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PONTINE RETICULOSPINAL AND OTHER BULBOSPINAL PROJECTIONS IN THE NEWBORN MOUSE

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LIST OF ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
CaGDA	Calcium Green-1-conjugated dextran amine
CNS	Central nervous system
DF	Dorsal funiculus
EMS	Emotional motor system
HMC	Hypaxial motor column
GABA	Gamma-aminobutyric acid
IN	Interneuron
DLF	Dorsal part of the LF
LF	Lateral funiculus
LMC	Lateral motor column
MN	Motoneuron
MLF	Medial longitudinal fascicle
MMC	Medial motor column
MRF	Medullary reticular formation
mRS	Medullary reticulospinal
P0, P1, …	Postnatal day 0, 1, etc.
PRF	Pontine reticular formation
pRS	Pontine reticulospinal
RF	Reticular formation
VF	Ventral funiculus
VLF	Ventral part of the LF

INTRODUCTION

1.1 THE BRAIN STEM

The central nervous system (CNS) consists of several parts: Spinal cord (medulla spinalis), brain stem, diencephalon, forebrain (cerebrum) and cerebellum. The brain stem is positioned at the hub of this conglomerate – all the other parts of the CNS border on it, and signals from one part to another can not get there without passing through the brain stem – unaltered or not.

The brain stem is traditionally subdivided into several compartments – medulla oblongata (usually referred to as medulla), pons and mesencephalon based on gross anatomical landmarks on the surface. The medulla is closest to the spinal cord. It is bounded caudally by the pyramidal decussation and rostrally by the pontine nuclei. The pontine nuclei are easily identifiable on the ventral surface by superficial axon bundles that run from the pontine nuclei to the cerebellum. The rostrocaudal extent of these superficial bundles defines the limits of the pons. The subsequent part, still heading rostrally, is the mesencephalon. It is most easily distinguished by two mounds on each side of the midline on its dorsal surface; the inferior and superior colliculi. Sometimes the diencephalon, which is almost completely surrounded by the forebrain, is counted as part of the brain stem. We use Brodal's definition of the brain stem, which does not include the diencephalon.

The brain stem is largely made up of clusters of neurons with specific functions, called *nuclei*. These are too many to all be listed here[1], but fall into four categories:

1. Nuclei related to what are known as cranial nerves – nerves that emerge directly from the brain (mainly the brain stem), not from the spinal cord as other nerves do. These nerves carry afferent (incoming) sensory information – either from the musculoskeletal system and skin (somatic afferent) or from the internal organs (visceral afferent), or they carry efferent (outgoing) impulses - again either somatic (to the musculoskeletal system) or visceral (to internal organs, blood vessels, glands). Within

[1] A full list may be found in text books such as Kandel, E.R., Schwartz, J.H., Jessel, T.M., Siegelbaum, S.A. and Hudspeth, A.J., 2013, Principles of Neural Science, 5th edn., McGraw Hill Professional, USA.

the brain stem the neuron types serving each of these four different purposes (two afferent, two efferent) are typically separate. The cranial nerves, however, normally carry more than one of these information categories, collecting axons from several separate areas. As an example, the trigeminal nerve carries both sensory information from the face (somatic afferent fibers) and executive signals to the chewing muscles (somatic efferent fibers), while within the brain stem the motor trigeminal (somatic efferent) and various sensory trigeminal nuclei (somatic afferent) are separate entities with different locations.

2. Nuclei that have relay functions for the sensory afferent fibers that ascend from the spinal cord, analogous to the sensory cranial nerve nuclei.

3. Nuclei relaying information to the cerebellum. It should be pointed out here that the word "relay" does not really cover the role of any of the neuron groups I have described so far. These groups, and indeed any neuron group in the CNS, do not simply pass a signal on unchanged. In the CNS, any time a signal is transmitted from one neuron to another via synapses, this signal will be combined with signals from other neurons that synapse onto the same neuron, so that the signal that is passed on to the next neuron in the chain will contain more salient or appropriate information. A synapse also affords the possibility to *weight* the arriving information according to circumstance – e.g. so that sensory information from the surroundings that is not relevant at the moment can be suppressed, or likewise so that bodily movements can be suppressed if the situation demands it.

4. Nuclei that project very widely and use diffusely acting transmitter substances such as serotonin, noradrenaline and dopamine. They are able to affect large parts of the CNS at once, but without the precision or speed of synaptic transmission. They accordingly have general functions like modulating mood, alertness or the sleep/wake cycle.

The remainder of the brain stem is made up of axon bundles and a structure called the reticular formation (RF), which is the main focus of this thesis.

1.2 THE RETICULAR FORMATION

The RF occupies the entire rostrocaudal extent of the brain stem, from the medulla to the mesencephalon, and fills the remaining space not occupied by the nuclei whose categories were summarized in the previous section. These nuclei mostly have circumscribable functions and are identifiable histologically as aggregates of similar-looking cells. Not so for the RF: it has very diverse and wide-ranging functions which cannot be summed up in a few words. Nor does it form orderly groups that are easily delineated in classical histological sections. That is not to say that the RF does not have a typical histological appearance. A rather regular criss-cross of axons and dendrites gives it a net-like appearance – hence its name (Neo-latin: reticularis; net-like). The RF is phylogenetically old (it appeared early in evolution), and is present also in lower vertebrates (Metcalfe et al., 1986). This may explain why it seems to fill up the space between the other nuclei: The more specialized, phylogenetically newer nuclei may have sprung up within it during evolution like holes in a swiss cheese - leaving the RF to occupy the remaining space.

1.2.1 Subdivisions

The RF forms a continuous mass along the whole length of the brain stem, all the time relatively homogenous in appearance. There are, however, some variations in cell size and density, which enabled the pioneers in the field to make up a system of subdivisions (Table 1). The Olszewski-Brodal nomenclature is the one that is currently used in anatomical atlases, with some variation between species. The borders between the subdivisions defined in this manner are not sharp - changes in appearance are subtle and gradual. Consequently, the organization into groups is to some extent arbitrary, and it would probably not surprise the pioneers to learn that differences in function do not necessarily coincide with the anatomically based delineation. The different subdivisions are called nuclei, which is somewhat misleading, given the relatively homogenous, unbroken nature of the RF. Some authors prefer to replace the prefix “nucleus” with “formatio” when referring to the different subdivisions (Jones and Yang, 1985).

One aspect of RF organization that was noted early on (Torvik and Brodal, 1957) is that RF axons that project beyond the limits of the brain stem mainly originate in the medial 2/3 of the RF. The origin of the long projection axons coincides with the location of the so-called giant neurons. The large size of these neurons can probably be partly explained by the higher demands for functional maintenance resulting from a long

Subdivisions of the Reticular Formation		
Region	Johnston-Tretjakoff Nomenclature	Olszewski-Brodal Nomenclature
Medulla	N. R. inferior	Raphe group N. R. gigantocellularis N. R. paragigantocellularis N. R. magnocellularis N. R. parvocellularis N. R. dorsalis N. R. ventralis N. R. lateralis
Pons	N. R. medius and N. R. superior	Raphe group N. R. pontis caudalis N. R. pontis oralis N. R. tegmenti pontis N. R. pedunculopontinus Nucleus cuneiformis Locus coeruleus
Mesen-cephalon	N. R. mesencephali	Nucleus subcuneiformis
N. R. = Nucleus reticularis	Adapted from: Comparative Vertebrate Neuroanatomy, by Ann Butler	

Table 1. Two systems of subdivision of the reticular formation.

projecting axon – these axons quite often run the length of the spinal cord and send off numerous collaterals (branches) along the way (Matsuyama et al., 1997; 1999), innervating multiple target neurons and thus maintaining a large number of synapses, resulting in a far greater cell volume and surface and more synaptic machinery than neurons that only project locally. Neurons in the remaining, lateral 1/3 of the RF mainly

project locally and do not include giant neurons. This lateral part receives somewhat more afferent input than the medial part (Brodal, 1957; Torvik and Brodal, 1957). The medial, far-projecting part of the RF has the most direct relevance for motor function, and will be the focus of this thesis.

1.2.2 Connections

The RF has very extensive connectivity – both afferent and efferent. It receives afferent information from all sensory modalities, integrating information about the surrounding world and the body's relation to it. This information comes from sensory fibers (touch, pain, temperature, position of joints) or from the brain stem nuclei that receive visual input from the superior colliculus, auditory input from cochlear nuclei and information about linear and rotational acceleration from the vestibular nuclei.

RF also receives input from numerous other CNS areas including the spinal cord, basal ganglia, hypothalamus, periaqeductal grey and motor areas of the cortex. Most parts of the RF receive input from most of these sources, albeit with regional differences in the prevalence of the different projections (Brodal, 1957).

Efferent connections of the RF are similarly extensive. The main recipients of RF axons are the spinal cord (in particular motoneurons (MNs) and motor related interneurons (INs)), brain stem nuclei, the cerebellum and the thalamus. The reticular nuclei that project to the cerebellum (N. R. tegmenti pontis, N. R. lateralis and N. R. paramedianus) do not seem to project to the spinal cord (Torvik and Brodal, 1957). From early studies of projection using methods based on degeneration of neurons after localized lesions, it was reported a tendency that caudal RF parts preferably projected rostrally, whereas rostral parts tended to project caudally (Brodal, 1957). This was seen to be an elegant arrangement, because the rostrally coursing axons could then communicate with the caudally projecting neurons as they passed them, and vice versa. However, this view has been challenged by more recent experiments – which suggest the opposite situation: Rostral, mesencephalic RF areas tend to project rostrally to the thalamus and hypothalamus/subthalamus, and caudal, pontine and medullary RF areas tend to project caudally into the spinal cord (Jones and Yang, 1985). There are neurons that project the way Brodal reported, but they do not dominate quantitatively. And there are certainly numerous reticulo-reticular connections (between RF neurons) that could

provide the suggested crosstalk (Basbaum et al., 1978; Hempel et al., 1993; Matsuyama et al., 1993). In fact, Jones and Yang (1985) present an interesting concept: RF-*like* areas that together with the RF proper form a continuous band throughout the CNS below the forebrain. The groups that constitute the RF-like areas receive heavy projections from true RF areas. Through the basal forebrain connections, the RF can influence large parts of the forebrain. RF-like IN pools in the spinal cord extend this system in the caudal direction, in effect forming a continuous, heavily interconnected system of RF-like nervous tissue that stretches all the way from the basal forebrain, through the brain stem and throughout the length of the spinal cord.

1.2.3 Function

What does the RF do? The profuse projections from the RF to spinal cord MNs and motor related INs, along with incoming connections to the RF from sensory modalities and motor areas of the cortex gives an indication as to what may be the main role of RF – it seems to have something to do with motor function.

Such a role is supported by the identification of several areas within the RF that have been found to have relatively specialized functions, all related to motor control. In the ventrolateral medullary RF, areas have been located which has been suggested to govern the rhythmic activity of the diaphragm and other respiratory muscles (De Troyer et al., 2005). In the rostroventrolateral medulla, a relatively extensive area has been localized which is involved in regulating the blood pressure, by regulating the stroke volume and stroke frequency of the heart and the diameter of blood vessels via the smooth muscle cells in their walls (Bourassa et al., 2009). Adjacent to this area, an area can be found which is directly responsible for the atonia (absence of muscle activity) during REM sleep. This group again is governed by another RF area in the dorsal pons (Clement et al., 2011; Chase, 2013). Several areas governing eye movement have also been identified, among them a horizontal gaze center in the paramedian pontine RF, immediately adjacent to the abducens nucleus (which innervates the abducens muscle of the eye), and a vertical gaze center in the rostral interstitial nucleus of the medial longitudinal fascicle; rostral to the oculomotor nucleus (which innervates four out of six external eye muscles) (Scudder et al., 2002; Cullen and Van Horn, 2011).

After thus pointing out several areas within the RF with relatively well defined functions, it is necessary to turn the coin and point out that the typical RF neuron does not necessarily only serve one function. In an interesting review (Siegel, 1979), Jerome Siegel surveyed the large body of work done to identify functions of RF neurons, mostly using single unit recordings. He showed how RF neuron discharge has been related to sensory stimuli, pain and escape behavior, conditioning and habituation, arousal, complex motivational states, REM sleep, eye movements, respiration and locomotion. The problem he pointed out, however, was that many of the fields of research, formed around the different properties, often studied the same areas, and quite often reported large proportions of the neurons in those areas as being related to one specific function – e.g. habituation neurons, arousal neurons, and so on. The sum of the percentages of RF neurons dubbed in this way in different studies by far exceeded a hundred percent. It appeared that many of the researchers in the different fields were studying the same neurons, so that the specialization they each suggested could not be true. Siegel suggested, as a solution to this conundrum, that what the RF neurons are actually and primarily related to is the excitation of muscles, and that their activity during the different more specific behaviors could be explained by their relation to muscle activity. This view, although probably too simplistic to satisfy researchers within many of the aforementioned fields, is nonetheless well suited to the connectivity of the RF. And the notion that a large proportion of the activity of the RF is related to muscle activity is widely accepted.

One ascribed function of the RF deserves special attention. Whenever I tell my medical doctor friends that I study the reticular formation, the once diligent students immediately cry out “Consciousness!” And indeed, the RF has been ascribed an important role in consciousness (understood as the opposite of unconsciousness - not as “sense of oneself”). Moruzzi and Magoun (1949) discovered what they called the “ascending reticular activating system” or RAS. They found that electrical stimulation of the RF led to desynchronization of the EEG, with disappearance of the synchronous wave pattern seen in the EEG of a sleeping animal – identical to the changes seen during naturally occurring arousal from sleep or drowsiness. Other results contributing to the RAS concept has been the discovery of an area within the mesencephalic RF, the bilateral lesioning of which leads to irreversible coma (Lindsley et al., 1950). A role for the RF in consciousness is not universally accepted, however. According to Siegel (1979), RF activity during sleep/wake transition should be seen as motor related, since

an increase in mental arousal would more likely than not be accompanied by increased muscle activation, and hence an increase in RF neuron activity. Of relevance to the discussion, when bilateral lesions of the mesencephalic RF are made sequentially, one side at a time, with an interval of two weeks, coma is not induced – animals so lesioned continue to feed, move around and have diurnal sleep-wake cycles (Adametz, 1959; John, 2006).

1.3 MOTOR APPARATUS

1.3.1 Muscles

When we move an arm, a finger, or an eyebrow, it is muscles at work. A muscle is a bundle of muscle cells (also known as muscle fibers) encased in a connective tissue capsule. At each end the muscle has a tendon or tendons that attach to bone, skin (as is the case for the eyebrows), or other fibrous tissue. For skeletal muscles, two attachment points need to be on two different bones, the muscle passing at least one joint on the way, so that when the muscle contracts, movement can occur. And for a controlled movement to occur, several muscles with different attachment points need to work at the same time. Through the action in concert of many muscles throughout the body, we can perform coordinated movements where every body part operates in harmony. But for that to be possible the muscles need something to coordinate their actions. There is need for motor control.

1.3.2 Motoneurons

Muscles are controlled directly by MNs in the spinal cord or brain stem. MN axons contact each muscle cell through a large conglomerate of synapses – the motor end plate. An action potential initiated at, or close to, the soma of a MN is transmitted along its axon and then to the axon terminals, which release the neurotransmitter substance acetylcholine. Binding of acetylcholine to acetylcholine receptors on the postsynaptic side of the motor endplate leads to depolarization and contraction of the muscle cell. The axon of any given MN branches and makes contact with several muscle cells. The group of muscle cells together with the MN that contacts them is called a motor unit, and is the smallest part of the musculoskeletal system that can be individually controlled.

In the spinal cord the MNs are located in the ventral horn (Figure 1, lamina 9). MNs controlling the same muscle are grouped into pools. Pools of MNs controlling muscles

with synergistic actions around a joint are often grouped together into larger functional pools (McHanwell and Biscoe, 1981). A yet more general organizational level is the grouping of the muscle pools into motor columns. The MNs innervating skeletal muscles are organized in three motor columns: The medial motor column (MMC), the lateral motor column (LMC) and the hypaxial motor column (HMC) (Philippidou and Dasen, 2013). MNs in the MMC innervate axial muscles (neck, trunk), MNs in the

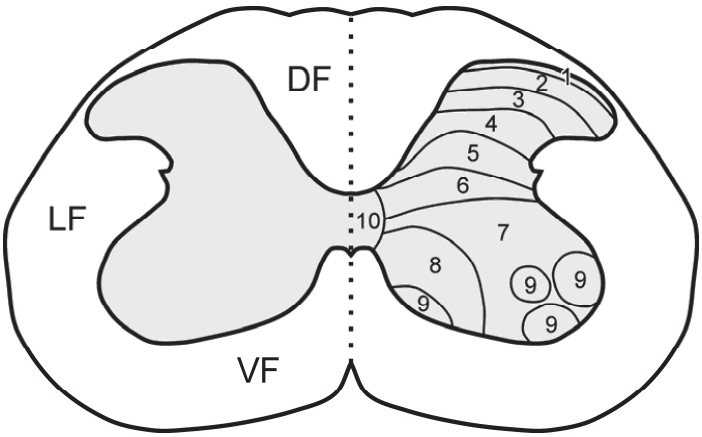

Figure 1. The spinal cord: White matter funiculi and Rexed's laminae. Left: The white matter of the spinal cord is made up of projecting axons and supporting tissue. It surrounds the grey matter, and is separated into three funiculi by the ventral and dorsal horns of the spinal grey matter. The path of the axon within the spinal white is an important distinguishing feature between different projection neurons. Right: The spinal grey matter, consisting of spinal cord neurons, is divided into 10 lamina, defined by Rexed (1952). Lamina 9 contains, by definition, clusters of MNs, of which there are several in the ventral horn. Where within the spinal grey the axons terminate is an important distinguishing feature between different projection neurons. Dotted line: Midline. DF: Dorsal funiculus, LF: Lateral funiculus, VF: Ventral funiculus. The illustration represents a transverse section from cervical level 5/6 in adult rat and is based on a figure in Jones and Yang (1985).

LMC innervate limb muscles while MNs in the HMC innervate intercostal and body wall muscles. The extremities are only innervated from certain segments of the spinal cord; arms from cervical segments (C5 to T1) and legs from lumbar and sacral segments (L1 to S2). It follows that the LMC is only present in these segments, whereas the MMC runs the length of the spinal cord. The larger amount of MNs (and INs, see below) needed for extremity innervation and control leads to corresponding

increases in spinal cord diameter. These are known as the cervical and lumbar enlargements.

1.3.3 Interneurons

Interneurons (INs) are neurons that only send their axons to other neurons within one part of the CNS. INs are thus by definition different from *projection neurons*, which send axons to other parts of the CNS, e.g. from the brain stem to the spinal cord. In the spinal cord, a large population of INs are essential to motor control (Jankowska, 2008). These motor-related INs are located in the intermediate zone and the ventral horn of the spinal cord grey matter (Figure 1). They receive sensory input and input from the descending motor pathways (see below). They make synaptic contact with MNs and other INs. With the exception of the monosynaptic reflex, INs are constituents of all reflex pathways between sensory inputs and motor outputs. Descending control of MN activity is to some extent the result of direct connections onto the MNs themselves, but indirect connections via INs also play an important role (Floeter and Lev-Tov, 1993; Alstermark and Ogawa, 2004). The IN networks probably serve to integrate incoming information with ongoing motor activity, so that the effect of descending signals can be adapted to the phase and context of the ongoing movement (Drew et al., 2004).

1.4 DESCENDING MOTOR PATHWAYS

A number of distinct areas in the cortex and the brain stem contribute to motor control. They do so through neurons extending axons that descend into the brain stem and spinal cord, making contact with MNs or motor-related INs.

Apart from their origin, the different motor pathways can be characterized by a number of characteristics (Lemon, 2008). Some of the more distinguishing characteristics are:

- Path taken by the descending axons within the spinal white matter (spinal trajectory, Figure 1).
- Number of spinal cord segments the axons contact by sending off branches (degree of axon collateralization).
- Location of the axon terminals within the spinal grey matter (termination pattern, Figure 1).

There are notable differences between species in the characteristics of each individual pathway, in particular when it comes to the phylogenetically young corticospinal pathway.

1.4.1 Corticospinal

Several cortical areas contribute to the corticospinal pathway. In monkey, these are: primary motor cortex (M1), dorsal and ventral premotor cortices, supplementary motor area (SMA), cingulate motor area, primary somatosensory cortex (S1), posterior parietal cortex, and the parietal operculum. It is mainly a crossed pathway; the axons cross at the junction between the brain stem and the spinal cord. A small proportion of the axons continue on the side of the neurons of origin (ipsilaterally). In the spinal cord the axons run dorsally in the lateral funiculus (LF, see Figure 1) in cats and primates, but in the dorsal fascicle (DF) in rodents. The cortical pathway is phylogenetically younger than the other descending pathways, and is relatively more important in evolutionary "higher" species, in particular in primates (Lemon, 2008).

1.4.2 Rubrospinal

Nucleus ruber is located in the central mesencephalon. Its axons all cross at the caudal end of the nucleus itself, and course contralaterally through the brain stem and through the lateral fascicle of the spinal cord. This pathway, while of functional importance in rodents and cats, is much diminished in primates, and rudimentary in humans (Brodal, 2010).

1.4.3 Vestibulospinal

The vestibular nuclei receive afferent input from the vestibular organs, which sense rotational and linear acceleration of the head. There are two separate pathways, emanating from different parts of the vestibular nuclei: The medial vestibulospinal tract (MVST) and lateral vestibulospinal tract (LVST) pathways. The MVST innervates MNs at cervical level, while the LVST innervates MNs both at cervical and lumbar level. Both mediate reflexes that counteract unexpected movement: the MVST by action on neck muscles that control head movement, the LVST by action on fore- and hind limbs (which affect body position) (Kasumacic et al., 2010). The axons of both the MVST and LVST run in the ventral fascicle.

1.4.4 Reticulospinal

The RF and its role in motor control are treated extensively elsewhere in this introduction.

1.4.5 Emotional motor system

In the interest of making a complete list of descending motor pathways, the emotional motor system (EMS) (Holstege, 1998) should also be mentioned. This is a concept that refers to motor related pathways that emanate from or are related to the limbic system. It consists of the lateral and medial emotional motor systems. The lateral EMS refers to fore- and midbrain areas related to basic emotions that work via the descending motor pathways that have already been listed. The medial EMS refers to spinally projecting brain stem nuclei employing the neurotransmitters serotonin (raphe nuclei, located at or close to the midline) and noradrenaline (locus coeruleus, in mid-pons). These neurotransmitters are not released at synapses (with immediate effects on one neuron), but upon release diffuse to several neurons in an area around the release site (with delayed effects on many neurons). With such a diffuse area of effect, these projections do not readily fit into a connection diagram of motor control networks, but they can strongly modulate the excitability of the MNs and INs that make up the networks.

1.4.6 Other spinal pathways

Several more spinal pathways exist, but are not treated separately here. These include the tectospinal and interstitiospinal pathways, and the spinal projections of dopaminergic and histaminergic neurons.

1.4.7 Functional considerations

The different motor control pathways have different functions, which can be inferred from their anatomical connections and tested in different ways, for example: 1) Stimulate neurons in a region of origin and observe the effects on MNs and muscles. 2) Record descending neuron activity during natural movement. 3) Sever descending pathway axons and observe the resulting impairments. 4) Compare different species that lack one or the other pathway (although all vertebrates have a reticulospinal pathway and most have some version of a vestibulospinal pathway). Using these and other methods, it has been concluded that cortico- and rubrospinal pathways are involved especially in voluntary control of movement and in hand and individual digit movement. The corticospinal motor connections have increasing importance in "higher" vertebrates like cats, monkeys and humans. Heffner and Masterton (1983) established a linear relationship between the size of the corticospinal projection and level of hand dexterity, the implication being that this projection has evolved together with increased fine motor abilities of the paw/hand.

The vestibulospinal system is important in adapting to and counteracting unexpected movements of the body as a whole or of the head in relation to the body, both while standing still or as on-the-go corrections of ongoing movement, for example during walking.

The reticulospinal system has an important role in automatic movement, that is, movement that we humans do not need to think actively about performing. These are often rhythmic, like walking, breathing or chewing, and often involve combined movement of many body parts in fixed patterns, as for example in reaching or gaze shifts.

Many species do not have a corticospinal pathway but still have a diverse and highly functional repertoire of movements. It is therefore natural to assume that even in the species that do have a corticospinal motor connection, the other descending pathways are responsible for the bulk of movements. This also entails that these systems can produce any and all movements that animals without a corticospinal pathway can do. There seems to be a tendency, however, that in species with a strong cortico-motoneuronal connection (humans, in particular), this connection has taken over some functions.

It should be stressed that it is not the case that one descending system controls any movement alone, while the other systems are silent. Rather, they work all at the same time, in concert, and the overlap and integration between systems is extensive. Thus, it is misleading to speak of any particular movement or behavior as belonging to one particular system. Rather, the sum of the descending systems should be seen as one big system, perfected through millions of years of evolution to the task of controlling bodily movement.

1.5 THE RETICULOSPINAL SYSTEM

1.5.1 Anatomy

The reticulospinal system is made up of neurons within the RF that send axons into the spinal cord. To determine the location, trajectory and termination pattern of these neurons several methods are available (Lanciego and Wouterlood, 2011). Most of these methods depend on some form of axonal transport. Historically, neuronal degeneration

has also played a major role. These methods can be applied either to gain information about the projection areas and characteristics of the axons and their terminations (anterograde degeneration/labeling), or to determine the location and the morphology of the neurons that project to a specific area (retrograde degeneration/labeling). Anterograde and retrograde techniques thus provide complementary information.

1.5.1.1 Spatial distribution of reticulospinal neurons

Retrograde tracing and degeneration studies largely agree on the distribution of reticulospinal neurons. Minor disagreements occur, which is to be expected since the experiments have been performed on different species using varying methods. Most studies agree that the reticulospinal neurons are more numerous in the medulla than in the pons, and are few in the mesencephalon (Figure 2). (Cat: Kuypers and Maisky, 1975a; Hayes and Rustioni, 1981; Rat: Leong et al., 1984a; Jones and Yang, 1985; Newman, 1985a; Newman, 1985b; Mouse: Vanderhorst and Ulfhake, 2006; Liang et al., 2011). Some researchers have found more mesencephalic reticulospinal neurons in rat than in cat (Basbaum and Fields, 1979). Reticulospinal neurons project to the spinal cord either ipsilaterally (same side as their somata) or contralaterally (opposite side), with a larger number projecting on the ipsilateral side. This ipsilateral preponderance is more obvious in the pons than in the medulla, but this is not a universal rule. Some researchers find a more even distribution (Liang et al., 2011) and some find a contralateral preponderance in the pons (Figure 2 (Kuypers and Maisky, 1975b)). Furthermore, a cluster of contralaterally labeled neurons is consistently found ventrolaterally in the rostral pons, in a position where few neurons are labeled on the ipsilateral side. Neurons that project both ipsi- and contralaterally, sending collaterals into both halves of the spinal cord, seem to be very rare (Glover and Petursdottir, 1991).

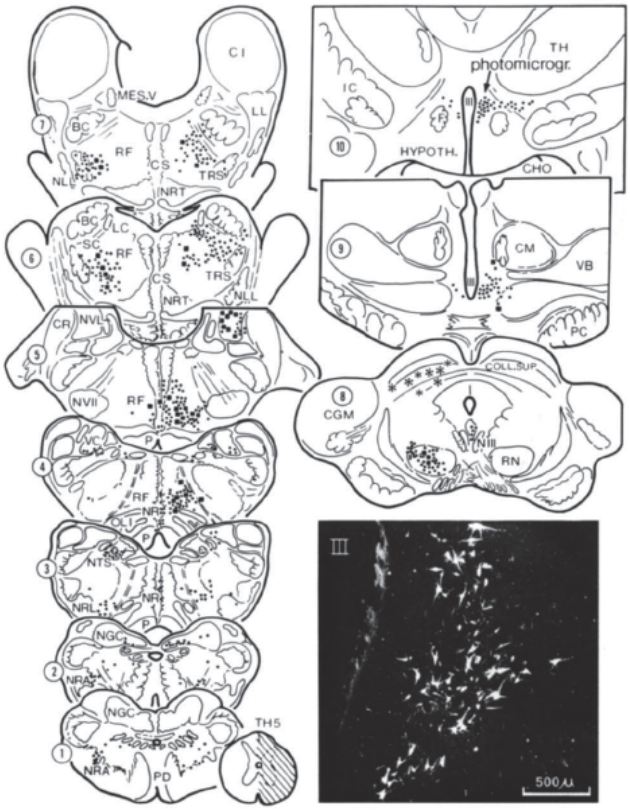

Figure 2 Example of labeling of spinally projecting brain stem neurons, in cat, from Kuypers and Maisky (1975). One half of the spinal cord was labeled with horseradish peroxidase at thoracic level 5 (small icon, bottom middle). The cartoons illustrate labeled neurons at different rostrocaudal levels of the brain stem. The reticular formation was labeled bilaterally all levels, with an ipsilateral preponderance at medullary levels (1-5) and more equal ipsi- vs. contralateral distribution at pontine levels (6-7).

1.5.1.2 Spinal trajectory of the reticulospinal axons

In the spinal cord, long-projecting axons travel in the outer layer, which is called the white matter. When the axons reach their target segments they enter the grey matter, the inner layer of the spinal cord where the somata of the spinal cord neurons (MNs and INs) are localized. The grey matter forms an X in the transverse plane, and the white matter is usually subdivided into three compartments, or funiculi, formed on each side by this X and the midline. Thus, on each side, there is a dorsal funiculus (DF), a ventral funiculus (VF), and a lateral funiculus (LF) (Figure 1). The LF is often further

subdivided into two parts of roughly equal size, the ventral LF (VLF) and the dorsal LF (DLF). Information about the position of specific groups of descending axons within the white matter can be obtained both by retrograde and anterograde tracing. Retrograde and anterograde tracing experiments indicate that reticulospinal neurons in the medulla, in N. R. gigantocellularis, project ipsilaterally in the VF and the VLF and contralaterally in the VF. From the caudal pons (N. R. pontis caudalis), axons run mainly ipsilaterally in the VF. From the rostral pons (N. R. pontis oralis) axons run ipsilaterally in the VF and VLF, and contralaterally in the LF (Retrograde: Kuypers and Maisky, 1977; Martin et al., 1979; Anterograde: Basbaum et al., 1978; Jones and Yang, 1985).

1.5.1.3 Termination patterns of reticulospinal neurons

The spinal cord grey matter has a uniform organization throughout the spinal cord, with some specialization at different levels. The commonly used subdivision, introduced by Rexed (1952), divides the spinal grey matter into ten laminae, named I-X (Figure 1). Laminae I to VI form the dorsal horn and contain INs that are heavily targeted by sensory afferents. Laminae VII and X form the intermediate zone and, together with lamina VIII in the ventral horn, contain many of INs involved in regulating MNs. Lamina IX in the ventral horn contains the somata of the MNs. In the thoracic segments, a specialized region, the intermediolateral column, is found lateral to the intermediate zone, forming a lateral horn in these segments. It contains the "motoneurons" of the sympathetic system, the sympathetic preganglionic neurons.

Information about where in the spinal cord grey matter the axons from the different reticulospinal regions terminate have been obtained mainly by anterograde tracing (Basbaum et al., 1978; Holstege and Kuypers, 1982; Jones and Yang, 1985) or degeneration (Nyberg-Hansen, 1965), but has also been mapped using electrophysiological methods (Peterson et al., 1975). The termination pattern from medullary levels is not easily summed up, because there are many different medullary areas that seem to terminate differently. There is also some disagreement among different authors. Some medullary areas terminate mainly in laminae VII-VIII, with some termination also in IX, while others terminate mainly in the intermediate zone and dorsal horn. Some ventral medullary areas also terminate in areas related to the autonomic system, like the intermediolateral column (Basbaum et al., 1978). From the pons, the ipsilateral reticulospinal neurons have a relatively restricted termination

pattern, mainly distributed between laminae VII and VIII. The contralateral, ventrolaterally located cluster terminates quite differently, distributing axons in laminae IV-VII, and possibly also in laminae I-II (Holstege and Kuypers, 1982).

1.5.2 Embryology

The reticulospinal axons are among the first descending axons to reach the spinal cord during embryonic development. In the north-american opossum, which is born at a very immature stage, 12 days after conception, reticulospinal axons are already present in the spinal cord at birth (Martin et al., 1988). In the chicken, the first axons reach the spinal cord from the RF about three days after fertilization, one and a half days before the first vestibulospinal axons arrive (Glover and Petursdottir, 1991). In the following days the descending projections develop quickly, reaching an essentially mature organization on embryonic day 9 (Glover and Petursdottir, 1991). In rodents, where the embryological time-scale is different from that in chicken, the first reticulospinal axons reach the spinal cord at embryonic day (E) 11 in mice (the average gestation period of mice is 20 days) and E12.5 in rats (Auclair et al., 1999). In rats, the lumbar spinal cord is reached around day 14 (Kudo et al., 1993). The reticulospinal groups increase in size and complexity of organization through subsequent embryonic stages (Glover and Petursdottir, 1991; Auclair et al., 1999). By birth, reticulospinal populations in the mouse exhibit essentially the same labeling pattern as in adults (Leong et al., 1984a).

How this pattern is specified and obtained during embryonic development is still unresolved. Differentiation of different neuron types arises through complex sequences of transcription factor expression and signaling molecule gradients. Several studies have linked cytoarchitectonic and functional characteristics in both the medulla and pons to segmental (Narita and Rijli, 2009; Philippidou and Dasen, 2013; Di Bonito et al., 2013a; Di Bonito et al., 2013b) or mediolateral and dorsoventral (Cepeda-Nieto et al., 2005; Gray, 2013) patterns of transcription factor expression. However, mapping of transcription factor expression has only rarely been compared to hodological (projection-based) organization, although it has been commenced for the vestibular brain stem areas (Pasqualetti et al., 2007). Transcription factor mapping of the RF areas is on the whole far from complete (Gray, 2013). For the reticulospinal population, the transcription factors Lhx3, Lhx4 and Chx10 have been implicated (Cepeda-Nieto et al., 2005), but most of the players in the differentiation process remain unknown (Perreault and Glover, 2013).

1.5.3 Evolution

The reticulospinal system is a common feature in vertebrate species that are otherwise very different and separated on the evolutionary tree. The general pattern of organization of the reticulospinal neuron groups, in terms of localization within the brain stem and ipsi- vs. contralateral descent, is similar in all mammals (Nudo and Masterton, 1988). Virtually the same pattern has also been found in marsupials (Martin et al., 1979). In fact, reticulospinal neurons sharing some basic organizational features can be found in all vertebrates (Metcalfe et al., 1986; Nudo and Masterton, 1988). This indicates that the reticulospinal system is phylogenetically ancient, inherited from a common ancestor living several hundred million years ago (Glover and Petursdottir, 1991).

1.5.4 Functional connections and activity patterns

As we saw when considering termination patterns, most reticulospinal neurons contact areas containing motor related INs, and some also appear to contact the MNs directly. These relationships have been further elucidated in electrophysiological studies. While activating reticulospinal neurons by stimulating the brain stem electrically, synaptic responses have been recorded in lumbar MNs at latencies indicating both direct (monosynaptic) connections and indirect connections where one (disynaptic) or more (polysynaptic) INs are intercalated in the pathway (Grillner et al., 1968; Wilson and Yoshida, 1969; Peterson et al., 1979; Floeter and Lev-Tov, 1993; Alstermark and Ogawa, 2004; Riddle et al., 2009). Although results differ, it seems that di- or polysynaptic connections are more common than monosynaptic ones.

Reticulospinal axons traverse much of the length of the spinal cord, and have extensive projections, sending out axon collaterals at many levels along the way both on the ipsilateral and contralateral side (Peterson et al., 1975; Hayes and Rustioni, 1981; Matsuyama et al., 1997; 1999). This suggests that individual reticulospinal neurons may be able to simultaneously activate MNs at different spinal levels (e.g. both cervical and lumbar enlargements). To my knowledge this has never been tested experimentally. But although it has not been demonstrated for individual reticulospinal neurons, the reticulospinal projection has been shown capable of activating MNs controlling muscles in either of the four extremities, and the trunk, monosynaptically (Peterson et al., 1979). The reticulospinal system does not seem able to control individual finger

movement, but can activate finger muscles en bloc during concerted movement, for example during climbing (Lawrence and Kuypers, 1968a; 1968b; Baker, 2011).

The reticulospinal axons descend into the spinal cord either ipsilaterally or contralaterally, and in some areas of the RF the reticulospinal neurons of origin are intermingled. Accordingly, reticulospinal stimulation can result in activation of muscles both ipsilateral and contralateral to the stimulation side (Davidson and Buford, 2006). Reticulospinal stimulation in intact, awake animals also demonstrates the widespread connections of the reticulospinal neurons, as near-threshold stimulation typically results in combined movements of several body parts at once (Drew and Rossignol, 1990a; Drew and Rossignol, 1990b). Though highly variable, a typical response to such stimulation is turning of the head towards the stimulated side, accompanied by flexion of the forelimb on that side. In freely moving cats, single cell recording from identified reticulospinal neurons have shown that reticulospinal neurons discharge during locomotion, with and without gait modifications (Prentice and Drew, 2001), and during reaching (Schepens and Drew, 2004). Reticulospinal neurons are often active during several phases of these movements, and some display the same activity during the same movement phase irrespective of the side performing the movement (for example whether the left or right forelimb perform reaching). Many reticulospinal neurons are also active during both gait modification and reaching tasks (Schepens and Drew, 2000). These data indicate that reticulospinal neurons do not always elicit the same pattern of muscle activity, but rather activate different sets of muscles depending on the behavioral context. How this context-dependent differential activation is achieved is uncertain. Changes in the activity patterns of the reticulospinal neurons have been suggested, as have changes in the excitability of target neuronal networks in the spinal cord (Drew et al., 2004).

It has been commonly held that the reticulospinal system activates flexor muscles more strongly than extensors. This notion has been based on imbalances between flexors and extensors in stroke survivors and on early electrophysiological studies (Baker, 2011). However, electrophysiological experimentation has shown a more differentiated pattern. In cat, stimulation of reticulospinal neurons results in flexion in the ipsilateral forelimb and extension in the contralateral forelimb, while there is a more mixed picture for the hind limbs (Drew and Rossignol, 1990a). In monkey, Davidson and Buford (2006) found that for the wrist, elbow and shoulder there is a situation of

reciprocal flexor/extensor activation between the two sides – activation of the reticulospinal projection tends to facilitate flexors and suppress extensors ipsilaterally, and suppress flexors and facilitate extensors contralaterally, compatible with the findings for forelimbs in cat.

1.5.5 Neurotransmitters

The bulbospinal (from brain stem to spinal cord) projection systems are predominantly glutamatergic, i.e. the transmitter substance released at their axon terminals is the excitatory neurotransmitter glutamate (Du Beau et al., 2012). This does not mean that the only effect of descending signals is excitatory. During microactivation studies (Davidson and Buford, 2006) 58 % of effects on muscles were suppressive (inhibitory), while 42 % were facilitatory (excitatory). But inhibitory effects are more often mediated through inhibitory interneurons in the spinal cord than by inhibitory descending axons (Grillner et al., 1968; Jankowska et al., 2003). Furthermore, while the corticospinal and rubrospinal systems are more or less exclusively glutamatergic, the vestibulospinal system and the reticulospinal projection have a more mixed transmitter make-up. In a study investigating the transmitter substance of the different descending systems (Du Beau et al., 2012), 63 % of the terminals of MLF axons (of which many are reticulospinal axons) at lumbar level were glutamatergic, 20 % utilized the inhibitory neurotransmitters GABA or glycine (GABA: 8%, Glycine: 9 %, GABA + Glycine: 3 %), while about 20 % were not positive for any of these neurotransmitters. Investigations utilizing retrograde labeling have indicated that GABAergic and glycinergic reticulospinal neurons are concentrated in the ventromedial region of the medulla (Hossaini et al., 2012). GABAergic and glycinergic reticulospinal neurons project to the dorsal horn, where they probably contribute to pain regulation, and to the ventral horn, where they are probably involved in the atonia seen during REM sleep (Chase, 2013).

1.5.6 Perspective of the present project

The function of the RF is still far from fully understood, offering a selection of unanswered questions for researchers in the field. For example, while the activity of single neurons has been studied, in the RF during natural movement (Prentice and Drew, 2001) or in spinal MNs (Alstermark and Ogawa, 2004) or INs (Jankowska et al., 2003) during RF stimulation, how they together are able to produce the natural movements seen in the living animal remains unresolved. From this perspective it is

important to know not only how single MNs act in response to descending reticulospinal signals, but also how the MNs react as a population. In this thesis I simultaneously recorded calcium signals in many spinal MNs while stimulating the pontine RF (PRF) in an isolated brain stem-spinal cord preparation. This made it possible to record the activity of whole populations of MNs at once and to compare, in different recordings, the response to the same RF stimulus in MN populations on opposite sides of the spinal cord or at different spinal levels.

The calcium imaging method as applied in this thesis can only be performed on neonatal mice, as preparations from older animals cannot be kept alive ex-vivo for extended periods of time. The neonatal mouse has proved to be a useful research platform also in other applications within biological research (Locomotor rhythm: Whelan et al., 2000; Respiratory rhythm: Kobayashi et al., 2010; Perinatal hypoxia-ischemia: Yamagata et al., 2013).

As research on spinal projections in the neonatal mouse continues, exact anatomical knowledge of the projecting populations in the brain will be important. In this thesis I have therefore carried out an anatomical characterization of bulbospinal neurons in the newborn mouse. The immediate motivation for this characterization was that during our calcium imaging work, we searched in vain for exact information on the location of the pontine reticulospinal neurons in the newborn mouse. The spinally projecting brain stem neurons in newborn rodents have, to our knowledge, only been described once, in rat (Leong et al., 1984b), but we have found this presentation altogether too brief and too lacking in detail to be particularly useful to us. Presumably, this sentiment is shared by other researchers with similar interests to ours. The fact that Leong (1984b) describes the newborn rat and not the newborn mouse is symptomatic, as there is a relative scarcity of published descriptions also in the adult mouse - we only know of two (Vanderhorst and Ulfhake, 2006; Liang et al., 2011). Descriptions of bulbospinal neurons in the rat are more prevalent, describing either all bulbospinal neurons (Basbaum and Fields, 1979; Leong et al., 1984a; Kudo et al., 1993) or focusing on specific brain stem populations or specific functions (Rostral medulla: Basbaum et al., 1978; Reticular formation: Jones and Yang, 1985; Newman, 1985a; Newman, 1985b; Pontine reticular formation: Thankachan et al., 2012; Rye et al., 1988; Trigeminal areas: Diagne et al., 2006; Mesopontine tegmental anesthesia area: Reiner et al., 2008; Locus coeruleus: Chen and Stanfield, 1987; DLF-projecting: Watkins et al., 1981; Sacrocaudal projections: Masson, Jr. et al., 1991; Penile muscle control: Shen et al., 1990). A major concern with these papers is whether or not measures have been taken

to reliably discriminate between ipsi- and contralaterally descending neurons. Injections into the spinal cord are usually made unilaterally, so that labeled neurons in the brain stem found on the same side are mostly ipsilaterally descending and neurons found on the opposite side mostly contralaterally descending. However, there is a chance that some dye will seep into and be taken up by nerve endings in the opposite half of the spinal cord, so that some ipsilaterally projecting neurons will be interpreted as contralaterally projecting and vice versa. And even if dye does not contaminate the opposite hemicord, some axons have collaterals that cross the midline at spinal levels (Matsuyama et al., 1993; 1997; 1999), again making unwanted labeling possible of axons descending in the opposite hemicord. A reliable way to avoid these two sources of error is to interrupt the axons of the opposite hemicord rostral to the injection site (Basbaum and Fields, 1979; Leong et al., 1984a; Leong et al., 1984b; Vanderhorst and Ulfhake, 2006; Reiner et al., 2008). Due to methodological constraints (e.g. concerns about the welfare, or even survival of the animal during the labeling period) such a restricting lesion have not been performed in many of the mentioned papers (Watkins et al., 1981; Jones and Yang, 1985; Chen and Stanfield, 1987; Rye et al., 1988; Shen et al., 1990; Masson, Jr. et al., 1991; Diagne et al., 2006; Liang et al., 2011). In the present thesis, a lot of effort went into ensuring an effective contralateral control lesion rostral to the labeling site.

We have also performed experiments where labeling has been restricted to smaller parts of the spinal white matter, in order to gain information on funicular trajectory of the axons of the bulbospinal neurons. This is valuable information, in humans due to diagnostic and clinical implications, and in all animals because funicular trajectory to some extent determines termination pattern in the spinal gray matter. Furthermore, this information can inform (electro-)physiological experimenters that want to interrupt specific descending pathways. Funicular trajectory information based on retrograde labeling has not previously been available in mice, but has been gathered in several other vertebrates (Cat: Kuypers and Maisky, 1977; Opossum: Martin et al., 1979), including rat (Basbaum and Fields, 1979).

2 AIMS

The aims of this Ph.D.-project were:

1. Determine whether pRS neurons have functional synaptic connections to spinal MNs in the newborn mouse, and describe the organization of these connections in terms of laterality and different spinal levels (Paper 1).

2. Characterize the internal organization of pRS neurons in the newborn mouse - their geographic distribution, the relationship between ipsi- and contralaterally projecting neurons, and the funicular trajectory of their axons within the spinal cord, using retrograde tracing (Paper 2).

3. Describe the organization of all bulbospinal neurons in the newborn mouse according to laterality and axon trajectory in the white matter, thus creating an atlas that can be used to more effectively investigate the structure and function of these neurons (Paper 3).

3 METHODS

3.1 THE BRAIN STEM SPINAL CORD PREPARATION

All the experiments presented in this thesis were performed on brain stem-spinal cord preparations dissected from newborn mice. The preparations were maintained ex vivo in artificial cerebrospinal fluid (ACSF) for the duration of the experiments. Brainstem-spinal cord preparations from rats have been in use for the last twenty years, but the mouse preparation was recently developed in our lab (Szokol et al., 2008; Szokol and Perreault, 2009).

The dissection procedure is as follows: Newborn mice are anesthetized using Isofluran inhalation. After ensuring that the mice are deeply anesthetized, the skull is opened from the dorsal surface, and the mice are decerebrated by separating the forebrain from the rest of the nervous system using a sharp spatula, in an oblique plane from above the superior colliculi on the dorsal surface to above the corpora mamillaria on the ventral surface. The mice are then immersed for the rest of the dissection process in oxygenated ice-cold dissection-ACSF (dASCF). In this solution all the NaCl content of the normal ASCF has been replaced by glycerol with the same osmolarity, an augmentation that has been shown to improve neuron viability in brain slice preparations (Ye et al., 2006; Tanaka et al., 2008). The mice are then euthanized by severing the great vessels above the heart, quickly eviscerated (the heart and other inner organs are removed) and the brain stem and spinal cord are carefully dissected out in one piece, from the ventral side. When the dissection is complete, the brainstem-spinal cord preparation is transferred to oxygenated, room temperature ACSF, where it remains for the rest of the experiment.

3.2 RETROGRADE NEURON LABELING

In all the experiments in this thesis we made use of different applications of retrograde labeling using fluorophore-conjugated dextran amines (Glover and Petursdottir, 1991). This method is based on cutting bundles of axons with sharp dissection scissors while the preparation is submerged in ACSF, quickly applying pre-made crystals of fluorophore-conjugated dextran amine to the freshly cut surface, and replacing with new crystals periodically to maintain the tracer concentration as high as possible for about 3 minutes. The tracer is taken up at the axon ends, and is transported retrogradely to the neuronal somata of origin during an incubation period that depends on axon length (3-12 hours).

3.3 MICROELECTRODE STIMULATION

In the calcium imaging experiments (see below), electrical current was delivered to the PRF using a monopolar microelectrode and a digital stimulator coupled to a stimulus isolation unit. The microelectrode was inserted into the brain stem from the ventral surface at a reproducible angle. Prior to electrode insertion we removed the meninges to avoid tissue dimpling and electrode damage. To establish an area from which responses could reliably be evoked in spinal cord MNs, we made electrode insertions over a broad range covering most of the volume of the PRF and including nearby surrounding structures. The responsive area thus delineated, which was consistent with PRF-specific stimulation, was targeted in later experiments.

The standard stimulation delivered via the electrode was 200 μs square pulses at 2 times the threshold (2T) for evoking MN responses, delivered at 10 Hz for 1 or 2 seconds. These parameters were based on initial experience with the parameters used for stimulation of the medullary RF (MRF) in previous projects (Szokol et al., 2008; Szokol and Perreault, 2009) and control experiments where the frequency was varied.

3.4 CALCIUM IMAGING

Spinal MN activation in response to PRF stimulation was recorded using calcium imaging. MNs can be labeled selectively by dye application to ventral roots, which contain only the MN axons. MNs in cervical segment (C) 6, thoracic segment (T) 7, and lumbar segments (L) 2 and 5 were labeled retrogradely with Calcium Green-1-conjugated dextran amine (CaGDA). MNs in both the medial and lateral motor columns (MMC and LMC) were labeled. The dye was allowed a transport time of 3 hours before calcium imaging commenced.

Calcium Green is a fluorescent dye that reacts to an increase in the concentration of calcium with a proportionate increase in fluorescence. When MNs are depolarized in response to signals from descending axons, an influx of calcium occurs, and any Calcium Green present within the MNs will increase in fluorescence. Calcium Green fluorescence is thus an indirect but reliable measure of MN response to descending signals (Lev-Tov and O'Donovan, 1995).

During experiments, regions in the brain stem were electrically stimulated as previously described, and the responding MNs were visualized using a water immersion objective and an epi-fluorescence microscope equipped with a halogen lamp, and with excitation and emission filters appropriate to the Calcium Green fluorescence absorption and

emission spectra. Video images were acquired using a couple-charged device (CCD) camera and specialized acquisition software.

3.5 RETROGRADE LABELING OF BULBOSPINAL NEURONS

An important part of this project was to map the locations and axonal trajectories of bulbospinal neurons in the brain stem. In these experiments, tracer was applied to cut axon ends at high cervical level. The axons from bulbospinal neurons will necessarily all pass the cervical spinal cord, so applying tracer there will ideally label all bulbospinal neurons in the brain stem, depending on the completeness and efficiency of tracer uptake. In our project, the level at which the C2 ventral roots leave the spinal cord was selected as the standard application site. The spinal cord white matter on one side was transected at this level, and fluorophore-conjugated dextran amine was applied as previously described. The standard incubation time for these preparations was 12 hours.

The brain stem and spinal cord are symmetrical around the midline. Labeling one half of the spinal cord is therefore sufficient to get a full picture of the bulbospinal neurons, and affords the advantage that neurons projecting ipsilaterally or contralaterally are easily distinguished, as they will be labeled on opposite sides of the brain stem.

The labeling intensity of labeled neurons gradually diminishes with increasing distance from the application site. This is probably a result of increasing distribution volume due to longer axons, and limited time for transport. This means that the pRS neurons, located relatively far from the labeling site, may be labeled faintly. To counter this problem we used one of two methods of signal enhancement. One involved using a mix of tetramethylrhodamine-conjugated dextran amine (RDA) and biotin-conjugated dextran amine (BDA) during labeling. During histological treatment, the sections were treated with a red fluorescent dye-conjugated streptavidin. Streptavidin binds to biotin with a strong bond, thus labeling BDA/RDA-containing cells with higher intensity than does the RDA alone. The other method involved enhancing RDA labeling with immunohistochemistry, using an anti-rhodamine primary antibody and a red-spectrum secondary antibody. These two methods of signal enhancement were about equally effective, and ensured strong and consistent labeling in the whole extent of the medulla and pons.

3.6 RESTRICTED SPINAL FUNICULUS LABELING

Axons from different groups of bulbospinal neurons have different positions within the spinal white matter. To gain information about these positions in the newborn mouse, we labeled a series of brain stems with standardized, restricted tracer application sites. We first took a picture of a transverse section from the C2 level and drew lines that divided the combined ventral and lateral funiculi into three parts of about equal size. During labeling experiments, cuts to establish the tracer application sites were aimed to match one of these zones, and during histological analysis the cuts were compared to the "master figure". Brain stems in which the tracer application site did not match the intended labeling zone (with a margin of error of about 20% in either direction) were excluded from further analysis. The bulbospinal neurons selectively labeled in this way had reproducible distributions that were very different between the three zones. This provided valuable information about the typical axon trajectories of the different clusters of bulbospinal neurons.

3.7 3D RECONSTRUCTION

For papers 2 and 3, 3D models of the brain stem with the locations of bulbospinal neuron clusters in relation to different brain stem nuclei and cytoarchitectonic areas was constructed. This was achieved by combining the information from two differently treated series of alternating transverse sections from the same preparation. One series underwent signal enhancement as previously described, the other was labeled with Methylene blue, a histological stain that enables identification of different brain stem nuclei.

The sections were visualized in a specialized microscope setup that was interfaced with 3D reconstruction software. In this software, the labeled neurons, along with outlines and midlines of the sections, were traced in every sixth section of the enhanced retrograde labeling sections. In the adjacent Methylene blue section, the outlines of identifiable brain stem nuclei were likewise traced. The two sets of outlines and markers were intercalated digitally, and the neurons in different clusters were assigned names according to their correspondence to brain stem nuclei and areas. The differently named neurons were then counted automatically. The counts provided an overview of the locations of bulbospinal neurons. In the software, the digitally traced sections could be used to reconstruct 3D models of the labeled brain stems (see Paper 2, Figure 5 and

Paper 3, Supplementary video). The markers could also be exported into drawing software, allowing us to make overview figures on transverse section cartoons.

3.8 TRANSVERSE SECTION CARTOONS

To present the results of the labeling experiments in an accessible way, we needed transverse section cartoons that illustrated the cytoarchitectonic structures. The method for producing these cartoons is illustrated in Paper 3, Supplementary figure 1. The existing atlas of the newborn mouse CNS (Paxinos et al., 2007) presents the brain stem in a sectioning plane that is very oblique to the natural transverse plane of the brain stem, in particular in the pons. To transfer the information in this atlas to our sectioning plane, we divided a set of transverse sections with our preferred sectioning plane into several dorsoventral segments. Matching dorsoventral sectors from crossing sections in the Paxinos atlas were identified, and the nuclear borders transferred. In this way we ended up with a series of dorsoventral segments with correct anatomical information. Based on mosaics of these dorsoventral segments the borders of brain stem nuclei in our own sections were inferred, and the cartoons drawn according to this.

4 SUMMARIES OF INDIVIDUAL PAPERS

4.1 PAPER 1

Organization of pontine reticulospinal inputs to motoneurons controlling axial and limb muscles in the neonatal mouse

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In this paper we used calcium imaging to study the connections between pontine reticulospinal (pRS) neurons and spinal motoneurons (MNs) in the neonatal mouse. The aims were: 1) To establish whether there were functional synaptic connections between pRS neurons and spinal MNs at birth and to map the distribution in the brain stem of the pRS neurons. 2) To characterize and compare the ipsilaterally and contralaterally descending components of the pRS projection.

In initial experiments the area containing pRS neurons, and surrounding areas, were probed for the ability to evoke responses in lumbar MNs, and a response-eliciting region was demarcated. The activation properties of the pRS neurons themselves were analyzed with a specialized application of the calcium imaging method. The response latencies of MNs at spinal levels C6, T7 and L2 were then compared. L2 responses occurred significantly later, on average, than C6 and T7 responses. The calcium response waveforms displayed a saw-toothed response pattern, with individual spikes in response to each of the pulses within the stimulus train. Responses were of similar magnitude in both ipsilateral (i) and contralateral (c) MNs in both the medial (MMC) and the lateral (LMC) motor columns (which contain trunk- and limb-innervating MNs, respectively) and in both L2 and L5 (which contain MNs innervating preferentially hind limb flexors and extensors, respectively). A cervical hemisection on the same side as the stimulation resulted in large reductions in response magnitudes in all four motor columns (iLMC, iMMC, cMMC and cLMC), but more on the ipsilateral side. It also strongly increased response latencies and eliminated the saw-toothed response pattern. A cervical hemisection on the side opposite to the stimulation likewise led to large reductions in response sizes in all four columns, but did not affect response latencies, or the saw-tooth response pattern. We concluded that the pRS projection influencing

spinal MNs comprised two pathways: A more direct and faithfully transmitting ipsilateral pathway and a less direct contralateral pathway. The two different pathways demonstrated in this paper, and the multisegmental synaptic connections that are indicated by our results provide new and interesting perspectives on the pRS-MN connection.

4.2 PAPER 2

Pontine reticulospinal projections in the neonatal mouse – internal organization and axon trajectories

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In Paper 1, we documented the existence of two pRS pathways, one ipsilateral and one contralateral, which could activate spinal MNs. During this work we found that a thorough anatomical description of the pRS neuron population in the newborn mouse was missing in the literature, and would be beneficial for future investigations.

In Paper 2 we therefore investigated the topographic distribution and axon trajectories of the ipsilateral and contralateral pRS populations, using retrograde labeling from the spinal cord with fluorophore-conjugated dextran amines. The aims were to: 1) Describe the ipsilateral and contralateral pRS neuron populations in terms of location, numbers, size and density patterns. 2) Characterize the initial pRS axon trajectories within the brain stem and the distribution of the descending axons in the spinal white matter at the cervical level.

PRS neurons were found at all rostrocaudal levels in the pons, both ipsilaterally and contralaterally. Both populations were found at all rostrocaudal levels of the pons, corresponding to the nucleus reticularis pontis oralis (PnO) and caudalis (PnC). The ipsilateral population was numerous in both regions, whereas the contralateral population was scarce in the PnC. The contralateral pRS neurons were located more ventral and lateral than the ipsilateral pRS neurons, so that the two populations formed adjacent and continuous, but largely separated clusters. Concerning cell size, the ipsilateral pRS neurons were larger on average than the contralateral pRS neurons, and they were larger on average in the PnC than in the PnO. Within the large-celled caudal population of ipsilateral pRS neurons, the largest neurons were found in the ventrolateral part.

The initial axon trajectory of the ipsilateral pRS neurons could be followed and was in accordance with that described in the adult mouse (Mitani et al., 1988b). The axons first headed in a dorsomedial direction in the transverse plane, before taking an abrupt turn in the caudal direction, continuing within or parallel to the medial longitudinal fascicle (MLF). The contralateral pRS axons followed a more tortuous initial trajectory.

Like the ipsilateral pRS axons, they first headed in a dorsal and slightly medial direction in the transverse plane, but unlike the ipsilateral pRS neurons they turned abruptly mediad, continuing in the same transverse plane in a medial and slightly ventral direction across the midline, before turning abruptly caudad to descend to the spinal cord. Selective labeling of pRS axons in the different zones of the upper cervical white matter revealed different distributions for the ipsilateral and contralateral pRS populations. The ipsilateral pRS axons showed a preference for the VF, and, for those originating from the PnC, the medial VF in particular. The contralateral pRS axons were concentrated in the LF, but with some scattered axons throughout the VF.

This study provides a description of the pRS neuron populations with a scope and level of detail that has not previously been available in the newborn mouse.

4.3 PAPER 3

Atlas of spinally projecting brain stem neurons and their axon trajectories in the newborn mouse

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Manuscript

Paper 2 provided a detailed atlas of the pRS neuron populations, and demonstrated that with the same set of tools we could produce an atlas of all bulbospinal neurons in the neonatal mouse. Reference material of this type is something that I have sorely missed during my Ph.D. research period and which if produced would benefit future researchers using the neonatal mouse model to study the descending regulation of spinal networks. It would also bridge a gap in the knowledge about the development of the descending pathways in the mouse, which has previously been described at embryonic stages (Auclair et al., 1999) and in the adult (Vanderhorst and Ulfhake, 2006; Liang et al., 2011), but not in detail in the newborn.

The aims of this paper were to: 1) Provide an overview of all clusters of bulbospinal neurons present in the newborn mouse, presented in an accessible form for practical use by other investigators, and 2) Characterize the distribution of axons from the different bulbospinal clusters as they enter the cervical spinal white matter.

Brain stems from newborn mice were retrogradely labeled as in Paper 2, with tracer applications targeting either all of the ventral and lateral funiculi (VF + LF), or one of the three defined restricted zones. Transverse and parasagittal sections were prepared. Representative labeled brain stem preparations were depicted by constructing digital overlays of adjacent transverse sections. Digital overlays were presented for four brain stems, one each for unilateral labeling of the complete VF+LF labeling and the three restricted white matter zone labelings.

Counts of labeled neurons in 3D reconstructions of 9 brain stems provided estimates of the relative numbers of bulbospinal neurons found within the different brain stem nuclei and cytoarchitectonic areas, discriminated according to axon trajectory in the cervical white matter. Four clusters contained numerically comparable neurons projecting to all three white matter zones, fifteen clusters were made up mainly of neurons projecting to two adjacent zones, while three clusters were made up mainly of neurons projecting to one zone, in all cases the medialmost zone. The results from these

reconstructions are also presented in figures, as raw data and transformed into neuron densities.

This paper provides detailed information about the topographic distribution and axonal trajectory of all bulbospinal neurons in the newborn mouse, presented in an accessible way, hopefully making it a useful tool for future researchers in the field.

5 DISCUSSION

In this section I will discuss overriding or particularly important aspects of the methods and results of this project. Some of the discussion found in the individual papers, for example pertaining to specific details about technical issues, will not be repeated here.

5.1 METHODOLOGICAL CONSIDERATIONS

5.1.1 Extracellular electrical stimulation in the pons.

In Paper 1, we made use of extracellular electrical stimulation with a monopolar electrode. This is a relatively crude stimulation method, in the sense that it exposes all the potentially excitable tissue in a field around the electrode tip to electrical current, which diminishes with radial distance to the electrode, creating a sphere of influence if the conductive properties of the surrounding tissue are homogeneous. This means that the stimulation potentially can elicit a depolarization in any soma, dendrite or axon within this sphere. Which of these neuron parts that is most easily stimulated is the subject of an extensive literature that I will not discuss here. It suffices to say that their excitability is comparable, meaning that a stimulus intended to stimulate one type of tissue (in our case pRS neuronal somata and dendrites) will always run the risk of stimulating the other types of tissue as well. The main fear is that the stimulus will excite passing axons that originate from other neuron populations that are not the subject of study.

To address this concern somewhat, we adapted the calcium imaging method to study the local activation of pRS neurons in response to our standard stimulus train (Paper 1, Figure 2). This showed that the pRS neurons were strongly activated by the stimulus, the responses diminishing rapidly with increasing electrode tip distance. This established that we at least excited what we wanted to excite. Whether passing axons were also activated remained undetermined. During the mapping of areas in the pons that elicited responses in lumbar MNs (Paper 1, Figure 3), we found that stimulation close to the MLF activated MNs strongly – indicating that the stimulus indeed had the potential to activate passing axons. However, the mapping also indicated that the MLF activation diminished rapidly with increasing distance from the MLF. Aside from this, the pattern of activation generated by our effective stimulation sites closely matched the area in which pRS neurons reside. In this area, there are relatively few axons of passage that continue into the spinal cord (Paper 2, Figure 9A-B), other than the axons of the pRS neurons themselves (Paper 2, Figure 9C-D (ipsilaterally projecting pRS neurons)

and Figure 9E (contralaterally projecting)). In sum, these investigations convinced us that the main source of the MN responses we saw during extracellular electrical stimulation in this area was activation of pRS neurons and their axons. Still, other minor contributions cannot presently be ruled out.

5.1.2 Age range of mice used in calcium recording experiments

In the previous papers from our lab using the brainstem – spinal cord preparation for calcium imaging, the age of the neonatal mice ranged from postnatal days (P) 0 to P4 (Szokol and Perreault, 2009) or P5 (Szokol et al., 2008; Kasumacic et al., 2010; Szokol et al., 2011; Kasumacic et al., 2012). In my studies focused on the pRS projection, it turned out that responses could rarely be evoked in animals from the older end of this range. We therefore settled on a more limited age range, using only P0-P2 mice. We have not investigated further why the older mouse pups were unsuited in this respect, but we suspect that the main reason is the deeper position of the pontine reticulospinal neurons compared to the populations studied in the two previous projects (Szokol: medullary reticulospinal (mRS) neurons, Kasumacic: Vestibulospinal neurons). In the ex vivo situation, the tissues receive oxygen and nutrients solely by diffusion from the surface (not from nearby blood vessels, as is the situation in the intact animal), so it is logical that a deeper location in the tissue could lead to poorer oxygenation and nutrition. The same concerns made us decide to routinely remove the cerebellum in our preparations, as this leads to significantly shorter diffusion distances from the dorsal surface of the brain stem in the pontine region. We have not performed dedicated studies to document the benefits of either of these changes in method, and can only assert that experiments were much more reliable after the changes were made. Other explanations for this improvement in reliability, for example gradual improvement in technical proficiency, cannot be ruled out.

5.1.3 Studying pRS – MN connectivity using calcium imaging

The calcium imaging method for studying neuronal activation is a potentially very useful method with distinct advantages and disadvantages. In the study of electrical activity in neurons, the gold standard is the patch recording method, which measures the electrical activity directly. Calcium imaging, on the other hand, utilizes the influx of calcium in conjunction with depolarization. This is an indirect measure of the electrical activity in the cell, and carries with it clear limitations. Calcium concentration varies on a different timescale compared to that of electrical activity. Thus, while electric

potential varies in very short spikes, depolarizing more or less instantaneously and returning to baseline within a very few milliseconds, the calcium concentration, and thus the fluorescence signal, increases more slowly, and decreases only gradually after each electrical spike, using about a second to return to baseline. Many spikes in quick succession will lead to gradual build-up of intracellular calcium levels that take even longer to clear out. Using filtering and deconvolution, the calcium signal can be "translated back" with a reasonable success rate (74 % fraction of variance recovered (Yaksi and Friedrich, 2006)), but is still inferior to patch recording in this respect. Another disadvantage is that since the calcium influx results from depolarization at the cell membrane, calcium imaging can only give information about depolarizing inputs. Inhibitory influences, which work by hyperpolarizing the cell membrane, are thus not available for study using calcium imaging.

The advantage, on the other hand, of calcium imaging is that many neurons can be recorded from at once. Patch clamping can only be performed on one cell at a time, with considerable time spent per neuron. Thus, in assessing the effect of a stimulation on a field of neurons, as we did with the spinal motor columns in Paper 1, the calcium imaging method is the method of choice.

It should be mentioned that there is one method that combines the advantages of the two aforementioned methods. Voltage sensitive dye imaging visualizes voltage changes directly, thus providing excellent temporal resolution and the visualization of both depolarizing and hyperpolarizing events, while still enabling recording from many neurons at once. However, another requirement in our application was the ability to record selectively from specific neuron populations, which was achieved by delivering the dye using retrograde labeling (thus excluding surrounding neurons with axons that do not pass the labeling site). Such selective labeling is not yet possible with voltage sensitive dyes.

In Paper 1 we use the calcium imaging method to assess response latencies. Given the relatively slow dynamics of the calcium concentration, this is not ideal. We could not use the latency information to infer reliably whether connections were monosynaptic versus polysynaptic. Electrical methods are better suited for this purpose, although the calculation of synaptic latencies requires the recording of descending volleys, which has proven difficult in the newborn rodent (Szokol et al., 2008). We were nonetheless able to draw some conclusions by comparing the calcium response latencies obtained under different conditions, showing marked differences in latency when different axon paths were interrupted.

5.1.4 Ensuring that the retrograde brain stem labeling was truly unilateral

As the brain stem, by all accounts, is symmetrical around the midline, labeling one half of the spinal cord is sufficient to get a full picture of the descending neurons. Unilateral labeling is preferable to bilateral labeling because it allows neurons projecting ipsilaterally versus contralaterally to be easily distinguished, as they will be labeled on opposite sides of the brain stem. It follows that it will be a goal to label all of the axons in one half of the spinal cord, but none in the other. Several different approaches were attempted to achieve this, with varying success (see lengthy discussion in Paper 3). The method we arrived at involved the following procedure prior to labeling: Carefully separate the two spinal cord halves from each other along the midline, and remove a long stretch of the half not intended for labeling, up to the medulla/spinal cord junction (Paper 2, Figure 1A). This way, only the half intended for labeling was present at the level where the tracer was applied. This method still involves the risk that stray tracer will diffuse to the cut surface on the contralateral side, and a risk of damaging the ipsilateral axons closest to the midline. Careful inspection of the labeled preparations indicated that the former is a negligible problem. The reality/impact of the problem of damaged ipsilateral axons is uncertain. Histological inspection indicates that the separation along the midline is accurate and reliable. It seems that during the dissection, which largely is performed bluntly (without cutting), the spinal cord naturally separates at the midline. The process nevertheless must involve some mechanical stress to the axons closest to the midline. Whether this stress affects the axons' ability to take up and transport dye is uncertain.

5.2 RESULTS IN PERSPECTIVE

5.2.1 Does the pRS projection merit individual study as a discrete entity?

In Paper 1 we studied the connection between pRS neurons and MNs in the spinal cord, and found a pattern of activation that differed from what has previously been found in the mRS population using similar methodology (Szokol et al., 2008). Unfortunately, the methodologies differ enough that direct comparisons cannot safely be made. Furthermore, the mRS study investigated two mediolaterally segregated populations within the mRS population that displayed different activation patterns in spinal MNs. A similar mediolateral segregation could hardly be made in the pons, as the ipsilateral pRS population (which is probably responsible for the bulk of the spinal MN responses, see section 5.2.3. The nature of…) is relatively narrow in the mediolateral aspect,

without any apparent segregation (Paper 2, Figure 3A). As we show in Paper 2, a more obvious segregation in the pons is that between the caudal (PnC) and rostral (PnO) parts of the pRS population. These two portions of the ipsilateral pRS population differ in average cell size, and although they are continuous with each other, their junction is marked by a shift in average mediolateral position. In Paper 1, we did find a tendency for diminishing response sizes, moving from caudal to rostral, but a thorough analysis of differences along the rostrocaudal axis could not be performed due to a scarcity of stimulation sites in the rostral pons. One can only lament that we did not have the knowledge provided by Paper 2 when we collected the data for Paper 1. On the bright side, this shows a potential in the results presented in Paper 2 for raising new and interesting research questions.

In the literature, the RF in the pons and medulla is often referred to as one entity, the pontomedullary reticular formation. In some studies, the MLF has been stimulated (Grillner et al., 1968; Floeter et al., 1993; Riddle et al., 2009) or injected with tracers (Du Beau et al., 2012) as a convenient proxy for this entity. The implicit assumption is that the PRF and MRF form one more or less homogenous group. And indeed, when different properties, like limb activation pattern (Drew and Rossignol, 1990a; Drew and Rossignol, 1990b) or monosynaptic activation of limb, trunk or neck MNs (Peterson et al., 1979) is mapped out, the different properties each cover large portions of the PRF and MRF, with considerable overlap between areas where the different properties occur. However, the overlaps are rarely complete, so some regional differences do exist. That this large brainstem area should be completely homogenous in function and connectivity also seems very unlikely. However, the fact remains that the MRF and PRF do not display a pattern of connectivity and functionality that is obviously topographically compartmentalized. This poses a challenge to researchers, technically and intellectually.

Another factor pertaining to the aforementioned studies of the pontomedullary reticular formation is that there is often very litte “ponto-“ involved. There is a tendency that the areas that other studies have mapped out only cover the medulla and the very caudalmost part of the pons, so that what is studied is really almost only the MRF. This is probably due to technical difficulties. The pons is in the intact animal covered dorsally by the cerebellum, and for this reason hard to access - e.g. with a stimulating electrode. In studies where significant pontine areas are included, some indication that the pRS population actually does differ from the mRS population can be found. In the aforementioned studies of monosynaptic connections to spinal MNs, no monosynaptic

connections were found from pontine areas (Peterson et al., 1979). And in a study analyzing reticulospinal cell activity during a trained task, only one out of four categorized activity patterns was prevalent in the pons, while all four were well represented in the medulla (Schepens et al., 2008), suggesting that the pRS neuron population is more homogenous, with a narrower range of functions, than the mRS neuron population. This would not be entirely surprising, as the pRS population is numerically much smaller than the mRS population (Paper 3). One function for which the pRS population seems to play a crucial role is that of the startle reponse: A reflex, well known also in humans, to strong, sudden sensory input consisting of instantaneous stiffening of the limbs, body wall and dorsal neck, probably as a defense mechanism that protects vital organs in the brief period before directed evasive or defensive action can be performed (Yeomans and Frankland, 1995). Anatomical and electrophysiological experiments indicate that the main central pathway mediating startle involves three synaptic relays connecting the sensory nerve fiber and the responding muscle: 1) Cochlear nuclei neurons, 2) giant neurons in the caudal pons and 3) MNs in the brain stem or spinal cord (Lingenhohl and Friauf, 1994). The giant neurons were probably responsible for part of the responses evoked in spinal MNs in Paper 1. That these neurons are supposed to be particularly large in size fits well with the finding in Paper 2 that caudal pRS neurons are larger on average than rostral pRS neurons.

Thus, in answer to the initial question; “Does the pRS projection merit individual study?”, the answer would be yes. But at the same time it seems clear that a complete understanding of the RF and the reticulospinal projections is not going to be reached by searching for a topographical organization alone, since different functionalities overlap to a large degree. It would seem that other kinds of reasoning are called for, ones that focus less on topographical organization.

5.2.2 Implications of using a newborn mouse model

For the applications used in the present thesis, the isolated brain stem-spinal cord preparation is only possible to use in the first days after the mouse is born (See section 5.1.2 Age range…). This means that all data obtained only applies directly to the newborn mouse. The unique advantages offered by this preparation (See section 5.1.3 Studying pRS…), which are not available in adult preparations, makes it tempting to use it as a model for the situation in the adult. This should be done with caution. There are major differences between the newborn mouse and the adult. For example, the

sensory apparatus is not fully developed. Newborn mice are deaf until day 9, probably due to an undeveloped internal hearing apparatus before this time (Ehret, 1976). This would, however, not present a difference from the adult in the case of the brain stem-spinal cord preparation, as in this preparation all sensory nerves are cut. However, the mice are relatively undeveloped motorically as well: Weight-bearing locomotion is not present at birth, but improves substantially between the first and second postnatal weeks (Geisler et al., 1993; Gramsbergen, 1998). There is, however, a hypothesis that this delay is an evolutionary adaptation. This is suggested by the finding that in rats, with proper motivation and facilitation, levels of locomotor efficiency normally not seen before P14 can be exhibited at P4. The evolutionary reward of this delay in behavior would be straightforward - for newborn mice it is very dangerous to leave the nest, so it is safer not to roam (Jamon and Clarac, 1998). The implication is that the neural networks may be more mature at birth than what is revealed in the behavior of the animals. However, there are differences between the newborn and the adult on the neural network level as well. For example, the pattern of atonia during REM sleep and activation during wakefulness is reversed until about two weeks of age in the cat (Chase, 2013). Another example is that in the cat spinal cord, commissural INs have bilateral connections in the newborn, but only contralateral connections in the adult (Jankowska, 2008).

There are, however, some features that are established in the newborn that are unlikely to change radically during further development. As previously noted (1.5.2 Embryology), the general organization of the descending neuron groups is already established in the newborn mouse (Leong et al., 1984a; 1984b), and, reticulospinal axons reach the lumbar spinal cord already on day 14 (in rats (Kudo et al., 1993)). Functional reticulospinal (Szokol et al., 2008; 2011) and vestibulospinal (Kasumacic et al., 2010; 2012) connections to INs and MNs have been demonstrated at birth, some of which are organized according to the known adult patterns. And although mice are behaviorally very limited at birth, neural networks by this time can produce left-right and flexor-extensor alternation and interlimb coordination, which can all be manifested under non-weight-bearing conditions (Gramsbergen, 1998; Clarac et al., 2004; Jamon, 2006). This indicates that much of the basic motor connectivity is in place at birth.

The bottom line, then, is that the brain stem-spinal cord preparation from the newborn mouse can provide direct information only about the situation in the newborn mouse, but that some inferences about the situation in the adult may be cautiously made, in particular concerning basic connectivity. Relating this to our results, we believe that the

conclusions drawn in Paper 1 concerning synaptic connectivity to cervical, thoracic and lumbar level and the greater directness of the ipsilateral pRS-MN pathway versus the contralateral one probably applies to the adult as well. Other aspects, for example the details concerning the relative contributions of the two pathways to the four different motor columns, cannot be extrapolated to the adult with confidence.

Considering Papers 2 and 3, these are anatomical analyses of the newborn mouse, undertaken to provide information about this particular developmental stage, partly because of the research utility of the newborn mouse model. Extrapolation to the adult is therefore not an issue in the case of these papers, although on comparison to studies in the adult there are substantial similarities.

5.2.3 The nature of the two pRS projection pathways to spinal MNs

In Paper 1, we documented that two separate pathways with different properties contribute to spinal MN responses elicited by PRF stimulation. We found an ipsilateral pathway, the activation of which led to fast and reliable responses, and a contralateral pathway, the activation of which led to slower and more erratic responses, which were however of comparable magnitude to the ipsilateral pathway responses. For the ipsilateral pathway, the anatomical substrate is almost certainly the ipsilaterally projecting pRS neurons. Many of these neurons have been shown to project along the whole length of the spinal cord (Vanderhorst and Ulfhake, 2006), and to terminate in areas of the spinal grey matter that contain MNs and motor-related INs (Matsuyama et al., 1997; 1999). Our stimulation mapping experiments in the spinally intact (no cervical hemisection) preparation showed that the areas that were most effective in evoking responses in lumbar MNs closely matched the distribution of the ipsilateral pRS neurons (Paper 1, Figure 3), indicating that these neurons were the main source of the MN activation. In the case of the contralateral pathway, the anatomical substrate is less obvious. The simplest and most immediate explanation is that this is provided by the contralaterally projecting pRS neurons. Accordingly, that is the explanation that we focused on in Paper 1, whilst pointing out that other explanations are possible. One problem with this explanation is the distribution of the contralateral pRS population. The stimulation sites used in Paper 1 were concentrated in the caudal pons, and only partially matched the location of the contralateral pRS neurons, which, as we show in Paper 2, are largely restricted to the rostral pons, with only a sparse population of small neurons in the caudal pons. Again, we can only lament that the information presented in Paper 2 was not available to us when we collected the data for Paper 1. Furthermore, as

we suggest in Paper 1, the contralateral activation pathway is probably less direct than the ipsilateral pathway. It is therefore not unlikely that one or more synaptic relays within the brain stem are involved. On the nature of these relays, one can only speculate. One interesting possibility is that of connections from the contralateral pRS area to ipsilateral pRS neurons. Anatomical studies certainly open for this possibility: At all levels of the brain stem, contralateral homologous areas on the same rostrocaudal levels are major targets for RF afferents (Basbaum et al., 1978; Jones and Yang, 1985; Mitani et al., 1988a; Hempel et al., 1993). Considering electrophysiological studies, researchers studying the startle response have been searching for the way in which *unilateral* sensory input is able to produce the typically very *symmetric, bilateral* startle response, even though the giant cells that transmit the response to the spinal cord mainly terminate unilaterally. They have found synaptic connections between the caudal PRF areas on the two sides that are fast and strong enough to be a likely explanation (Hempel et al., 1993). So both anatomical and physiological data suggest that there exist pRS-pRS connections between the two sides that could be part of the contralateral pathway found in Paper 1. However, this remains speculative for the time being.

An interesting finding pertaining to the nature of these pathways is the relative similarity between responses seen in ipsilateral and contralateral MNs after stimulation of either pathway. Response sizes were comparable in all four motor columns in either pathway isolation situation. And the one-to-one response pattern between pulses in the stimulation train and spikes in the response waveform, typical of the ipsilateral pathway, was present in all four motor columns, both ipsi- and contralaterally. The reason that this is noteworthy is that ipsilaterally descending pRS axons, as pointed out in the previous paragraph, seem to mainly terminate ipsilaterally in the spinal grey matter (Matsuyama et al., 1997; 1999). One possible explanation could be that in the newborn, more of these axons terminate bilaterally. Examples exist of terminations that are present in the developing animal, but have disappeared in the adult (Glover and Petursdottir, 1991; Jankowska, 2008). Another, perhaps more likely, possibility is that the responses, both ipsilateral and contralateral, are mainly the result of transmission via INs, and not of direct synaptic contacts between descending pRS axons and MNs. This would be in agreement both with the preferred areas of termination of reticulospinal axons, and with electrophysiological data indicating that polysynaptic connections play a greater role than monosynaptic connections in transmission between reticulospinal pathways and MNs (Floeter and Lev-Tov, 1993; Alstermark and Ogawa,

2004; Riddle et al., 2009). Incidentally, it also suggests an alternative explanation for the bilateral startle response to unilateral sensory input presented in the previous paragraph.

5.2.4 Future perspectives

Future perspectives can be both short term and long term. And really long term. The shortest term perspective relating to this thesis is that of the experiments and analyses that are directly available in the continuation of it - that are just waiting to be performed, so to speak. The detailed analysis of position and size of pRS neurons performed for Paper 2 (Figures 3, 6 and 7) revealed patterns that had not previously been described and in some instances took us by surprise. Motivated by this, we plan in the near future to perform the same kind of analysis on the mRS and the vestibulospinal populations. In Paper 1 we applied the calcium imaging method to pRS neurons, in order to assess the ability of our normal stimulation train to activate these neurons. The experiments indicated that this method, with small modifications, could be used to study the interconnections between the pRS populations on either side of the brain stem (discussed in the previous section). We have already performed pilot experiments where we could reliably evoke this connection, indicating that this is a viable avenue of research.

Another perspective is that of questions arising directly from our results. In paper 1, we demonstrated the existence of two qualitatively different pathways by which lumbar MNs could be activated by stimulation of the pRS population. Our results also suggested that single pRS neurons can synaptically activate MNs in widely separated spinal segments, something which is also suggested by anatomical studies (Matsuyama et al., 1997; 1999). Both these findings invite corroboration and further description using complementary methods.

Finally, a look at what other methods are available and which I think have the most potential in terms of relieving the reticulospinal projection of its secrets. Transsynaptic, viral labeling studies are being widely performed and will probably in the near future determine the prevalence of mono- vs. di- and polysynapticity in the reticulospinal-motoneuron connection as well as other motor pathways (Tang et al., 1998). Optogenetic tools offer the opportunity to single out specific inputs to MNs or to the pRS neurons (Tye et al., 2011) and will enable us to dissect this and other motor pathways and analyze their separate components. Finally, I am becoming increasingly convinced that motor control systems are just a bit too complicated for the human mind

to understand. To remedy this I suggest to involve computers more in the analysis of experiments. In my dream project, experiments are performed where multiple single-unit recordings are simultaneously acquired from many regions involved in motor control, at different hierarchical levels, on a freely moving animal (Fan et al., 2011). High-speed cameras and motion analysis provides a complete picture of the animal's movements, which is broken down to movements across single joints, but also synthetized into several different levels of abstraction ("move leg", "walk", "walk over there", "eat that morsel of food over there", etc.), and translated into virtual sensory information (proprioceptive and vestibular). All this data is then fed into the computer and subjected to sophisticated analysis algorithms, which correlates all the different elements, applying different schemes of timing, interconnections, hierarchy and so on, until it all clicks into place. And out comes the answer to everything. Which may or may not be 42. I am very much looking forward to reading that paper one day.

6 SUMMARY

In this Ph.D.-project I have investigated the properties of the pontine reticulospinal (pRS) neuron population in the newborn mouse, and have characterized the internal organization and axon trajectory profiles of all the bulbospinal neuron groups labeled retrogradely in the pons and medulla.

In Paper 1, we used calcium imaging to establish that there are synaptic connections between pRS neurons and MNs in the newborn mouse, and distinguished two pathways with different properties that both contributed to this connection, an ipsilaterally descending pathway that elicits MN responses quickly and faithfully, probably through only one or a few synapses, and a contralaterally descending pathway that elicits MN responses more erratically and with longer latency, probably through more synapses than the ipsilateral pathway.

In Paper 2, we characterized the internal organization and projection trajectories of the ipsilateral and contralateral pRS neuron populations in the newborn mouse, demonstrating rostrocaudal patterns in mediolateral position, soma size and number of neurons comprising the two populations.

In Paper 3, we described the spatial organization of all the bulbospinal neuron clusters in the pons and medulla of the newborn mouse, and compared the locations of these clusters to a previously published map of cytoarchitectonically defined brainstem nuclei and areas. We also characterized the profile of axon trajectories in the spinal white matter for each cluster. These anatomical findings were documented in the form of an instructive atlas.

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8 PAPERS