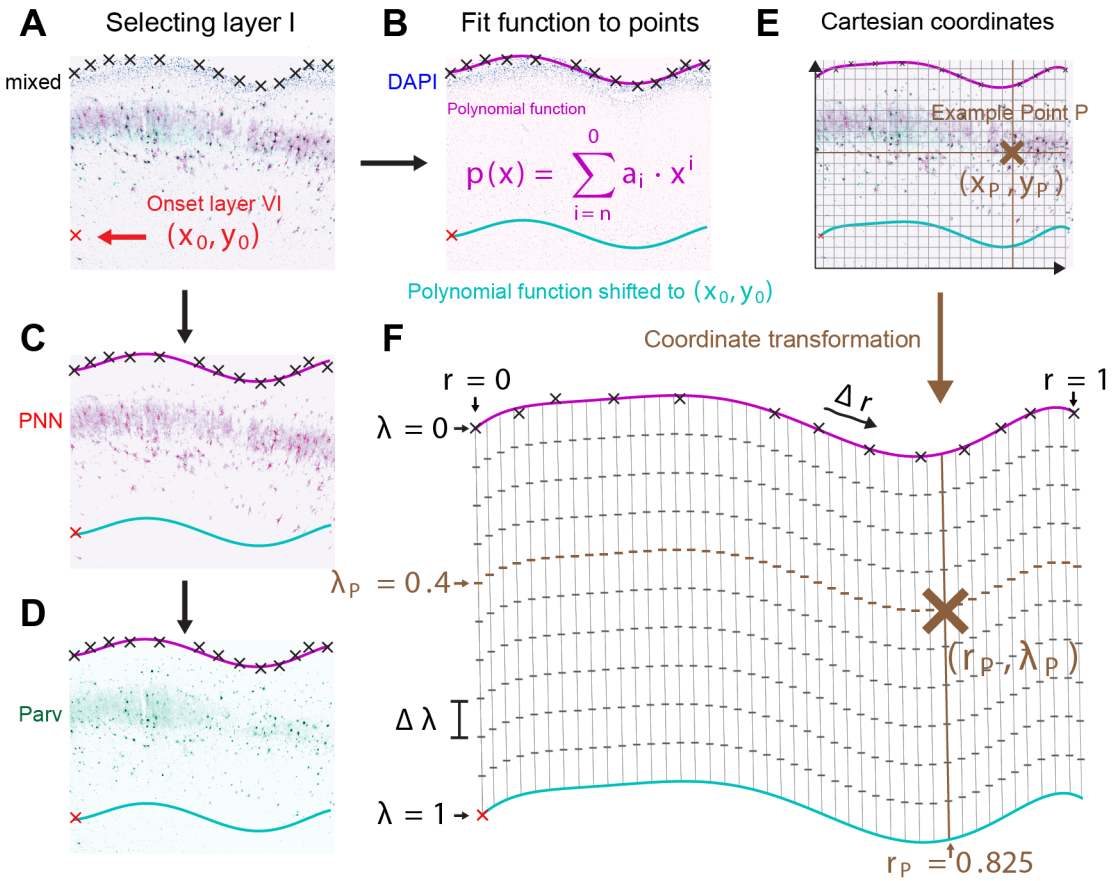

Figure 5.6: *Cortical layer determination and coordinate transformation.* (A) Defining the shape and extent of cortical layer I by choosing characteristic points k_i with $i \in \{1, \ldots, n\}$ (black crosses) and the onset x_0, y_0 of layer VI (red cross). (B) A polynomial function $p(x) = \sum_{i=n}^{0} a_i x^n$ was fitted to points and shifted by x_0, y_0. (B-D) Apply polynomial function and shift to DAPI, PNN and Parv. The intensity of each colour channel indicates the amplitude of the luminance (Equation 5.1). (E) Representation of one example point P (brown cross) in Cartesian coordinates and its transformation to a new laminar / layer specific coordinate system (F). The laminar depth λ and the constant along the polynomial function r are normalised. This coordinate transformation was performed for every pixel of each (colour-specific) image and for each cell (and PNN) position.

Defining cortical surface and layer extend using a layer-specific coordinate transformation

The laminar specific extent was determined by manually choosing several characteristic points k_i with $i \in \{1, \ldots, n\}$ along layer / laminar I using the mixed

luminance images containing PNN, Parv and DAPI (Figure 5.6A) for each ROI. Subsequently, the onset (x_0, y_0) of cortical layer VI was chosen. The mixed luminance image matrix provided indication of the laminar extend from layer I to VI. A polynomial function of the form

$$p(x) = \sum_{i=0}^{n} a_i x^i \tag{5.2}$$

was fitted to these points k_i by minimizing the squared error

$$E(x) = \sum_{i=n}^{0} \left(k_i - p(x_i)\right)^2 \tag{5.3}$$

with $\frac{\partial E}{\partial x_i} = 0$ for n chosen points along the cortex. The degree of the polynomial function characterised by n was chosen depending on the complexity of the cortical shape: n was typically between 3 and 5. The polynomial $p(x)$ was then shifted by (x_0, y_0) for each colour-specific luminance image (Figure 5.6B-5.6F, cyan).

Each pixel position in every $n \times m$ luminance matrix was transformed from Cartesian coordinates (x_i, y_i) to new laminar position specific coordinates (r_i, λ_i) (Figure 5.6E-5.6F).

The coordinate system (r, λ) was defined by constant steps on the polynomial function Δr (Note that r does not exactly match the idea of cortical columns because the projection of each point on the polynomial function to its shift is not necessarily perpendicular to the cortical surface) and by its cortical depth λ. Both r and λ were normalised to $0 < r \leq 1$ and $0 < \lambda \leq 1$, respectively. Therefore, the value $\lambda = 0$ represents the onset of layer I and $\lambda = 1$ represents the end of cortical layer VI (Figure 5.6F). The luminance laminar position $L_r(\lambda)$ was used as a measure of staining density.

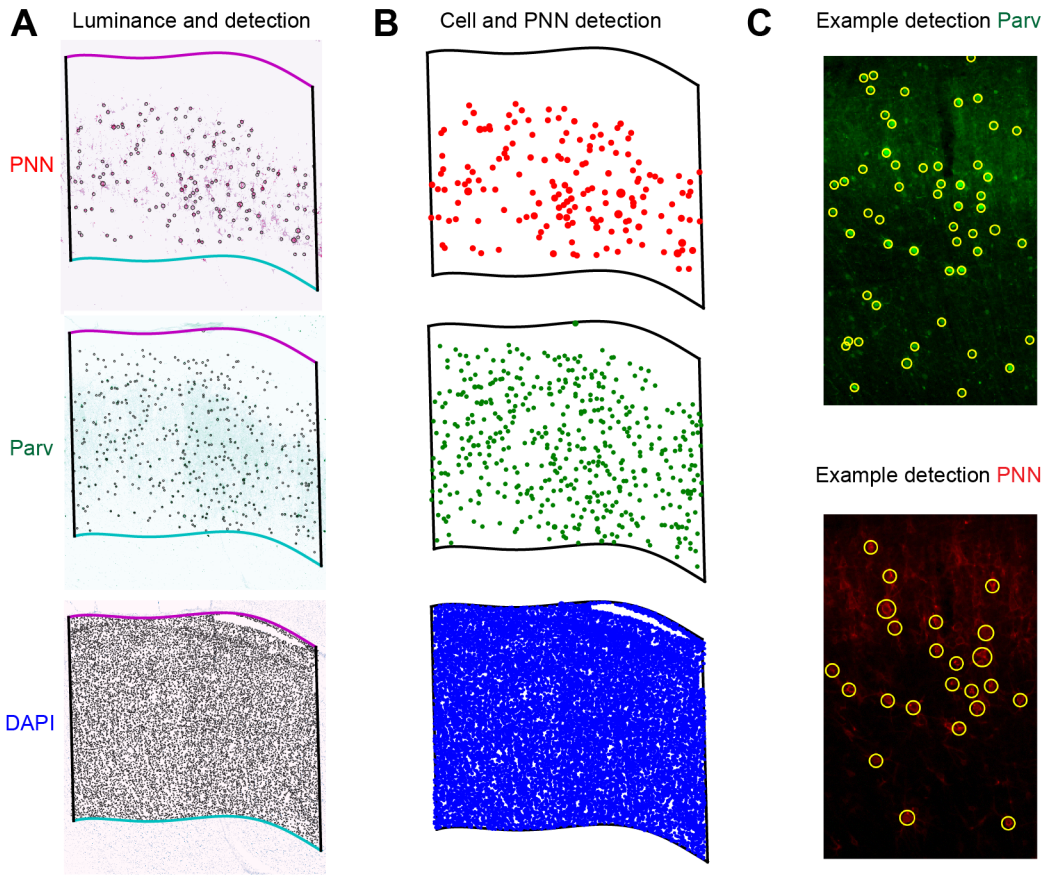

Figure 5.7: *Automated cell and perineuronal net detection*. (A) Luminance plot of an example ROI (new M1) for PNN, Parv and DAPI with automated cell and net detection (black circles). Note: The size of the circles indicates the size of the detected cells and nets. (B) Automatically detected cells for Parv and DAPI and automatically detected perineuronal nets for PNN. (C) Example Parv and PNN staining with cell and net detection with higher magnification. The detected cells (yellow circles) and nets are showed on the original RGB matrix for better comparison.

Automated cell count and perineuronal net detection

An automated cell (Parv), nucleus (DAPI) and an automated PNN detection was performed. This yielded an unbiased and automatic determination of the number of Parv- and DAPI–positive cells as well as a count for the PNNs.

The automated cell detection was performed using the Laplacian of Gaussian (LoG) method (Lindeberg, 1993, 1998). This method combines a Laplacian filter

of the form

$$L\left(x,y\right)=\bigtriangledown^{2}\cdot f_{\mathtt{IMG}}\left(x,y\right)=\frac{\partial^{2}\cdot f_{\mathtt{IMG}}\left(x,y\right)}{i}\partial\cdot x^{2}+\frac{\partial^{2}\cdot f_{\mathtt{IMG}}\left(x,y\right)}{\partial\cdot y^{2}} \tag{5.4}$$

with a Gaussian filter

$$G\left(x,y,\sigma_{i}\right)=\frac{1}{2\cdot\pi\cdot\sigma^{2}}\cdot e^{-\frac{x^{2}+y^{2}}{2\cdot\sigma^{2}}} \tag{5.5}$$

with Equation 5.4 follows

$$L\left(x,y\right)=\frac{1}{\pi\cdot\sigma^{4}}\left(1-\frac{x^{2}+y^{2}}{2\cdot\sigma_{s}^{2}}\right)\cdot e^{-\frac{x^{2}+y^{2}}{2\cdot\sigma_{s}^{2}}} \tag{5.6}$$

for an input image $f_{\mathtt{IMG}}(x,y)$ and a scale σ_{s}.

The second derivatives of the LoG filter lead to the detection of areas in the image with rapid luminance changes (Canny, 1986). This detection approach for identifying cells is implemented in the `skimage.feature.blob_log` method of the scikit-image Python libraries (Walt et al., 2014). The Gaussian kernel (Equation 5.5) is appropriate for detecting circular cells of a certain size with $s=2\cdot\sqrt{\sigma_{s}\cdot 2}$.

For PNNs however, this method would lead to false-positive matches due to its non-circular and distinctive shape. Therefore, I modified the Gaussian kernel (Equation 5.5) to shifted Gaussian function of the form

$$G\left(x,y,\sigma_{i}\right)=\frac{1}{2\cdot\pi\cdot\sigma^{2}}\cdot e^{-\frac{(x+c_{\mathtt{s}})^{2}+(y+c_{\mathtt{s}})^{2}}{2\cdot\sigma^{2}}} \tag{5.7}$$

where $c_{\mathtt{s}}$ is the shift of the Gaussian function. For both detection methods the optimal cell size $s_{\mathtt{opt}}$ for detecting a cell or PNN was determined.

Parv cells were excluded if their cell body size was smaller than 10 or bigger than $60\,\mu\mathrm{m}$ (Defelipe and Jones, 1991) and PNNs were only accepted between a diameter of 10 and $100\,\mu\mathrm{m}$. No restriction to the size of the DAPI-nuclei was applied.

The quality of the automated cell detection was assessed using the overlay images of the ROIs and the detected cells and nets with its corresponding luminance matrix (Figure 6.1A).

Once the automated PNN, Parv and DAPI detection was finished each cell and net was stored in laminar-depending coordinates (Section 5.3.5).

5.3.6 *Quantification of cells, nets and density distributions*

A total number of 40 slices (20 ipsi- and 20 contralateral) for monkey S and U were analysed for stimulation induced differences on the ipsi- and contralateral side. Some ROIs were not intact due to blade or cutting artefacts and therefore excluded. There were no samples for area 3a of S1 for monkey S. Furthermore, only one intact sample for old M1 for monkey S (contralateral side) could be included in further analysis. In all other ROIs equal number of slices on the ipsi- and contralateral side were used for the population analysis. In Figure 5.8 and 5.9 averages over all samples per ROI were shown. The shaded area in those plots denotes the SEM. The laminar position of the peak of luminance curves was determined as the λ value of the maximum luminance $L_{\mathtt{max}} = L(\lambda_{\mathtt{peak}})$. The width of the luminance distributions was defined as the width $\Delta\lambda$ measured at $50\,\%$ of the maximum normalised luminance. Statistical significance tests were performed using Monte-Carlo permutations. The Null-Hypothesis H_0 states that both samples were taken from the same distribution. Shuffling of the samples was performed by taking random permutations from the samples $n_{\mathtt{sim}}$ =10 000 times. H_0 was rejected if the value of interest was not within $99\,\%$ of the

simulated distribution (significance level of $p < 0.01$). To compare the cell count distributions of both hemispheres, the cell count density (number of cells per ROI) was calculated. The terminal distribution was analysed by subtracting automatically detected Parv-positive cells and PNNs from the corresponding RGB matrix and determining the luminance afterwards. Although, I used the term terminal distribution, this denotion is not completely accurate: In fact, the remaining distribution (after subtracting cellular and net entities) consists of terminals, axons, and possibly weakly or partially stained cells and nets. Nevertheless, I assume that the major contribution to this luminance density distribution originates from terminals. Furthermore, the terminal distribution also contains cells not detected by the cell detection algorithm because of an irregular shape or due to very low luminance values.

Cellular colocalisation was analysed as the proportion of automatically detected cells which were both Parv and PNN. Population effects were analysed area- and laminar-specifically. The total number of all detected cells and perineuronal nets is summarised in Table 5.1.

5.4. Results

5.4.1 *Consistent cell detection for all ROIs*

The parvalbumin-positive cell, DAPI-nuclei and the perineuronal net (PNN) detection led to consistent results for all regions of interest (ROIs). The detection algorithm with its constant parameters for each ROI enabled an evenly distributed sampling and coverage (cf. Figure 5.3.5) within the target areas. In total, the cell and net detection algorithm detected 24 703 Parv-positive cells, 17 858 PNNs and 958 019 DAPI-nuclei (Table 5.1).

	Monkey S						Monkey U								
	contra[b]			ipsi[c]			contra[b]			ipsi[c]			∑ (monkey S and U)		
ROIs[a]	Parv	PNN	DAPI	Parv	PNN	DAPI	Parv	PNN	DAPI	Parv	PNN	DAPI	Parv	PNN	DAPI
M1 (old)	224	198	13 953	1650	665	48 082	1321	1407	46 359	2146	2161	81 181	5341	4431	189 575
M1 (new)	864	1060	81 778	2581	1129	61 092	1928	1767	76 291	2669	2730	129 577	8042	6686	348 738
S1 (area 3a)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	471	295	11 631	600	437	23 729	1071	732	35 360
S1 (area 3b)	475	583	59 205	1642	539	39 091	1315	912	42 404	1600	1633	69 384	5032	3667	210 084
S1 (area 1)	1059	617	51 397	1245	476	33 727	1682	364	42 247	1231	885	46 891	5217	2342	174 262
∑ (all areas)	2622	2458	206 333	7118	2809	181 992	6717	4745	218 932	8246	7846	350 762	24 703	17 858	958 019

[a] Regions of interest (ROIs); [b] contralateral hemisphere; [c] ipsilateral hemisphere

Table 5.1: *Number of automatically detected cells.* The total number of automatically detected parvalbumin-positive (Parv), perineuronal nets (PNNs) and DAPI–positive nuclei for each ROI[a] for monkey S and U. Furthermore, the cell counts were divided between the contra[b]- and ipsilateral[c] hemisphere.

The majority of cellular entities were detected in new M1 (8042 Parv-positive cells, 6686 PNNs and 348 738 DAPI-nuclei) and the minority of objects were detected in area 3a of S1 (cf. Table 5.1). The latter was the case because this area was not included into further analysis in monkey S. Area 3a of the somatosensory cortex was not present in monkey S because slices were resectioned and trimmed (cf. Figure 5.2). Furthermore, area 3a (monkey U) was significantly smaller than new M1 and therefore less likely to contain the same number of cells assuming that the cell density did not change. Thus, cell and net counts were expressed as density (number of nuclei, cells or nets per square millimeter) in the following.

5.4.2 *Characteristic laminar specific luminance profile for each ROI*

The normalised average luminance profiles for each ROI showed a characteristic laminar profile for Parv-positive neurons and perineuronal nets (PNN). The staining for DAPI however did not show a consistent laminar specific profile between areas.

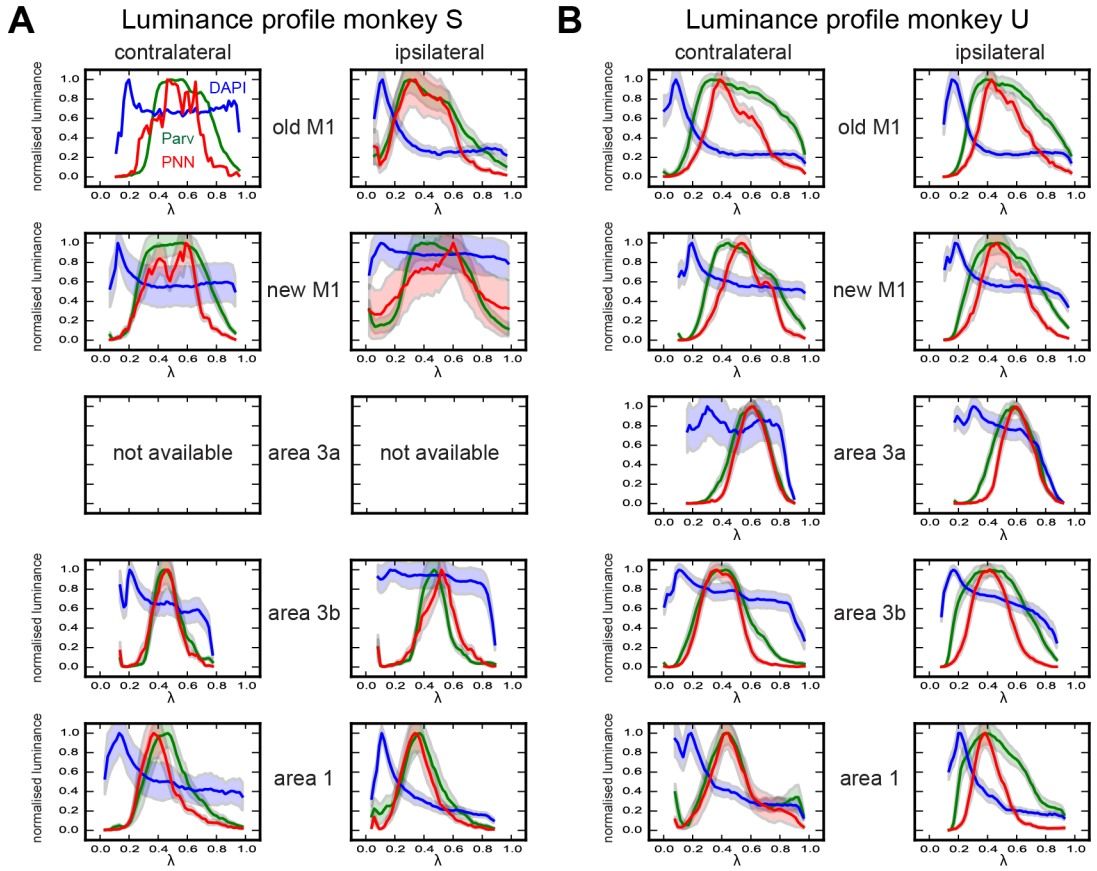

Figure 5.8: *Luminance staining distribution.* (A) Luminance profile for monkey S. (B) Luminance profile for monkey U. The blue traces show the normalised relative luminance (cf. Equation 5.1) for DAPI, the red traces the laminar luminance profile for PNNs and the green traces the relative laminar luminance profile for Parv. The shaded areas show the SEM. A λ value of 0 represents the beginning of cortical layer I and a λ of 1 the end of layer VI.

The only consistent characteristic of the DAPI profile was that the first peak of the normalised average luminance occurred consistently at a λ value smaller than 0.2 (Figure 5.8). This peak in superficial lamina (layer I and II) was merely a staining characteristic but rather a staining artefact. This was caused by the way the DAPI dye was applied: The mounting medium with DAPI was applied on slides to the whole slice. The perfusion of the dye was strongest at the transition between the mounting medium and the slice. Therefore, the peak in DAPI staining occurred exclusively in superficial layers (no transition zone between medium and slice at layer VI, cf. Figure 5.5).

	Monkey S				Monkey U				Monkey S and U			
	λ peak[b]		λ width[c]		λ peak[b]		λ width[c]		Mean λ peak		Mean λ width	
ROIs[a]	Parv	PNN	Parv	PNN	Parv	PNN	Parv	PNN	Parv	PNN	Parv	PNN
M1 (old)	0.43	0.38	0.46	0.40	0.35	0.40	0.66	0.34	0.39 ($\pm$ 0.05)	0.39 ($\pm$ 0.02)	0.56 ($\pm$ 0.05)	0.37 ($\pm$ 0.01)
M1 (new)	0.50	0.395	0.52	0.47	0.45	0.49	0.53	0.35	0.47 ($\pm$ 0.02)	0.54 ($\pm$ 0.02)	0.52 ($\pm$ 0.01)	0.41 ($\pm$ 0.04)
S1 (area 3a)	n.a.	n.a.	n.a.	n.a.	0.59	0.59	0.31	0.24	0.59 ($\pm$ 0.01)	0.59 ($\pm$ 0.01)	0.31 ($\pm$ 0.01)	0.24 ($\pm$ 0.01)
S1 (area 3b)	0.45	0.48	0.19	0.20	0.42	0.39	0.44	0.29	0.43 ($\pm$ 0.01)	0.43 ($\pm$ 0.01)	0.31 ($\pm$ 0.06)	0.25 ($\pm$ 0.02)
S1 (area 1)	0.41	0.35	0.28	0.23	0.41	0.40	0.38	0.23	0.40 ($\pm$ 0.02)	0.38 ($\pm$ 0.01)	0.33 ($\pm$ 0.04)	0.23 ($\pm$ 0.01)

[a] Regions of interest (ROIs); [b] λ value of peak luminance; [c] λ value of width at 50% luminance

Table 5.2: *Peak luminance and laminar width for area-specific luminance distributions.* The laminar position λ of the peak in the normalised average luminance and laminar with for each ROI is shown for Parv (green) and PNNs (red). Note that there are significantly broader luminance distributions for Parv and PNN in motor areas as opposed to somatosensory areas.

This also explained why the peak was smaller for area 3a in S1 (and to a weaker extend for area 3b and new M1 as well) since there was less mounting medium in between the central sulcus. Without this peak in the relative luminance caused by the DAPI perfusion gradient, there were no laminar specific luminance differences. Thus, DAPI was not further analysed. The luminance profiles for Parv and PNNs however possessed a laminar specific profile with a peak amplitude at characteristic laminar positions λ (Figure 5.8 and Table 5.2). Additionally, the width of the laminar luminance profile was significantly different between motor and somatosensory areas (Table 5.2). The laminar position of the average peak in old M1 for both Parv and PNNs was in laminar III ($\lambda = 0.39$, Table 5.2). The peak position for new M1 was deeper in layer V ($\lambda = 0.47$ for Parv and 0.54 for PNNs). In the somatosensory area 3a the peak laminar position was in layer V ($\lambda = 0.59$ for both Parv and PNNs). For area 3b and area 1 of S1, the peak in the average luminance for Parv and PNNs could be found in layer IV ($\lambda = 0.43$ for Parv and PNNs in area 3b and 0.40 for Parv and 0.38 for PNNs in area 1). In summary, when traversing all areas around the central sulcus from old M1 to area 1 of S1, the laminar peak position starts at layer III, changes to layer V for new M1 and area 3a and ends in layer IV for both area 3b and area 1 of S1.

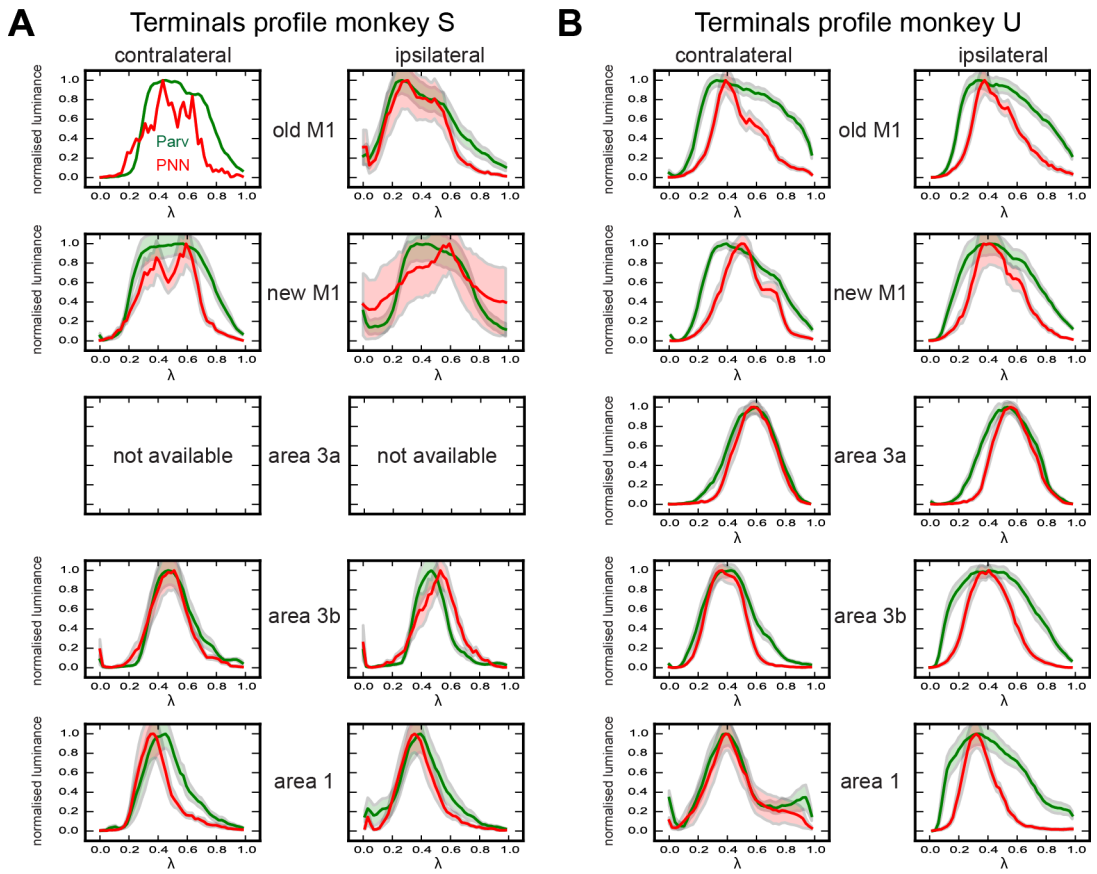

Figure 5.9: *Terminals staining distribution.* (A) Terminals profile for monkey S. (B) Terminals profile for monkey U. The blue traces show the normalised relative luminance (cf. Equation 5.1) for DAPI, the red traces the laminar luminance profile for PNNs and the green traces the relative laminar luminance profile for Parv. The shaded areas show the SEM. A λ value of 0 represents the beginning of cortical layer I and a λ of 1 the end of layer VI. Note that all traces represent the terminal density (which are the luminance values calculated after all detected cells have been removed from the image).

The width of the Parv and PNN distribution at 50% maximum luminance was significantly broader in motor than in somatosensory areas ($\lambda = 0.56$ and 0.52 for Parv and 0.37 and 0.41 for PNNs in old and new M1, respectively; cf. Table 5.2). The distribution width of the remaining somatosensory areas was very similar for Parv (λ between 0.31 and 0.31) and PNNs (λ between 0.23 and 0.25). The relative luminance and terminal luminance distributions were very similar between contra- and ipsilateral hemisphere in terms of their shape, laminar peak position and laminar distribution width (cf. Figure 5.8 and Figure 5.9).

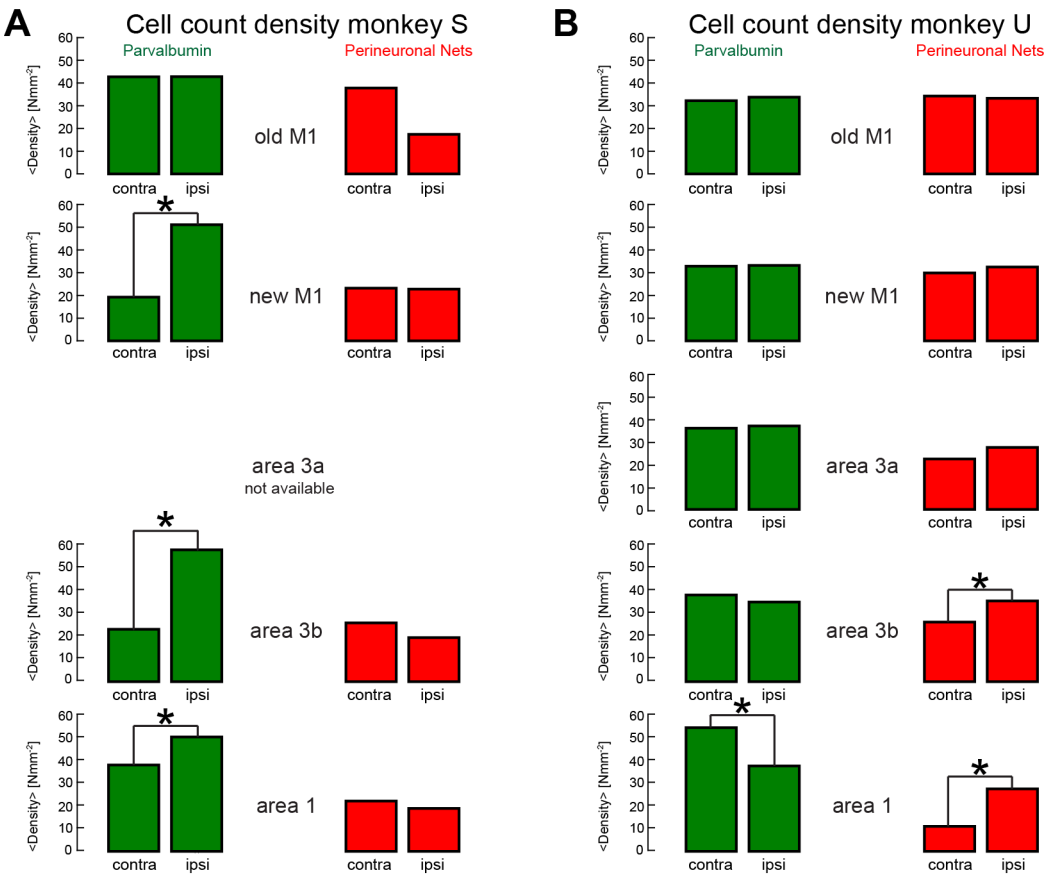

Figure 5.10: *Comparing average cell count density between contra- and ipsilateral sites.* (A) Cell count densities for monkey S for Parv-positive cells (green) and PNNs (red). (B) Cell count densities for monkey U. Each row represents one ROI. Significant differences are marked with an asterisk.

5.4.3 *The total area-specific cell count density did not change consistently between the stimulated and the non-stimulated hemisphere*

The average cell count density for Parv-positive neurons and PNNs did not change systematically between the stimulated and non-stimulated hemispheres (Figure 5.10). On the stimulated side, there was a significant reduction of the overall average cell count density for Parv-positive cells in new M1, area 1 and 3b of S1 in monkey S ($p < 0.01$, Monte-Carlo permutation test), but not for monkey U (Figure 5.10). Average net count density of perineuronal nets was significantly lower on stimulated hemisphere in area 1 and area 3b for monkey U but not S.

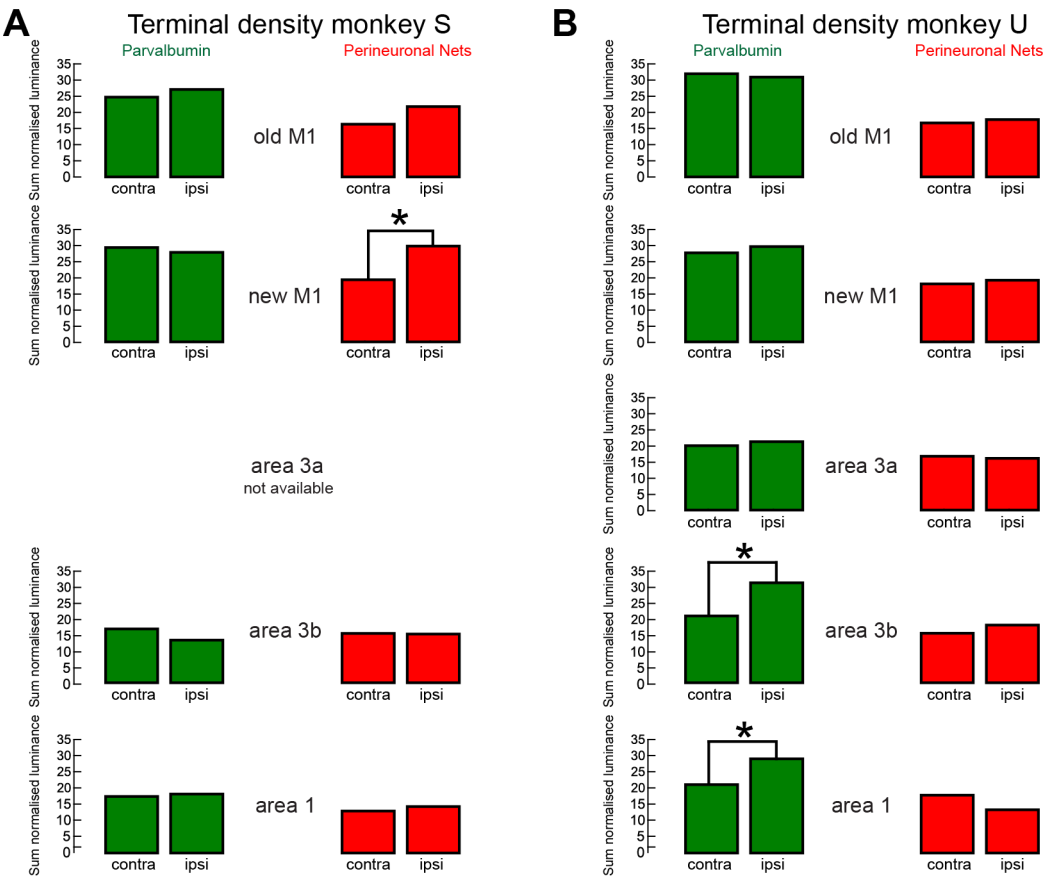

Figure 5.11: *Comparing terminal density between contra- and ipsilateral side.* (A) Terminal density of Parv-positive cells and PNNs for monkey S. (B) Terminal density for monkey U. Significant differences in terminal density ($p < 0.01$, Monte-Carlo permutation test) are marked with an asterisk.

5.4.4 *The total terminal density distribution did not change consistently*

The total cell and remaining net density distribution did not change in the same way between stimulated and non-stimulated hemisphere (Figure 5.11). There was a significant reduction in the overall PNN terminal density in new M1 for monkey S ($p < 0.01$, Monte-Carlo permutation test), but not for monkey U. In all other areas, there were no significant differences between contra- and ipsilateral hemispheres for PNNs. For monkey U, there was a significant decrease in Parv terminal density in area 1 and area 3b ($p < 0.01$, Monte-Carlo permutation test). There was no significant difference for Parv terminals in this area for monkey S.

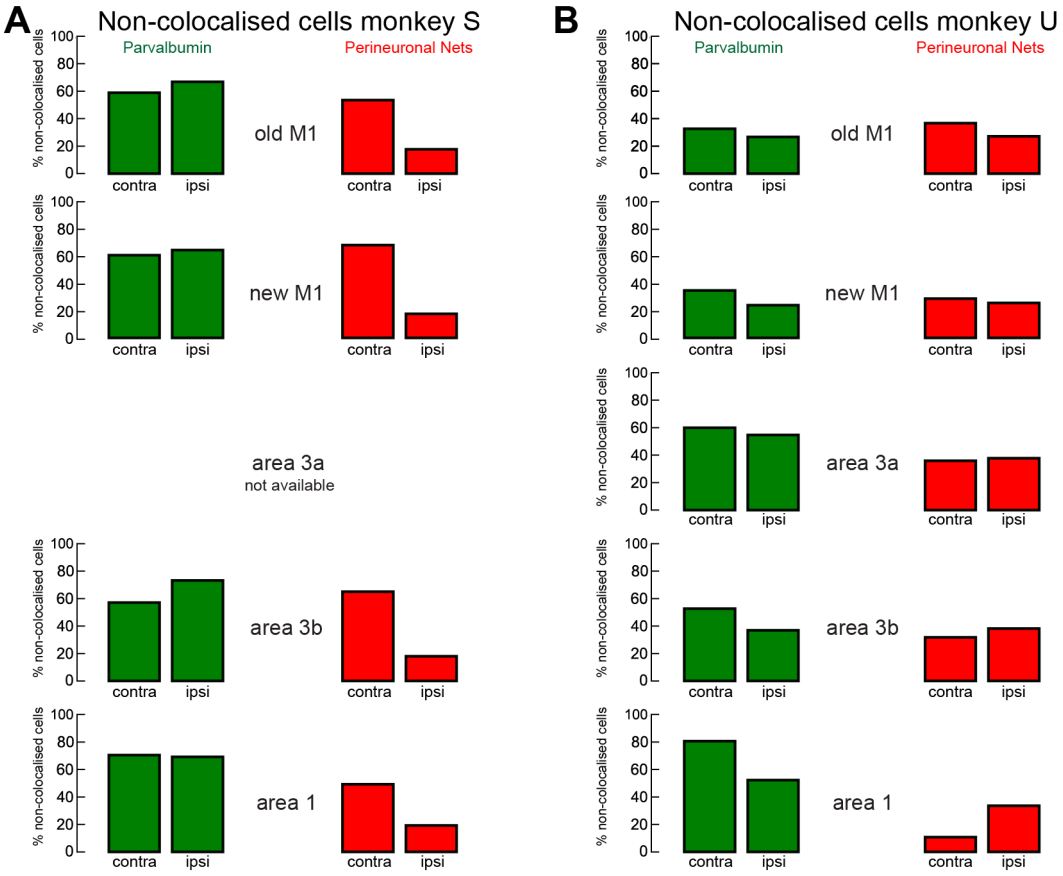

Figure 5.12: *Proportion of non-colocalised cells and perineuronal nets.* (A) Percentage of non-colocalised Parv-positive cells and PNNs for monkey S. (B) Proportion of non-colocalised cells for monkey U. For each ROI the percentage is shown for the contra- and ipsilateral hemisphere.

5.4.5 *Non-colocalised parvalbumin-positive cells and PNNs did not change consistently between stimulated and non-stimulated side*

Colocalisation was defined as the overlap between Parv$^+$ cells and PNNs (namely PNNs around Parv$^+$ cells, cf. Figure 5.3E). In total the percentage of non-colocalised Parv-positive cells did not change systematically between the stimulated and the non-stimulated hemisphere (Figure 5.12). The only consistent finding was that individual (non-colocalised) PNNs were present to a higher percentage on the contra- than on the ipsilateral side for monkey S. There were fewer non-colocalised PNNs on the contralateral hemisphere for monkey U.

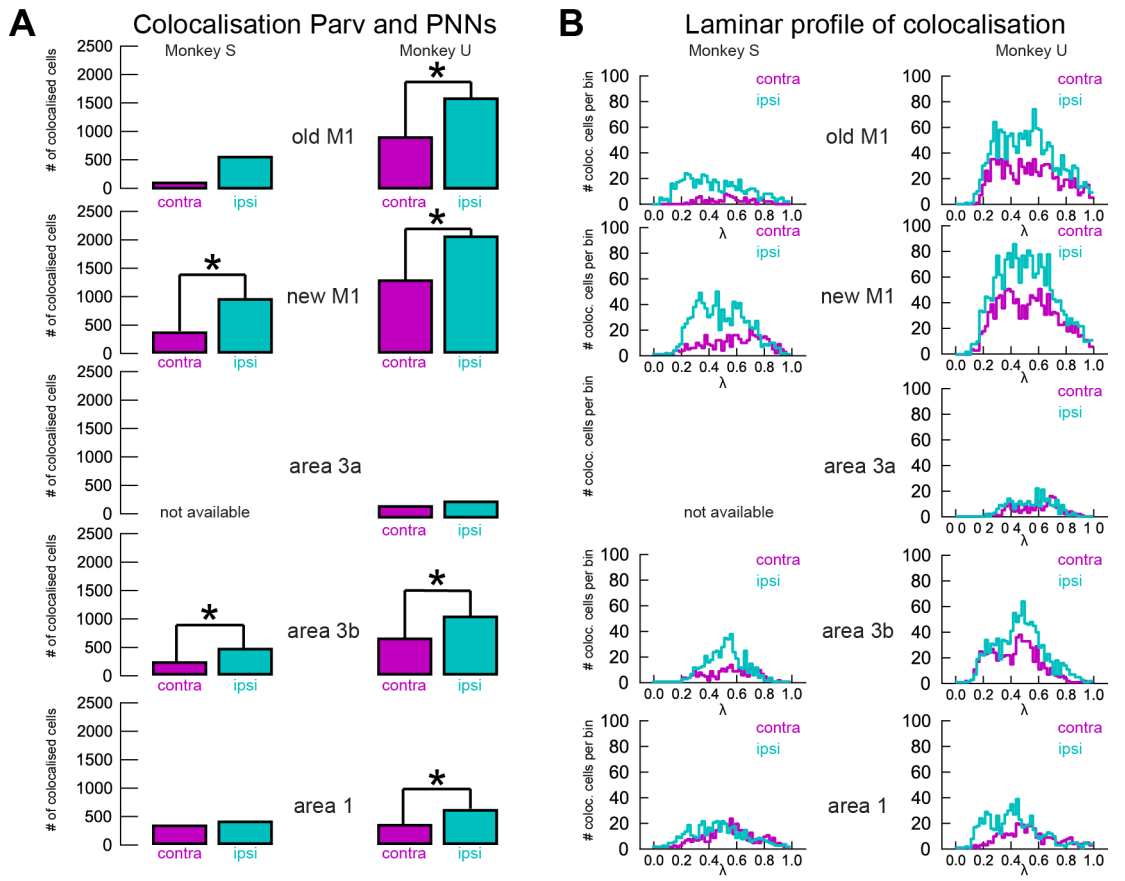

Figure 5.13: *Laminar specific changes in colocalisation profiles of Parv-positive cells and perineuronal nets*. (A) Comparison of the total number of colocalised cells and nets between stimulated (contralateral, magenta) and non-stimulated (ipsilateral, cyan) hemisphere. Significant differences are indicated by an asterisk ($p < 0.01$, Monte-Carlo permutation test). (B) Laminar profile of colocalised cells and nets.

5.4.6 *Less colocalisation of cells and nets on the stimulated hemisphere*

There were consistently fewer colocalised Parv-positive cells and perineuronal nets on the stimulated (contralateral) hemisphere than on the non-stimulated (Figure 5.13A). The absolute number of colocalised cells was significantly higher for both monkeys on the non-stimulated (ipsilateral) hemisphere for old M1, new M1 and area 3b ($p < 0.01$, Monte-Carlo permutation test). In area 1 of S1, there was a significant reduction of the total number of colocalised cells for monkey U ($p < 0.01$), and a non-significant decrease in monkey S (Figure 5.13A).

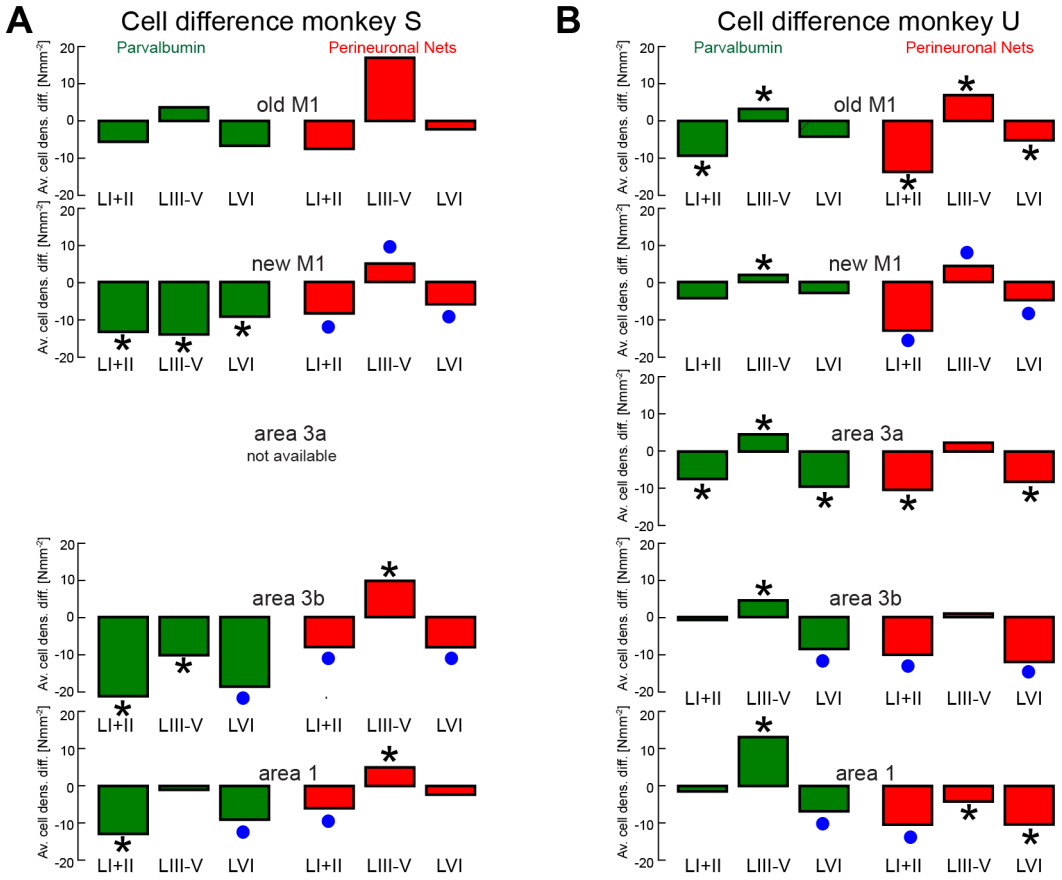

Figure 5.14: *Laminar specific differences in cell density between contra- and ipsilateral side*. (A) Differences in cell density for Parv-positive cells (green) and perineuronal nets (red) for monkey S. (B) Laminar-specific cell density differences for monkey U. Note that a positive difference value denotes a higher density on the contralateral as opposed to the ipsilateral side. The density values were separated by its laminar position: Layer I+II ($\lambda < 0.2$), Layer III-V ($0.2 > \lambda < 0.8$) and Layer VI ($\lambda > 0.8$). Significant differences are indicated by an asterisk ($p < 0.01$, Monte-Carlo permutation test). Blue asterisks denote significant and consistent effects between monkeys.

More specifically, the reduction in the colocalisation of Parv-positive cells and PNNs was caused by laminar specific changes (Figure 5.13B). In the motor areas (old and new M1), the difference in colocalisation occurred predominantly between layer III and V (λ between 0.2 and 0.8). The reduction was most pronounced in layer IV of the somatosensory areas 3b and 1 (cf. peak in laminar colocalisation profile, Figure 5.13).

5.4.7 *Differential changes in cell count density depending on its laminar position*

When considering total cell count densities, no consistent differences were found. Further analysis was carried out to investigate whether, if counts were compared in a laminar specific way, more consistent differences might emerge. Reanalyzing the cell count density depending on its laminar position, revealed laminar specific changes between stimulated and non-stimulated hemispheres (Figure 5.14A and 5.14B). The cell count density of all Parv-positive cells in layer I+II was reduced consistently, although not significantly in both monkeys. In layer III-V, these changes were more divergent but significantly different between contra- and ipsilateral hemispheres. In layer VI, there was a reduction in cell count density similar to layer I+II (although slightly more significant reductions: 6 out of 9 with $p < 0.01$ in contrast to 5 out of 9). The laminar specific cell count densities for PNNs were typically decreased on the stimulated (contralateral) side (8 out of 9 areas, $p < 0.01$, Monte-Carlo permutation test). The cell count density for PNNs was persistently increased on the stimulated hemisphere in layer III-V. The only exception for the latter, was a significant reduction of the PNN cell count density in layer III-V of area 1 for monkey U. The cell count density for PNNs was reduced in layer VI (7 out of 9 significantly reduced, $p < 0.01$) similarly to layer I+II.

5.5. Discussion

5.5.1 *Improved reliability in cell and perineuronal net detection*

The automated parvalbumin-positive cell and perineuronal net detection led to a high number of reliably detected cells and nets (cf. Figure 6.1 and Table 5.1). In contrast to manual counting approaches, this method eliminates quantitative errors (miscounting) and biases by the experimenter (Coggeshall, 1992; Guillery,

2002; West, 1999). Although the detection method might still miss some Parv-positive cells and PNNs due to the luminance threshold, the number of false-negative cells should be small and consistent for all ROIs. Since the threshold is comparable for all ROI, the same proportion of weakly stained cells should be missed. The automated PNN detection made it possible to study the nets quantitatively. Furthermore, this approach enabled the study of colocalisation of PNNs and Parv-positive cells. In addition, higher cell counts covering the whole ROI made it possible to study overall area-specific effects.

5.5.2 *Luminance and terminal density profiles resemble typical laminar and area profiles*

The area-specific luminance profile for parvalbumin-positive interneurons, perineuronal nets and DAPI cell nuclei showed typical laminar characteristics for each region of interest (Figure 5.8). This was the case for the immunohistochemical staining density as expressed by the total average luminance and for parvalbumin-positive and PNN terminals (Figure 5.9). In general, the luminance profile replicates particular features of Parv-positive and PNN staining. These include predominant Parv-positive interneuron localisation in the internal granular layer (Defelipe and Jones, 1991), high colocalisation of Parv and WFA expression (Balmer et al., 2009; Donato et al., 2015), and inter-area laminar differences in PNN and Parv expression (Kritzer, 2002). The high expression of parvalbumin and WFA in sensorimotor areas is not surprising considering the high transcriptional expression of these entities in sensorimotor areas (Hashimoto et al., 2008). The laminar profile of parvalbumin and perineuronal nets tends to be smaller in somatosensory areas and broader in motor areas. The maximal luminance concentration was highest in layer III-V for motor areas (new and old M1) and layer III-IV for somatosensory areas 1 and 3b. The peak of area 3a, as a transition zone between motor- and somatosensory areas, was slightly deeper than in its adjacent somatosensory areas.

DAPI-positive nuclei were homogeneously distributed across the lamina except for a peak in their corresponding luminance profile in superficial layers. This was presumably caused by a higher diffusion rate of the dye at the transition zone between mounting medium and cortical tissue (Figure 5.5).

The terminal distribution of Parv and PNNs is very similar to its total luminance profile counterparts. Although automatically detected nets and Parv-positive cells had been removed from the image samples before calculating the luminance, cells with very low luminance values (weak staining) might still contribute.

Even though the autofluorescence was blocked with Sudan Black B (Oliveira et al., 2010; Schnell et al., 1999), some fluorescent artefact remained. This should not be a critical confounding factor since this was the case equally for all samples, areas and lamina.

Long-term associative peripheral nerve stimulation did not affect the laminar profile of the luminance distributions comparing the stimulated (contralateral) with the non-stimulated (ipsilateral). These were comparable for each ROI and between monkeys.

5.5.3 *Peripheral nerve stimulation (PNS) did not influence the total area-specific cell and net distributions*

The entire cell densities in motor and somatosensory areas were not affected by PNS consistently (Figure 5.10). In rostral M1 and area 3a of S1 there were neither significant changes in cell nor perineuronal net density between the contralateral and ipsilateral hemisphere. In caudal M1, area 3b and area 1 of S1, there was a significant absolute reduction of the Parv-positive cell density in monkey S on the contralateral side, but no change for monkey U except for a significant increase in Parv-positive occurrences in area 1. PNNs were only significantly reduced in one monkey (monkey U) in area 3b and 1.

The Parv-positive cell density differences in caudal M1, area 3b and area 1 of S1 of monkey S, but not in rostral M1 could be explained in terms of area-specific sensory inputs. All of these three areas receive cutaneous inputs while rostral M1 does not (Pons et al., 1992). If this explanation would be true, why is there no significant change in monkey U? Another reason could be the difference in the tissue preparation between contra- and ipsilateral hemispheres in monkey S (cf. Figure 5.2). There are other possible explanations as well: In principle, the two monkeys might have received a slightly different contribution of cutaneous inputs due to minor differences in stimulation intensities, electrode position, and posture.

5.5.4 *PNS affected neither area-specific terminal distributions nor colocalisation of parvalbumin-positive cells and PNNs*

The aggregated parvalbumin terminal density was not different between the stimulated and non-stimulated hemisphere for both monkeys in caudal M1, rostral M1 and area 3a of S1 (Figure 5.9). There were no dissimilarities between Parv-positive terminals in area 3b and area 1 for monkey S, but there was a significant reduction in Parv terminal density for monkey U on the contralateral side in these areas. PNN terminal density was only significantly reduced in caudal (new) M1 for monkey S. All other areas did not show any distinct densities between contra- and ipsilateral hemispheres. The proportion of non-colocalised Parv-positive interneurons did not vary between the stimulated and non-stimulated hemisphere. The relative amount of non-colocalised PNNs for monkey A was higher on the stimulated side whereas monkey U did not show any hemisphere-specific variations in M1 and area 3a and 3b. There was a reduction of non-colocalised PNNs on contralateral area 1 for monkey U. The terminal density distributions, proportions of non-colocalised PNNs and Parv-positive interneurons did not lead to any coherent effects for both monkeys. The constant increase in PNNs on the contralateral side of monkey S raises the question whether this

(similar to the reduction in Parv-positive cell density in monkey S) might be caused by the cutting procedure before histochemical processing. However, it might be possible that differential laminar specific-effects lead to a confounded general view. Therefore, it is essential to investigate these criteria depending on its laminar position.

5.5.5 *Parvalbumin-positive interneurons and PNNs undergo laminar-specific modulation and are less colocalised after synchronous peripheral nerve stimulation*

The Parv-positive cell and PNN densities in putative neocortical lamina I, II and VI were typically decreased for monkey S and U (Figure 5.14). Parv-positive cell densities in layer III-V were significantly different between stimulated and non-stimulated hemispheres, although not in the same direction (decrease for monkey S and increase for monkey U) across the regions of interest. PNN densities were substantially increased for rostral M1, caudal M1, and area 3b of S1. Area 1 did show an increase in PNNs in laminar III-V for monkey S and a decrease for monkey U. Area 3a did not show any significant change between contra- and ipsilateral hemispheres. Parv-positive interneurons and PNNs are less colocalised on the stimulated hemisphere across all ROI. The difference is biggest in cortical lamina which are characteristic for each ROI (cf. Figure 5.8).

These findings are in agreement with my initial hypothesis. Structural changes induced by long-term synchronous PNS occurred both in motor- and somatosensory areas. These were quantifiable by changes in the laminar-specific Parvalbumin-positive cell and PNN density and changes in the colocalisation of these entities on the contralateral (stimulated hemisphere) as opposed to ipsilateral sensorimotor cortices.

5.5.6 *Putative mechanisms underlying laminar-specific parvalbumin-positive cell, PNN and colocalisation differences induced by PNS*

The differential laminar specific effect for cortical layer I, II and VI as opposed to layer III-V might reflect processing sensory inputs to sensorimotor areas preferentially in relevant locations responsible for inter-areal modulations and corticofugal outputs. These correspond to those layers possessing the highest density in Parv and WFA expression. The reduction in layer I, II and IV in conjunction with the predominant increase in PNNs accompanied by differential changes in Parv-positive interneurons in layer III-V might reflect a “shaping” mechanism to favour microcircuits in layers mostly associated with sensory afferent inputs and Parv-positive terminals (Defelipe and Jones, 1991).

How are these changes initiated? The majority of sensory afferents does project to the somatosensory cortex. Area 1 and 3b receive cutaneous (Pons et al., 1992), nociceptive (Vierck et al., 2013) from primary, and tactile information from secondary afferents (Qi et al., 2011). Moreover, these areas are highly linked (Negyessy et al., 2013) and influencing each other via horizontal connections (Friedman et al., 2008).

Area 3b receives, in addition to inter-cortical connections, thalamic (Padberg et al., 2009) input. Furthermore, area 3b obtains tactile (Thakur et al., 2012) information and is the major recipient of cutaneous afferents (Pons et al., 1992).

Area 3a as the transition zone between somatosensory and motor areas (Jones and Porter, 1980) has more divergent inputs than the latter regions (Padberg et al., 2009). Area 3a receives nociceptive via non-myeliniated fibres (Vierck et al., 2013), vestibular (Zarzecki et al., 1983), cutaneous (Pons et al., 1992), and mainly proprioceptive information (Phillips et al., 1971; Porter and Izraeli, 1993). Area 3a receives the majority of inputs from thalamic nuclei associated with motor systems and possesses several similarities with respect to its thalamic inputs to

M1 (Huffman and Krubitzer, 2001b). Area 3a is highly connected to M1 (Huerta and Pons, 1990).

M1 acquires multiple inputs from thalamic regions (Strick, 1975) and can be divided into a rostral (old M1) and caudal (new M1) part (Strick and Preston, 1978a,b). More recent studies however indicate that this separation might not be as strict (Kozelj and Baker, 2014). Neurons in M1 can be activated by cutaneous inputs (Lemon, 1979). M1 receives projections from premotor areas (Muakkassa and Strick, 1979). New M1 receives cutaneous input whereas old M1 receives input from deep somatosensory afferents (Strick and Preston, 1982; Tanji and Wise, 1981). Prolonged associative PNS might persistently depolarise inter-areal projection neurons in somatosensory areas which in turn might drive target specific signal to M1 (Yamashita et al., 2013). Somatosensory areas are capable of plastic reorganisation (Jain et al., 2008). Plastic changes can affect directly GABAergic synapses (Lu et al., 2014; Pieraut et al., 2014; Suzuki et al., 2014a; Suzuki et al., 2014b).

What role do Parv-positive interneurons and PNNs play in associative PNS induced plasticity and how do they interact? Although the exact mechanisms of how PNNs and Parv-positive interneurons interact and how these are affected by plastic changes remains unclear, there are some possible explanations for mechanisms leading to the increase in PNNs in layer III-V combined with a reduction in the colocalisation of these entities. The associative PNS leads to structural modifications of the input neuron's synapses accompanied by changes in extracellular matrix structures. This favours an increase of perineuronal nets. The increase in PNNs facilitates the internalisation of orthodenticle homeobox 2 (Otx2) proteins in Parv-positive interneurons (Beurdeley et al., 2012). This might lead to a closure of the critical period (Beurdeley et al., 2012) and therefore to reduced parvalbumin expression. Thus, Parv-positive interneurons are specifically reduced at these sites of the PNNs which might have initiated plasticity mechanisms at Otx2 GAG binding sites. The functional organisation of

microcircuits across areas is different (Ninomiya et al., 2015) and might therefore lead to differential effects. Further research to investigate functional and structural mechanisms induced by long-term associative and non-associative stimulation might lead to new therapeutic approaches to reorganise sensorimotor circuits.

Chapter 6. General discussion

6.1. Prolonged peripheral afferent nerve stimulation leads to neuroplastic changes in the sensorimotor cortex of the human and non-human primate and has behavioural consequences

A variety of non-invasive methods have been developed to induce plastic changes in the sensorimotor cortex. These rely on the stimulation of pairs of peripheral afferent nerves (McKay et al., 2002; Ridding et al., 2001) or motor points (McDonnell and Ridding, 2006; Pyndt and Ridding, 2004; Ridding and Uy, 2003; Schabrun and Ridding, 2007). Prolonged synchronous (associative) stimulation of two peripheral nerves stimulated during the intervention led to excitability changes in the motor cortex indicated by studies reporting changes in MEPs elicited by TMS (McKay et al., 2002; Ridding et al., 2001). Furthermore, the motor-cortical representations of the muscles stimulated during the intervention (cf. Ridding et al., 2000) are extended (increase of active sites and volume as assessed by motor cortical mapping procedures using TMS) and overlap (Ridding et al., 2001; Schabrun and Ridding, 2007).

In addition to these findings regarding the motor-cortical physiology, a number of behavioural assessments after prolonged synchronous (associative) and asynchronous (non-associative) stimulation have been performed. These encompass hand function tests including writing, cyclic drawings, grip force, object manipulations, peg board tasks, and a number of strength assessments

(McDonnell and Ridding, 2006; Rosenkranz et al., 2009; Schabrun et al., 2009; Sorinola et al., 2012).

In the present study, I investigated the neurophysiological effects on identified and unidentified neurons in M1 after one hour of synchronous and asynchronous median and ulnar nerve stimulation (Chapter 4), structural changes in M1 and S1 induced by long-term associative median and ulnar nerve stimulation (Chapter 5), and the behavioural effects affecting selective finger movements (Chapter 3) induced by either prolonged associative or non-associative median and ulnar nerve stimulation.

The non-human primate is an excellent model to study underlying physiology and functional implications of neuroplasticity inducing stimulation protocols. Monkeys and humans share a number of neuroanatomical similarities. For example the existence of direct corticomotoneuronal connections (Lemon, 2008). Monkeys can further be trained to perform skilled motor tasks (Brochier et al., 2004; Castiello et al., 1993; Novak et al., 1993). The ability to move fingers independently is another feature that human and non-human primates have uniquely in common (Sasaki et al., 2004; Schieber, 1991; Soteropoulos et al., 2012). Therefore, two macaque monkeys were trained to perform a skilled finger abduction task involving the independent and dexterous movement of either the thumb or the index finger (Chapter 2) using refined training methods and by using positive reinforcement training (PRT) techniques. Qualitative and quantitative training analysis, and the use of familiar objects and routines can enhance the training progress and yield advanced behavioural assessments for a number of neurological conditions (Chapter 2).

Studies on human subjects report an improvement in a spatial discrimination task (Bliem et al., 2007; Godde et al., 1996; Hoffken et al., 2007) and grooved pegboard task (McDonnell and Ridding, 2006) after prolonged associative stimulation.Improvement in this context might refer to either yielding a higher

score / better result or decreased time necessary to accomplish the particular task. In terms of the underlying physiology, it might be feasible that for these tasks a co-activation of sensory afferents was actually beneficial (i.e. a co-activation of intrinsic thumb and index finger muscles might have increased the index finger-thumb coordination). Studies focussing on non-associative interventions found mostly an improvement in task performance in patients with focal hand dystonia (FHD) as opposed to healthy subjects (Rosenkranz et al., 2009; Schabrun et al., 2009). This is very interesting since the pathophysiology of FHD is considered to be a result of maladaptive (co-) activation of sensory afferents which might lead to aberrant motor-cortical plasticity (Quartarone et al., 2008, 2014; Schabrun et al., 2009; Tinazzi et al., 2000). Rosenkranz et al. (2009) explicitly found an improvements in subjects with musician's dystonia after applying non-associative muscle vibration to task-relevant digits. Combining the evidence that especially motor-cortical projections to muscles stimulated during the intervention are affected and overlap after associative stimulation protocols (Pyndt and Ridding, 2004; Ridding and Uy, 2003; Ridding et al., 2001; Schabrun and Ridding, 2007), raises the question what explicitly would happen to performance depending on selective finger movements after synchronous median and ulnar nerve stimulation. The present study, revealed that one hour of synchronous (associative) median and ulnar nerve stimulation diminished the selective finger movement performance (Chapter 3) as indicated by increased number of errors and decreased performance measures (Figure 3.1A, 3.1B, 3.2A, and 3.2B). One hour of asynchronous median and ulnar nerve stimulation led to decreased number of errors and increased performance measures (Figure 3.1A, 3.1B, 3.2A, and 3.2B). Thus, the selectivity of independent thumb and index finger movements was improved after the non-associative intervention.

Despite these behavioural effects of the interventions, what neurophysiological changes in M1 can be observed? A number of neurons in M1 were activated

by individual EDC, median and ulnar nerve stimulation (Table 4.1). A particular subset of those neurons changed their responses after the intervention, namely those with convergent inputs from both peripheral nerves stimulated during the intervention (Figure 4.12A and 4.13A). After the synchronous intervention, M1 neurons showed a significant suppression in the population response difference profile at 20 ms following by a facilitation (or return to baseline) between 30 and 40 ms (see e.g. Figure 4.8A) whereas the suppression profile was broader and temporally less precise after the asynchronous intervention (see e.g. Figure 4.8A). Investigations on human subjects found an increased MEP amplitude evoked by TMS after synchronous interventions (e.g. Ridding and Uy, 2003). How does this finding relate to the characteristic population response difference profile in M1? The profile implies that approximately 20 ms after the stimulation onset (of either EDC, median or ulnar nerve), an evoked (positive) response was suppressed at this latency. This suppression might arise from consistent co-activation of pre- and postsynaptic neurons. These presynaptic neurons might be inhibitory GABAergic interneurons forming horizontal connections with the recorded target neurons (e.g. pyramidal cells in layer II/III or projection neurons in layer V). A LTD-like mechanism might reduce the synaptic efficacy and thereby the inhibitory influence of horizontal interneurons on the target cells. Thereby, the target cells can be depolarised easier resulting in increased excitability. Another possibility would the strengthening of excitatory synapses onto the target M1 neurons via LTP-like mechanisms. Thus, the increased excitability would reflect an increased synaptic efficacy of translaminar excitatory connections. Hereby, strengthened translaminar connections onto PTNs might account for the increases in excitability (cf. increased MEPs in studies on human subjects). Inhibitory interneurons (e.g. basket cells in layer II/III and layer V) might mediate the suppression as seen in the changes of the evoked cell responses in M1. These and other possibilities are discussed in Section 6.2.

Schabrun et al. (2009) implicitly raised the question of what would happen after long-term application of non-associative stimulation (especially in the context of the treatment of patients with FHD). In the present study, I investigated the structural changes induced by long-term application of associative median and ulnar nerve stimulation (Chapter 5) applied with an electronic wearable device. To study structural changes, I used an automated cell and perineuronal net detection algorithm (Figure A-5C) to quantify laminar specific changes (Figure 5.14A and 5.14B) as well as colocalisation (Figure 5.13A and 5.13B) of parvalbumin-positive interneurons and perineuronal nets (PNNs). The expression of both parvalbumin-positive interneurons and PNNs was significantly reduced in layer I, II and VI. In layer III-V, PNNs were significantly increased whereas parvalbumin expression was divergently changed on the stimulated (contralateral) hemisphere. Colocalisation of nets and cells was significantly decreased on the stimulated hemisphere predominantly in layer III-V. This finding showed that only one week of synchronous (associative) median and ulnar nerve stimulation is sufficient to induce structural changes in the sensorimotor cortex. Both of the aforementioned intracortical signal-chains could have triggered structural changes in M1. An increased synaptic efficacy mediated by a LTP-like mechanism targeting M1 neurons would lead to increased (direct or indirect) activation of pyramidal tract neurons (PTNs). PTN collaterals might initiate intracortical (horizontal) signaling-cascades leading to increased perineuronal net activity and to reduced colocalisation (particularly a reduction of parvalbumin-positive interneurons which were surrounded by PNNs). A decreased synaptic efficacy between local inhibitory interneurons and the target cell (e.g. pyramidal tract neuron) caused by LTD-like mechanisms might make these horizontally projecting interneurons redundant causing increased extracellular matrix activity (i.e. perineuronal nets), internalisation of receptors on parvalbumin-positive interneurons, and finally the reduction of parvalbumin expression. Thus, the diminished influence of local inhibitory interneurons might lead to reduced inhibition of target M1 neurons, and therefore to increased

corticospinal excitability. Inputs from afferents originating from peripheral nerves and muscles would be enhanced as well. Therefore, less suppression of the evoked M1 neurons' responses could be observed. These and other mechanisms are discussed in Section 6.2 and 6.3.

These findings stress the capability of peripheral afferents in driving sensorimotor plasticity. The importance of peripheral afferent nerves for neocortical topography and reorganisation in the sensorimotor cortex has been confirmed by studies investigating the effects of peripheral nerve damage or amputation (Cohen et al., 1991; Doetsch et al., 1996; Florence et al., 1994; Kaas, 2000; Merzenich et al., 1983; Qi et al., 2000; Sanes and Donoghue, 2000), modifications in afferent inputs (Abbott and Chance, 2005; Allard et al., 1991; Recanzone et al., 1991), motor skill learning (Nudo et al., 1996, 2001; Plautz et al., 2000), and peripheral nerve stimulation (Hamdy et al., 1998; Ridding et al., 2000, 2001).

6.2. A LTP-like mechanism might underpin motor-cortical changes induced by repetitive associative peripheral nerve stimulation

The role of concurrent inputs to induce neuroplasticity changes has been examined in a number of studies describing synaptic strengthening and weakening depending on pre- and postsynaptic firing (Almaguer-Melian et al., 2010; Baranyi et al., 1991; Engert and Bonhoeffer, 1997; Gambino et al., 2014; Humeau et al., 2003; Remondes and Schuman, 2002; Takeda et al., 2015). The co-activation and coincidence of correlated inputs has been postulated by Hebb (1949) who stated that *[w]hen an axion of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cell firing B, is increased* (page 50). This co-activation paradigm is hypothesised to underly neuroplastic changes in the sensorimotor cortex induced by peripheral afferent nerve, motor point or mixed cortical and peripheral

stimulation (Amaya et al., 2010; Godde et al., 1996; McDonnell and Ridding, 2006; Ridding and Uy, 2003; Schabrun and Ridding, 2007; Stefan et al., 2000). In addition to the consistent co-activation of inputs, the relative timing of pre- and postsynaptic action potentials (APs) is critical for the effect and extent of synaptic modifications (Figure 1.6). This phenomenon is known as spike-timing dependent plasticity (STDP, Bi and Poo, 1998; Caporale and Dan, 2008; Dan and Poo, 1992; Gerstner et al., 1996; Song et al., 2000). If a presynaptic AP is preceding a postsynaptic one with a consistent relative timing, this will lead to the strengthening of the synapse between the presynaptic and postsynaptic neuron (Markram et al., 1997). This long term potentiation (LTP) has been linked to be the underlying mechanism in studies using pairing of peripheral afferent nerves (McKay et al., 2002; Ridding et al., 2001) or muscles (Ridding and Uy, 2003; Schabrun and Ridding, 2007). The authors of these and other studies using the pairing of cortical and peripheral stimuli (Stefan et al., 2000) usually refer to a LTP-like mechanism. An increased MEP could indeed arise from a strengthened synaptic connection to a pyramidal tract neuron mediated by a LTP-like mechanism. On the other hand, this increase in motor cortical excitability could also be mediated by a long term depression (LTD). Weakening of the synaptic connection occurs when a postsynaptic APs precedes a presynaptic one (Dan and Poo, 1992). Thereby, synaptic connections of local inhibitory interneurons to PTNs might be weakened. This reduction of (tonic) inhibition by e.g. GABAergic basket cells might increase the excitability as reflected by the increase in MEPs. In addition to these simplified examples, there are many more possibilities which might underly the change in MEPs. For example intracortical recruitment of chandelier cells, recurrent inhibitory and excitatory feedback loops, activation of intracortical horizontal connections via e.g. pyramidal cell collaterals, etc. Furthermore, TMS activates PTNs directly and indirectly. Thus, a substantial amount of intracortical circuitry will be recruited. The findings in the present study showed a significant suppression of the M1 population response difference profile at $20\,\mathrm{ms}$ post individual median or ulnar nerve stimulation (Figure 4.8A).

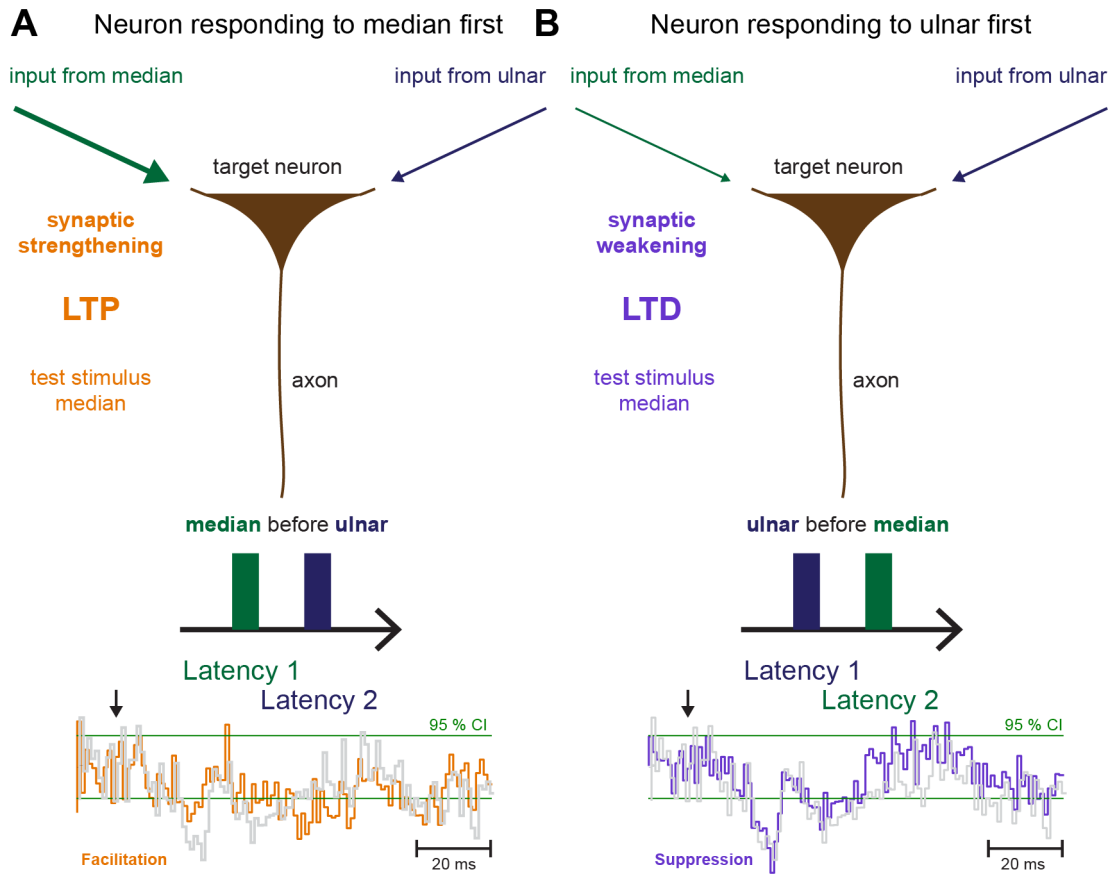

Figure 6.1: *Schematic illustration of spike-timing dependent effects on M1 population response differences.* (A) Schematic illustration of spike-timing dependent strengthening of the median nerve input synapse. (B) Schematic illustration of spike-timing dependent weakening of the median nerve input synapse. The bottom traces show the population z-score difference for all neurons which responded to median nerve stimulation first and subsequently to ulnar nerve stimulation (A, orange trace) and vice versa (B, purple trace). The M1 population response difference profiles to individual median nerve stimulation (test stimulus) are shown in grey (cf. Figure 4.14). The black arrow is indicating the stimulation onset of individual median nerve stimulation.

No change of the evoked EDC stimulation response was observed (control site). This is in agreement with studies on human subjects reporting changes predominantly in the muscles stimulated during the intervention (Ridding and Uy, 2003; Schabrun and Ridding, 2007). Here however, I report that changes in M1 neuron's discharge exclusively occurred in cell responses evoked by the peripheral nerves stimulated during the intervention (and not by the control stimulation site). Interestingly, these changes only occurred at M1 neurons

which received convergent input from both peripheral nerves stimulated during the intervention (Figure 4.12A and 4.13A).

This finding provides evidence for the importance of the co-activation of (peripheral) stimuli capable of driving neuroplastic changes in M1 neurons. The direction of the significant suppression at 20 ms post individual median nerve stimulation depends on the timing and order of the cell activation by the two peripheral nerve stimuli (Figure 6.1A and 6.1B). If a M1 neuron receives convergent activation from both median and ulnar nerves, and if this neuron receives further activation from median nerve first (response onset latency to median nerve shorter than ulnar) and subsequently from ulnar nerve, it can be hypothesised according to STDP that the synapse conveying sensory information from the median nerve should be strengthened (Figure 6.1A). This would imply that the median nerve stimulus leads to a presynaptic AP first followed by a postsynaptic AP of the target neuron. This would lead to LTP. After the synchronous (associative) intervention, all M1 neurons which were activated in this order showed less suppression (Figure 6.1A, orange trace). On the contrary, if a M1 neuron is activated by ulnar nerve first and subsequently by median nerve, this should lead to a weakening of the connections mediating information from the median nerve (Figure 6.1B). In this scenario the M1 neuron would elicit a postsynaptic AP first, followed by a presynaptic one (elicited by the median nerve stimulus). This would cause LTD. Therefore, the population response difference after the synchronous intervention is more suppressed (Figure 6.1B, purple trace). Although this timing dependency was not apparent after individual ulnar nerve stimulation (Figure 4.15A), M1 neurons tested by individual median nerve stimulation showed spike-timing dependent changes. Therefore, this study provides direct evidence for STDP-like changes in M1 neurons with convergent inputs from the peripheral nerves stimulated during the synchronous (associative) intervention.

Although the schematic illustration (Figure 6.1A and 6.1B) is appropriate to

explain the differences seen in the changes of the suppression, it does not provide an explanation for the increases seen in MEPs or for the effects after the asynchronous intervention. Hereby additional network components (e.g. intra- and inter-laminar interneurons) must be responsible for these phenomena.

The latency of the prominent change at 20 ms (time of the strongest suppression and influenced by the order of afferent inputs to the target neuron) in response to individual median or ulnar nerve stimulation after the synchronous intervention, might be related to the somatosensory evoked potential N20 in non-human primates (Allison et al., 1991). The human analogue response (N30) has been linked to dystonia (Kanovsky et al., 1997) and hypothesised to reflect sensorimotor integration (Cebolla and Cheron, 2015; Dancey et al., 2014).

In terms of the latency and the functional modulation of this response, it seems very likely that the response is mediated via S1. Further evidence is provided by a study reporting co-activation of S1 and M1 after peripheral electric stimulation (Schabrun et al., 2012). The electrical stimulation (ES) activates peripheral afferent nerves (presumable efferent nerves as well, see Chapter 1). Presumably mostly group I afferents (large diameter muscle fibres) are mediating the majority of the signals which are processed and relayed via the dorsal column system before they reach S1. From there on (long-range) cortico-cortical connections (Aronoff et al., 2010) connect mostly to layer II/III neurons in M1. The signal might be relayed over horizontal connections (Sanes and Donoghue, 2000), and intra- and inter-laminar instances. Projections from layer II/III might *amplify* the signal (Weiler et al., 2008) before it reaches the PTNs in layer Vb (Kiritani et al., 2012; Shepherd, 2009). Furthermore, the signal might be relayed over intracortical inhibitory interneurons (Katzel et al., 2011; Sanes and Donoghue, 2000). Since trans-laminar inhibition is rare in M1 (Katzel et al., 2011), the majority of inhibition on excitatory pyramidal cells must be transmitted via intra-laminar interneurons. Especially fast-spiking interneurons receive strong intralaminar (horizontal) excitation from

corticospinal (and corticostriatal) neurons (Apicella et al., 2012). These fast-spiking interneurons are GABAergic parvalbumin-positive interneurons (Hu et al., 2014). Parvalbumin-positive interneurons provide perisomatic inhibition and play thereby an important role in timing pyramidal cell's output (Chen et al., 2015; Fuchs et al., 2007). Therefore, parvalbumin-positive interneurons are a likely target of neuroplasticity effects induced by associative peripheral nerve stimulation. Even if a LTP-like mechanism might not affect the interneuron directly, effects of LTP have been shown to spread (Engert and Bonhoeffer, 1997) and recruitment of these indirectly via polysynaptic connections would also be feasible. Another line of evidence that inhibitory intracortical networks might be affected by co-activation of peripheral afferent nerves provide studies on dystonia patients. In these patients excessive representational plasticity (Hallett, 2011), abnormal cortical representations (Schabrun et al., 2009) and reduced intracortical inhibition (Butefisch et al., 2005; Hallett, 2011; McDonnell et al., 2007; Rona et al., 1998; Stinear and Byblow, 2004) was present. parvalbumin-positive interneurons are typically surrounded by perineuronal nets (Figure 5.3E). PNNs have frequently been shown to be affected by plastic changes (Wang and Fawcett, 2012). Thus, I studied the changes in parvalbumin-positive interneurons and PNNs after one week of synchronous (associative) median and ulnar nerve stimulation. The expression of both parvalbumin and Wisteria floribunda agglutinin (perineuronal nets) was significantly reduced in layer I, II and VI. In layer III-V, PNNs were significantly increased whereas parvalbumin expression was divergently changed on the stimulated (contralateral) hemisphere. This is interesting since especially the transition between layer II/III and layer V to VI possessed the biggest difference in parvalbumin and WFA expression. Both layer II/III and layer V have been shown to receive horizontal connections (potentially mediating plastic changes) in M1 (Sanes and Donoghue, 2000). S1 was equally affected by these changes (Figure 5.10A and 5.10B). Additionally, the colocalisation of parvalbumin-positive interneurons and PNNs was significantly reduced on the contralateral (stimulated) hemisphere

(Figure 5.13A and 5.13B). This finding supports the hypothesis that inhibitory cortical networks are affected by long-term plasticity inducing associative peripheral afferent stimulation. One putative mechanism which would affect the laminar distribution and colocalisation of these interneurons and the nets, would imply the involvement of LTD mechanisms resulting in synaptic weakening of the (intralaminar) synapse between parvalbumin-positive GABAergic interneurons and pyramidal cells in layer II/III or layer V. Both corticospinal neurons in layer Vb and corticostriatal pyramidal cells in layer Va might be affected by this. The long-term synaptic weakening might make these inhibitory interneurons redundant and might result in the recruitment of the PNNs which have a crucial role in maintaining and regulating the excitation-inhibition balance (Hensch, 2005) and homeostatic plasticity. The PNNs might *silence* parvalbumin-positive interneurons by internalisation of specific receptor proteins (Beurdeley et al., 2012). This might lead to the reduction in colocalisation and a reduction of parvalbumin-positive interneurons particularly on those surrounded by PNNs. The parvalbumin expression in layer III-V in the present study was however divergent although significantly increased or decreased on the contralateral sensorimotor cortex (Figure 5.14A and 5.14B). This heterogeneity might be due to the fact that particular subtypes of parvalbumin-positive interneurons were affected (cf. early versus late born parvalbumin cells, Donato et al., 2015). Parvalbumin-positive basket cells can be divided into sub-populations based on their pattern of neurogenesis (Donato et al., 2013, 2015). Late born Parvalbumin-positive interneurons are more likely to be located in superficial and late born in deep cortical layers (Ciceri et al., 2013; Donato et al., 2015). Donato et al. (2015) conclude that these sub-populations control different ensembles of excitatory cortical neurons. Therefore, it is feasible that these two types of Parvalbumin-positive cells account for the heterogeneity described in the context of laminar-specific Parvalbumin-positive interneurons. Other types of structurally and functionally distinct subtypes of Parvalbumin-positive interneurons exist as well (Varga et al., 2014). Their functional and structural significance needs to

be studied more extensively to be able to fully understand their role in gating neuronal information and in the context of neuroplasticity.

A number of studies reported changes mediated by LTD in inhibitory interneurons in the sensorimotor cortex. Lu et al. (2007) found that parvalbumin-positive fast-spiking interneurons showed mGluR-dependent LTD independent on whether the presynaptic AP followed or preceded the postsynaptic one. A highly linked network of divergent interneuron types can be modified by short- and long-term plastic mechanisms. Parvalbumin-positive interneurons can be coupled with somatostatin-positive interneurons (Ma et al., 2012) and interneurons linked by electrical synapses are capable of modifying each other by LTD (Haas et al., 2011; Kullmann et al., 2012). All of these studies provide evidence that an indirect or direct modification of parvalbumin-positive interneurons mediated by LTD might be feasable. The line of evidence supporting that predominantly horizontal connections and inhibitory intracortical (potentially intralaminar) networks in M1 are altered after concurrent (peripheral afferent) input, might explain why (adjacent) motor-cortical representations are less separated (e.g. lack of lateral inhibition) and why an input to the local circuitry is more likely to elicit responses in projection neurons (cf. increase in motor-cortical excitability).

6.3. Cortical changes after prolonged non-associative peripheral afferent nerve stimulation

In contrast to the consistently timed input to S1 and M1 after the synchronous (associative) intervention, what are the effects and potential mechanisms mediating changes in the sensorimotor cortex after asynchronous (non-associative) peripheral afferent nerve stimulation? The majority of evidence from human subjects relies on findings in subjects with FHD. Since the pathophysiology of FHD resembles the effects *artificially* induced by synchronous motor point of peripheral nerve stimulation (e.g. overlap of motor-cortical

representations, increased sensorimotor excitability, and co-activation of muscles), FHD is considered to arise from a maladaptive co-activation of peripheral afferent nerves resulting in aberrant neuroplasticity in the sensorimotor cortex (Hallett, 2011; Lin and Hallett, 2009; Quartarone et al., 2008, 2014; Schabrun et al., 2009; Tinazzi et al., 2000). Cohen and Hallett (1988) reported co-activation and overflow of EMG activity to the inappropriate (adjacent) muscles in subjects with FHD. More recent studies confirmed this finding by reporting the dystonia patients' decreased capability of performing independent finger movements (Rosenkranz et al., 2009). This is in agreement with the behavioural findings of the present study (Chapter 3). After one hour of synchronous (associative) median and ulnar nerve stimulation, monkeys were unable to move their index finger and thumb as selective as after the non-associative intervention (Figure 3.1A, 3.1B, 3.2A, and 3.2B). After asynchronous (non-associative) stimulation, the capability of dystonia patients to perform independent finger movements was increased accompanied with a temporarily *normalisation* of characteristic motor-cortical physiological features (e.g. less overlap of motor-cortical representations, Rosenkranz et al., 2009; Schabrun et al., 2009). Also in the present study, monkeys performed significantly better after the asynchronous in comparison to the synchronous intervention (Figure 3.1A, 3.1B, 3.2A, and 3.2B). The M1 population response difference profiles were predominantly characterised by a sustained inhibition (Figure 4.8B). That means that neurons in M1 which responded to median and ulnar nerve stimulation, showed reduced stimulation evoked discharges after the asynchronous intervention. Due to the fact that the sustained inhibition occurred at a variety of response onset latencies (Figure 4.8B) and was accompanied with a decreased facilitation, it might be possible that the influence of polysynaptic inhibitory networks was enhanced after the non-associative intervention. The interpretation of these electrophysiological effects is more difficult due to the nature of inconsistently timed inputs to M1 neurons. However, the idea of increased (tonic) inhibition in M1 would be in line with evidence of research on dystonia patients and (animal) models of

dystonia (Tinazzi et al., 2000). The lateral inhibition on pyramidal cells and the sensorimotor integration might be distorted in dystonia patients. But how can asynchronous muscle or peripheral afferent nerve stimulation (of adjacently innervated muscles) lead to a temporal increase or recruitment of these inhibitory neuronal populations? It would be possible that an input to a M1 pyramidal neuron would cause recurrent inhibition via intralaminar collaterals activating inhibitory interneurons. This would result in an inhibition of this pyramidal neuron before the subsequent stimulus to an adjacent pyramidal neuron arrives. This stimulus would cause a more selective output via PTNs. The activation of the PTNs themselves would trigger recurrent inhibition before the next (peripheral afferent) input arrives. The repetitive activation of recurrent inhibitory networks would result into more selective processing of subsequent inputs. In addition to recurrent inhibition, intralaminar interneurons inhibiting adjacent (pyramidal) neurons might be involved. This mechanism is very similar to those described in the context of of lateral inhibition and center-surround inhibition in the visual (Blakemore et al., 1970; Dacey et al., 2000; Eysel et al., 1987; Paffen et al., 2006; Stemmler et al., 1995; Xing and Heeger, 2001), auditory (Brosch and Schreiner, 1997; Pena and Konishi, 2001; Suga, 1995; Sutter et al., 1999) and somatosensory (Derdikman et al., 2003; Foeller et al., 2005; Moore and Nelson, 1998; Zhu and Connors, 1999) system. In the motor system, surround inhibition has been investigated by describing antagonistic changes in MEPs elicited by TMS (Sohn and Hallett, 2004) between two adjacent muscles (e.g. FDI and APB). Furthermore, tonic contraction of one muscle does influence the motor cortical representation of the adjacent muscle (Jono et al., 2015). Surround inhibition is further considered to be disrupted in patients with FHD (Beck and Hallett, 2011; Beck et al., 2008, 2009; Moore et al., 2012). A number of studies proposed that this mechanism is essential for the successful generation of independent finger movements (Beck and Hallett, 2011; Furuya and Altenmuller, 2013; Rosenkranz and Rothwell, 2003). Surround inhibition is affected by plasticity inducting repetitive pairing of a peripheral (median nerve) and cortical (TMS) stimulus (Belvisi et al., 2014).

Synchronous movements of the little and index finger reduce the surround inhibition between these fingers (Kang et al., 2012).

Interestingly, Schneider et al. (2002) reported that the iontophoretical application of the $GABA_A$ receptor antagonist bicuculline and the resulting disinhibition led to merging of two motor cortical muscle representations. A general increase in excitability induced by glutamate application did not lead to this effect. Therefore, it would be feasible that prolonged synchronous stimulation of two peripheral nerves does lead to a reduction in GABA-mediated surround inhibition. Asynchronous stimulation on the other hand would increase the direct and indirect recruitment of local inhibitory circuits in M1 leading to increased surround inhibition (and as a consequence to more selective finger movements). This might be the reason for the prolonged sustained inhibition observed in individual stimulation-site evoked M1 responses after the non-associative intervention (Figure 4.8B). The synchronous intervention would reduce the surround inhibition by weakening (potentially via LTD, see Section 6.2) inhibitory influence of GABAergic interneurons on pyramidal neurons.

Another line of evidence for altered intracortical inhibition due to maladaptive co-activation of peripheral afferents is provided by studies describing effects elicited by paired-pulse (PP) TMS on MEPs (Kujirai et al., 1993; Lazzaro et al., 1998; Reis et al., 2008). PP-TMS consists of a subthreshold conditioning stimulus (CS), followed by a suprathreshold (sub- and suprathreshold with respect to eliciting a response in the corresponding MEP) test stimulus (TS). Alternating the timing between these stimuli (inter-stimulus intervals), does lead to facilitation or suppression of the MEP (in comparison to the single pulse evoked MEP, for illustration see Figure C.1). Short inter-stimulus intervals (ISIs) between 1 and $6\,\text{ms}$ lead to suppression (SICI), intervals between 8 and $30\,\text{ms}$ to facilitation (ICF), and longer intervals between 40 and $200\,\text{ms}$ to suppression (LICI) of the test MEP (Berardelli et al., 2008). Neuropharmacological investigations led to the hypothesis that SICI is mediated by intracortical inhibitory networks using

the neurotransmitter $GABA_A$, whereas LICI is mediated by $GABA_B$ receptor dependent neurotransmission (Ziemann, 2004; Ziemann et al., 2008). In dystonia patients only minor effects of ICF (McDonnell et al., 2007; Rosenkranz et al., 2005) and LICI were found (Chen et al., 1997; Di Lazzaro et al., 2009; Espay et al., 2006; Meunier et al., 2012). Effects on SICI however, were quite divergent (Berardelli et al., 2008; Butefisch et al., 2005; Di Lazzaro et al., 2009). Di Lazzaro et al. (2009) reported a reduced SICI in dystonia patients. A study investigating the effects of individual finger training did not find changes in SICI (Zeuner et al., 2005). There are potentially several reasons for these divergent results. Zeuner et al. (2005) for example only trained individual finger movements. It would be possible that alternating finger movements would be necessary to involve intracortical inhibitory networks assessed by SICI. Furthermore, the effect of individual finger movements on SICI might dependent on the frequency, strength and consistency by which individual fingers are moved. Furthermore, it is possible that SICI despite the evidence in human subjects, might be mediated by intrinsic mechanisms of corticospinal projecting neurons (Figure C.1). Nevertheless, the increased recruitment of intracortical inhibitory interneurons enhancing mechanisms similar to GABA-mediated surround inhibition, could be increased after prolonged asynchronous (non-associative) median and ulnar nerve stimulation. This could explain why more selective finger movements would be enabled and why asynchronous peripheral nerve or motor point stimulation would be a viable option in the treatment of (focal hand) dystonia and other movement related neurological conditions.

6.4. Implications and future directions of non-invasive peripheral afferent nerve stimulation

I investigated the neuroplastic changes in the sensorimotor cortex induced by prolonged synchronous (associative) and asynchronous (non-associative) peripheral nerve stimulation. A number of behavioural, electrophysiological, and immunohistochemical assessments revealed structural and functional neurophysiological changes in M1 and S1. This is in agreement with my initial hypotheses (see Chapter 1). The changes in individual stimulation-site evoked M1 neurons' discharges after the synchronous intervention were analysed and discussed in the context of spike-timing dependent mechanism. The present study provides hereby direct evidence that neuroplastic changes occur in M1 neurons with convergent (afferent) inputs of both peripheral nerves stimulated during the intervention. Hereby, plastic changes occur potentially at the last order synapse. The direction and extend of the changes induced by the synchronous intervention, followed a characteristic time profile and depended on the timing of the relevant inputs to M1 neurons (Figure 6.1A and 6.1B). These M1 neurons might represent an important stage in the process of sensorimotor integration before (altered) muscle responses are generated. The sensorimotor integration is differentially altered after synchronous and asynchronous peripheral nerve stimulation by presumably polysynaptic recruitment of GABAergic intracortical inhibitory interneurons relayed via S1. Inhibitory neurophysiological responses in M1, altered parvalbumin, and increased WFA expression after long-term synchronous peripheral nerve stimulation in the relevant cortical lamina support this hypothesis. It is remarkable that only one week of peripheral afferent nerve stimulation was capable of inducing structural changes.

Although the present study identified important sites of neuroplasticity changes in the sensorimotor cortex, future investigations should extend the examination of effects induced by repetitive peripheral nerve or motor point stimulation to

additional cortical and subcortical locations. These include for example the spinal cord, cuneate nucleus, basal ganglia, and the cerebellum. Additionally, inter-hemispheric influences should be studied.

The hypothesis regarding the plasticity mediating mechanism within M1 could be verified by combining extracellular single cell recordings with the (e.g. iontophoretical) application of GABA antagonists. Optogenetic modifications of GABAergic interneurons and pyramidal cells (English et al., 2012; Kepecs and Fishell, 2014; Lee et al., 2012, 2014) would further allow to manipulate and isolate particular cell type specific effects. This would further advance our understanding of underlying neuroplasticity mechanisms induced by non-invasive peripheral afferent nerve stimulation.

Future immunohistochemical assessments should involve the investigation of alternative plasticity inducing stimulation protocols (e.g. long-term application of alternating peripheral nerve stimulation). The structural changes could hereby be studied in all of the above mentioned cortical and subcortical regions. An increased number of immunohistochemical marker (e.g. visualisation of dendritic spines) would extend the repertoire of structural assessments.

The monkey model is an excellent model to study non-invasively induced neuroplasticity (Amaya et al., 2010). Future research could focus on combined non-invasive (e.g. TMS) and invasive (e.g. single cell recordings) neurophysiological techniques assessing task performance of highly specialised and skilled motor tasks. A refinement of animal training routines (e.g. by optimising training strategies and the use of positive reinforcement training) would result in better and more advanced behavioural paradigms. This might lead to decreased stress levels and thereby to increased performance (Figure 2.10) and welfare. Along with advantages for the primate's behaviour and welfare, reduced (or consistent) stress levels are also important to avoid potentially confounding

effects of stress on the induction of neuroplasticity (Kehoe and Bronzino, 1999; Pittenger and Duman, 2008; Sale et al., 2008).

Although the present study revealed some comparable effects to those reported on subjects using TMS, the range of assessments could be extended. McKay et al. (2002) investigated the time course of the plasticity induction by synchronous (associative) radial and ulnar nerve stimulation. Plasticity effects as indicated by changes in MEPs reached a plateau level after approximately 60 min. The exact time course of changes in stimulation evoked M1 responses could be analysed. Furthermore, evidence regarding the time profile and similarities to SEPs would motivate to analyse cell discharges in conjunction with SEPs contained in the LFP signal. Stimulation evoked responses in the LFPs could also be used to construct a map of stereotaxically defined active sites. This could be used to estimate motor-cortical reorganisation. Extracellular single cell recordings of pairs of neurons could shed light on potentially altered connectivity patterns induced by prolonged peripheral nerve stimulation. The analysis of cell responses to the finger abduction task could identify neuronal mechanisms related to increased or decreased behavioural performance after the interventions.

Finally, these findings should be used to develop improved behavioural assessments and interventions. Intervention-specific parameter including intensity, frequency, stimulation sites, and duration should be carefully optimised. The usage of wearable electronic devices enables advanced intervention protocols. These could include the use-dependent stimulation of adjacent muscle sites in subjects with FHD. Long-term applications of these novel stimulation paradigms (incorporating evidence of the underlying neurophysiology) might lead to more robust therapeutic interventions and potentially long-lasting effects in the treatment of dystonia and related neurological conditions.

Appendices

A. Sample SQL queries for behavioural analysis

- How many training sessions were registered in total for monkey S in the database `trainings`?

```
SELECT COUNT(*)
FROM trainings
WHERE monkey_id=1;
```

- When was the first training session for monkey U?

```
SELECT sdate
FROM trainings
WHERE monkey_id=2
ORDER BY sdate ASC LIMIT 1;
```

- What was the average correct trial number in all arm abduction training sessions for both monkeys (where a minimum of 1 trial was performed)?

```
SELECT AVG(correcttrials)
FROM trainings
WHERE correcttrials>0
AND category_id=1
GROUP BY monkey_id;
```

- What are all average correct trial numbers (with a minimum trial number of 1) averaged between both monkeys ordered and displayed by session / procedure name?

```
SELECT categories.name, AVG(trainings.correcttrials)
FROM trainings, categories
WHERE trainings.correcttrials>0
AND trainings.category_id=categories.id
GROUP BY categories.name
ORDER BY categories.name;
```

B. Immunohistochemical procedures

B.1 *Dehydration, crystal and lipid removal*

Before mounting, slices were (in the following order)

- washed for $5\,\mathrm{min}$ in dH_2O
- washed for $5\,\mathrm{min}$ in $70\,\%$ ethanol
- washed for $5\,\mathrm{min}$ in $95\,\%$ ethanol
- washed for $2 \times 5\,\mathrm{min}$ in $100\,\%$ ethanol
- washed for $2 \times 10\,\mathrm{min}$ in histo-clear[1]
- dried for $10\,\mathrm{min}$
- covered with histomount[2] and cover glasses[3]

[1] Agar Scientific, Catalogue Number: AGR1353.
[2] Fisher Scientific, Catalogue Number: 12825996.
[3] Menzel, Catalogue Number: BB024050A1.

C. D-wave modulation by epidural paired-pulse stimulation

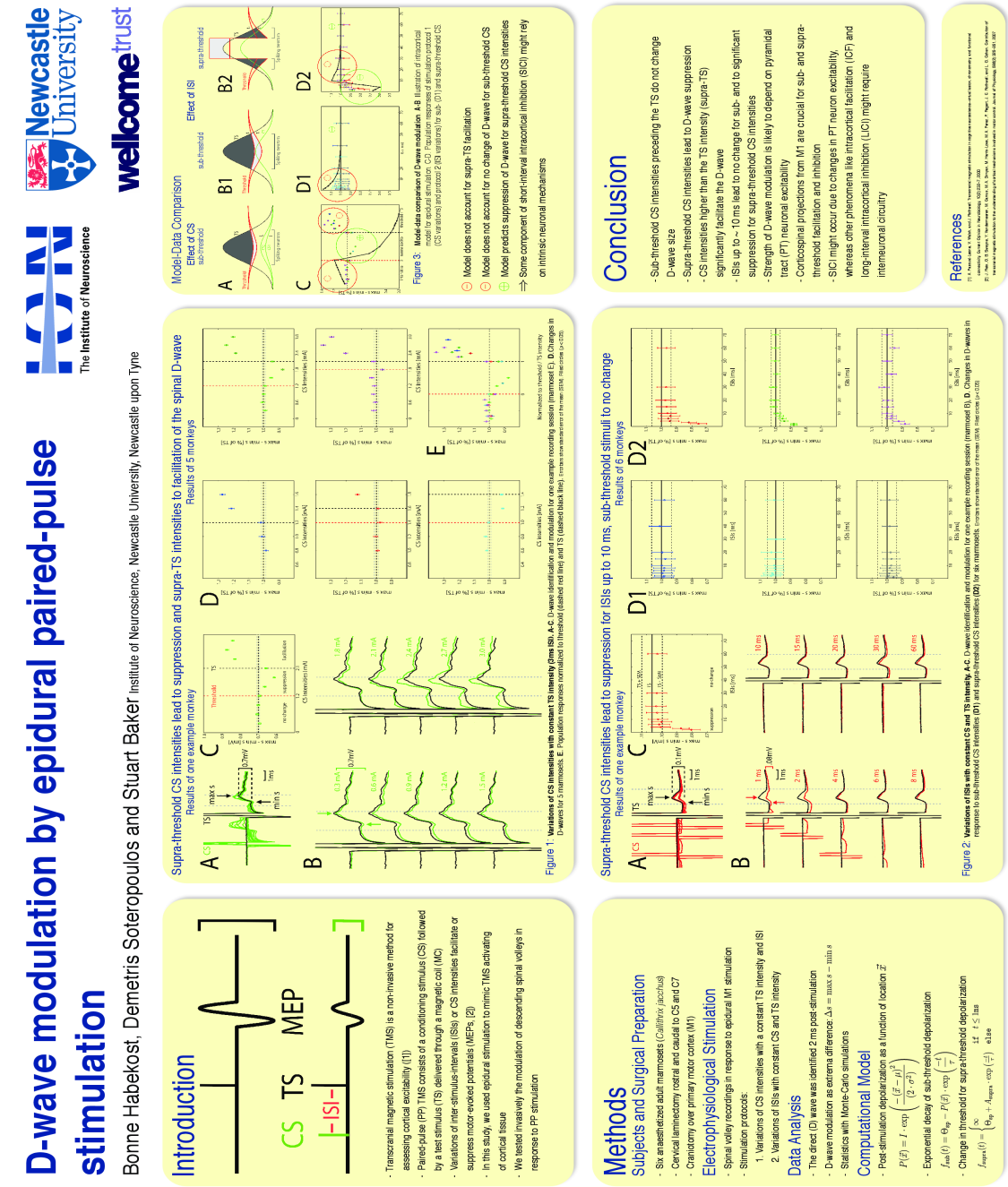

Figure C.1: *D-wave modulation by epidural paired pulse stimulation.* This poster illustrates the effects of paired-pulse stimulation paradigms on spinal D-waves. The majority of this work was conduced and analysed during my MRes studies at Newcastle University (additional data recorded and analysed at early stages of the PhD studies).

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